The Pancreatic Macrophage Compartment in Health and autoimmune Diabetes: a study on Maturation, Mobility and Matrix interactions

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Het macrofaag compartiment in de pancreas in gezondheid en autoimmune diabetes: een studie over maturatie, mobiliteit en matrix interacties

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Contents

Preface

Autoimmune insulin-dependent diabetes mellitus

Diabetes mellitus is a disease resulting from a disturbed glucose metabolism due to either an almost complete deficiency in insulin production (type 1 diabetes) or to impaired insulin sensitivity (type 2 diabetes). In this thesis we will only discuss type 1 diabetes (T1D). In T1D, also referred to as insulin-dependent diabetes mellitus (IDDM), insulin deficiency is the consequence of immune-mediated damage to the insulin-producing β-cells. The subsequent rise of blood glucose to clinically dangerous levels confines T1D-patients to life-long treatment with exogenous insulin to control this hyperglycemia.

The cause of T1D is multifactorial and largely unknown. The initiation and progression of the inflammatory process that destroys the β-cells involves the interplay of environmental factors with an autoimmune-prone genetic background. To clearly comprehend the process leading to T1D, it is best to subdivide its pathogenesis into distinct steps:

First, endogenous or exogenous triggers are believed to activate innate immune cells, like macrophages and dendritic cells (DC) that initiate the activation of T-cells with autoreactive capacity.

Second, genetic and environmental factors influence the regulation of this response and accelerate or delay the progression towards autoimmune disease by sustaining or suppressing a proinflammatory environment in the target organ.

Third, immune-mediated effector mechanisms are activated locally in the target organ, initiating the final phase: that of β-cell destruction and clinical manifestation of diabetes.

The variables involved in each step are numerous. Nevertheless, there is a certain consensus on the evolvement of the pathogenic process of T1D: at a certain point the regulation of the immune balance shifts towards a pro-inflammatory phenotype and if not counteracted, this will lead to a Th1-mediated autoimmune destruction of the islet β-cells.

The introduction of this thesis will first discuss the facts and figures of diabetes incidence and susceptibility in humans and animal models of IDDM. Defective immune regulation is importantly involved in T1D pathogenesis, therefore mechanisms controlling self-tolerance of the immune system will be introduced next. The diabetes pathogenesis itself will be discussed generally, since the questions and the related research presented in this thesis mainly focus on the period prior to the initiation of IDDM and in particular on the interaction of macrophages with the target organ. The end-stage of the inflammation responsible for the final destruction of the islets will be discussed first, followed by what is

currently believed to be the hallmark of IDDM onset in both mice and man: the process of insulitis.

Insulitis is characterized by the intra-islet infiltration of T and B-lymphocytes that mediate islet destruction (lymphocytic insulitis). T and B-cell infiltration is preceded by an accumulation of macrophages and DC at the islet periphery. This early peri-islet accumulation of antigen presenting cells (APC) probably identifies the first response of the immune system progressively heading for islet destruction. Macrophages and DC are suggested to be leading actors in this early process in mice, rats and in humans as well. Moreover, they are involved in each step of the diabetogenic process. Not only are they the first to arrive at the “site of action”, they also function as professional APC that are capable of activating naïve T-cells. Macrophages and DC that remain “on site” will stimulate the infiltrating activated $CD4^+$ T-cells. Subsequently stimulated $CD4^+$ and $CD8^+$ T-cells, as well as activated macrophages, will continue the process by producing a wide range of different damaging mediators, sustaining the inflammatory environment. Finally, when β-cells are destroyed, macrophages are also required to clear the debris of dead cells.

In T1D patients and diabetes-prone animal models, in particular the nonobese diabetic (NOD) mouse that develops IDDM spontaneously, aberrancies are not only observed in cells of the immune system, like T-cells, macrophages and DC, but in the target organ as well. In this thesis the mutual relationship between macrophages and the pancreas, as well as the distinct roles of these immune cells and the target organ in the process of IDDM, will be discussed in detail.

Chapter 1
Does macrophage malfunction lead to diabetes?

General introduction

1.1 Genes and environment: diabetogenic pressure comes from both sides

1.1.1 Genetic susceptibility of T1D

T1D is predominantly diagnosed in children and young adults and forms a major health concern in developed countries. The exact etiology of T1D is still unknown, but both genetic as well as environmental factors contribute to T1D development. Family studies show that the genetic background is a predisposing factor to the development of IDDM. About 6% of siblings of T1D patients develop T1D, compared to a prevalence in the general population of 0.5% [1, 2]. Monozygotic twins, sharing identical genetic backgrounds, show a concordance rate of 30-50%, while that of dizygotic twins is much lower, i.e. around 11%. Several genetic regions that confer susceptibility of diabetes have been determined and are associated with immune regulation as well as with the regulation of tissue specific gene expression. Among the 15 identified regions [3], the Major Histocompatibillity Class (MHC) II genetic region located on chromosome 6p21 is probably the most important region, responsible for 45% of the genetic susceptibility. Furthermore, the tissue-specific short class I variable number of tandem repeat minisatellites, within the regulatory region of the insulin gene on chromosome 11p5, accounts for approximately 10% of genetic susceptibility.

1.1.2 Environmental factors involved in the onset and progression of T1D

Additional to the genetic predisposition, environmental factors exert a substantial influence on diabetes development, as is indicated by the relatively low concordance rate in monozygotic twins. Nonhereditary transmission of diabetes receptiveness from diabetic mothers to their unborn children has been associated with the exposure to elevated levels of insulin in the amniotic fluid, suggesting that early disturbances in the glucose metabolism may influence the development of T1D later in life [4]. The early consumption of several food components such as cow's milk (containing bovine insulin) has been associated with an increased diabetes incidence at a later age as well [5]. However, since several reports have shown contradictive results, the significance of these correlations have been disputed [6, 7].

Several studies have related increased diabetes incidence with previous viral infection with Measles and congenital infection with Rubella [8, 9]. Importantly, in some cases of rapidly progressing T1D, viral material could indeed be isolated from pancreases of recently diagnosed patients [10]. The possibility of viruses being candidate causative agents of T1D has led to the proposition of several hypotheses explaining infection-related T1D onset. Viral infection of the β-cells can directly lead to their destruction, but β-cell death can also be the unfortunate consequence of the initiated anti-viral immune attack. In both cases infection will lead to a local sudden release of large amounts of β-cell specific antigens. The subsequent anti-β-cell autoreactivity possibly results from bystander activation, enhanced by the local pro-inflammatory environment. Massive β-cell death can additionally lead to the processing and presentation of epitopes of β-cell antigens that normally remain unexposed (cryptic

epitopes). Finally, molecular mimicry of the recognized viral epitopes with epitopes on β-cell-specific antigens has been proposed to initiate autoimmunity [11, 12]. In this case infection of the β-cells themselves is not imperative.

1.1.3 Does infection mediate protection?

In contrast to autoreactive activation, a protective effect of infection has also been reported. In one study the remission of T1D in newly diagnosed patients was associated with earlier administrated intracutaneous Bacille Calmette-Guerin (BCG) injections [13]. This protective effect of BCG in humans has not been verified in other studies thus far [14, 15]. In spontaneous animal models of diabetes complete protection against IDDM development was conferred by BCG and other infectious agents like streptococcal derivatives and the helminth Schistosoma mansoni [16,17]. The north to south gradient of diabetes-incidence additionally supports infection-related protection. The highest incidence of diabetes is observed in northern Europe (in Finland over 30/100,000 children under the age of 14 years are diagnosed each year) and decreases in southern, more tropical countries with lower hygienic standards. Moreover, the incidence in NOD mice and biobreeding (BB) rats [18], two inbred animal strains that spontaneously develop diabetes, is the highest (80-100%) when animals are housed under germ-free or specific pathogen-free conditions. The viral and microbial conditions possibly affect the immune profile either maintaining an immune balance or skew it to an autoimmune-prone setting.

Etiopathologic studies are not always feasible in humans and are also limited by ethical considerations. Most of our current understanding of the mechanisms involved in diabetes development has derived from studies in animals that develop diabetes. The NOD mouse or the BB-rat develop autoimmune diabetes spontaneously, whereas experimental intervention with chemical substances like streptozotocin or alloxan is used to induce diabetes in susceptible animal strains. It is noteworthy that animals used for scientific experimentation are usually inbred, in contrast to human populations that carry different genetic backgrounds. Therefore, studies in inbred animals must be regarded as single case reports, and only reflect a certain aspect involved in, or associated with the development of T1D on that particular genetic background. Although the data provided by animal studies are of great value, regular verification of the relevance of these experimentally obtained results to human diabetes remains very important. The data that will be discussed in the subsequent sections were derived from animal studies if not stated otherwise.

1.2 From T-cell priming to pathology

The process leading to T1D can be subdivided into distinct phases. Initiation of islet autoreactivity requires the breakdown of immune tolerance that under healthy conditions is regulated by distinct control-mechanisms. In the case of T1D pathogenesis, immune tolerance is broken and T and B-cells are primed and activated against islet-specific antigens. Whether this lymphocytic activation leads to the destruction of the β-cells depends on numerous factors and the progression of the process is critically determined by the action of different cytokines.

Importantly, the immune cells need to infiltrate the pancreas to reach and destroy the β-cells, adding an additional checkpoint in the progression towards disease. Their extravasation from the circulation to gain access to the pancreas parenchyma is regulated by a special type of signaling molecules called chemokines, and by receptors that are expressed by either the endothelial cells (addressins) or the leukocytes themselves (selectins and integrins).

1.2.1 Progression towards diabetes I: failure of mechanisms that control tolerance

The adaptive immune system is designed to mount adequate and specific responses towards invading environmental pathogens, thereby avoiding damage to the surrounding tissue or the induction of autoimmune disease. The presence of autoreactive T-cells in the periphery is not uncommon, but peripheral T-cells normally remain ignorant for self-tissue antigens since presentation is not accompanied by costimulatory signals [19]. This strongly suggests that inadequate regulation of immune tolerance underlies the initiation of autoimmune disease.

If peripheral tolerance is broken, the resulting autoimmunity involves different levels of faulty immune control. Two distinct systems of tolerance regulation are currently recognized: central tolerance regulated in the thymus, and peripheral tolerance that includes the non-responsiveness (anergy) of T-cells, activation-induced death of responding autoreactive T-cells (apoptosis) and regulation of the response by special subsets of T-cells: the regulatory T-cells. Furthermore, cytokines are vitally important for the resulting peripheral response, and main players in tolerance regulation are the pleiotropic cytokine transforming growth factor-β1 (TGF-β1) and interleukin-10 (IL-10).

1.2.1.1 Regulation of central tolerance

Early prevention of autoreactivity is orchestrated by the thymus. T-lymphocytes mature within the thymus while being subjected to a strict selection procedure: only T-cells that recognize self-MHC molecules with high affinity (positive selection) and respond with low affinity to self-MHC when complexed with self-peptide (negative selection) will leave the thymus. The negative selection procedure, i.e. apoptosis of T-cells that display a too high affinity for the MHC-self-peptide-complex, can be activated during all stages of T-cell maturation from the double-positive stage on. Positive selection of T-cells takes place upon the acquisition of T-cell receptor (TCR) expression, in the

deeper cortex of the thymus. Intrathymic antigen presentation for negative selection takes place in the medulla and corticomedullar areas. Here, antigen will be presented by thymic epithelial cells (TE) and/or APC with a phenotype similar to bone marrow-derived APC. Peripheral antigens are transported to the thymus or synthesized within the thymus for direct presentation. In the human thymus mRNAs of tissue-specific candidate autoantigens for T1D like (pro)-insulin, glutamic acid decarboxylase (GAD) and the tyrosine-phosphatase-like protein IA-2, are expressed at low levels and their corresponding proteins were identified in thymic macrophages and DC [20, 21]. The thymic expression level of these autoantigens was earlier implied as an important variable in the education of T-cells [22]. High levels possibly result in the deletion of higher numbers of autoreactive T-cells, while low levels of expression might predispose to autoimmunity by allowing more autoreactive T-cells to escape the thymus. Whether this proposed hypothesis is indeed valid remains to be determined. Thus far, it has been shown that the expression level of insulin mRNA in the thymus of NOD mice was indeed lower than that of BALB/c mice which do not develop diabetes spontaneously [23].

The NOD thymus structure is heavily disturbed and characterized by giant perivascular spaces and medulla-derived epithelial cells that are not only found in the medulla, but in the thymic cortex as well [24-26]. A defective function of the NOD thymus may predispose NOD mice to the generation of an autoimmune susceptible T-cell repertoire. The abnormal localization of medullary epithelial cells and possibly of APC in the cortex might initiate the negative selection procedure of NOD thymocytes at a premature state of their development. Importantly, pancreatic insulitis was induced in recipient C57BL/6 mice when the selection procedure of infiltrating C57BL/6 T-cell precursors was driven by embryonic NOD-derived thymic epithelial anlage [27]. Moreover, in addition to the autoimmune-prone TE, thymocytes of NOD mice are also defective, as they suffer from impaired TCR-signaling [28].

1.2.1.2 Regulation of peripheral tolerance

The majority of mature T-cells that leave the thymus display reactivity against foreign antigens and maintain our protective immunity. The thymus additionally produces a subpopulation of T-cells with regulatory capacity. Regulatory T-cells maintain self-tolerance by controlling the responses of autoreactive T-cells that have escaped the thymus. Either directly via cell-cell contact and induction of apoptosis, or indirectly via the production of several cytokines that will influence cellular activity [29, 30]. Although a certain level of diversity is observed, the larger part of regulatory T-cells show a distinct $CD25^+CD4^+$ phenotype and the expression of the transcription factor Foxp3 [31]. Regulatory T-cells are naturally anergic and comprise 5-10% of the total peripheral $CD4^+$ T-cell population. Different subsets of regulatory T-cells have currently been identified and some are characterized by the predominant production of high amounts of TGF-β or IL-10. Regulatory T-cell-derived TGF-β reduced or completely prevented damaging Th-1 mediated responses in either infection or autoimmune disease [32-34].

TCR-$\alpha\beta$ $CD4^-CD8^-NK1.1^+$ natural killer (NK) T-cells represent a specific subset of T-cells that is present in most peripheral tissues. A subset of these NKT-cells have a strong capacity to produce IL-4 and constitutively express Fas-ligand (Fas-L), enabling

them to recognize and induce apoptosis of autoreactive T-cells that express the death receptor Fas. Importantly, peripheral NOD $CD4^+$ and $CD8^+$ T-cells appear resistant to apoptosis due to a reduction in the expression levels of caspase 8 [35]. This additionally contributes to faulty tolerance regulation and possibly IDDM.

The precise role of regulatory T-cells in the control of inflammation and autoimmune disease is not exactly known. A reduction in $CD25^+CD4^+$ T-cell number or function can lead to the spontaneous onset of multiple autoimmune disorders [36]. Importantly, NKT-cells of T1D patients [37] and of NOD mice are functionally deficient and present in reduced numbers in the NOD thymus [38, 39] Alpha-galactosylceramide-mediated activation of NKT cells in NOD mice resulted in the suppression of B- and T-lymphocyte-mediated IDDM and protected against diabetes development [40]. The dramatic rise in the percentage of peripheral $CD4^+CD25^+$ T-cells upon systemic IL-10 administration also provided protection against diabetes in NOD mice [41]. Several other studies additionally imply that the shortage in number of regulatory T-cells in BB-rats and NOD mice contributes to their permissiveness to develop diabetes [42, 43]. Whether this is an intrinsic problem of constitutively low numbers of regulatory cells or related to a failing competition with the devastating magnitude of autoimmune activation is currently not clear.

1.2.1.3 Is the autoimmune process underlying IDDM organ-specific?

The first step in the autoimmune process of T1D is the activation of autoreactive T-cells following presentation of self-antigen by professional APC. It is still questionable whether IDDM sets off due to the initial recognition of islet and/or β-cell-specific autoantigens, since autoreactive antibodies and T-cells with other antigen specificities were isolated in diabetic individuals or spontaneous animal models for IDDM. Although recognition of islet-associated antigens, like insulin or GAD, appear to be predominant, not all recognized antigens are pancreas-specific and vary from nerve-associated antigens such as peripherin, and intracellular stress-proteins like heat shock protein-60 [44, 45]. This raised the question whether the underlying cause of autoimmunity in IDDM is indeed pancreas-specific.

Diabetic patients often suffer from multiple autoimmune disorders. Autoimmune thyroiditis and sialoadenitis are frequently diagnosed in T1D patients and are also observed in NOD mice and BB-rats [46-48]. Since defective immune regulation is an important aspect associated with IDDM, it is not surprising that other autoimmune disorders develop as well. Thus, it cannot entirely be excluded that IDDM is solely caused by general defects in immune regulation. Beta-cells are extremely sensitive to activation-induced cell death and do not easily regenerate [49]. This makes them easy victims of cytotoxic mediators, even when they were not the initial target. T-cell-mediated damage in the pancreas is restricted particularly to the β-cells, leaving disorganized islets devoid of β-cells. The autoreactive response does not appear to spread to other islet-cells that might share identical antigens with β-cells. However, peripheral activation of autoreactive cells might affect organs distant of the pancreas. Splenocytes of 6 months old NOD mice that were deprived of β-cells at 3 wks of age, were no longer capable of transferring diabetes to naïve NOD mice. Therefore, the presence of autoantigen is

required for the initiation and/or maintenance of β-cell-specific autoreactive T-cells [50]. However, these β-cell-deprived mice did develop sialoadenitis, suggesting that other autoimmune phenomena are regulated independently from the progression of IDDM in NOD mice, but may be initiated secondary to the initial insult in the pancreas.

1.2.2 Progression towards diabetes II: the role of cytokines

Cytokines have profound effects on immune-mediated processes, being an essential mode of communication used by the immune system. Despite extensive research, their exact role in the initiation and progression towards diabetes remains unclear. The broad repertoire of cytokines has been subdivided based on their response-related appearance in cytokines involved in a T-helper-1 (Th-1) response, a Th-2 response, or a Th-0/Th-3 response. The latter response rather denotes the phenotype of cells that produce high amounts of TGF-β, like the regulatory T-cells discussed above. The primarily macrophage-derived cytokines IL-1α, IL-1β, tumor necrosis factor-α (TNF-α) and interferon-α (IFN-α), form a distinct subgroup and are best described as pro-inflammatory cytokines. They are rapidly secreted following a damaging insult and often lead to the onset of Th-1-mediated immune reactivity. Typical Th1 responses are associated with cell-mediated immunity and usually involve cytotoxicity and inflammation mediated by T-cells, NK-cells and macrophages. Th-1 reactivity is further believed to stimulate anti-viral immunity and is regarded as the main type of response involved in organ-specific autoimmune diseases like T1D, autoimmune thyroiditis and Sjogren's Syndrome [51-53].

Th-2 responses involve humoral immunity and confer protection against parasitic infections. Th-2 responses are in general characterized by the activation and degranulation of mast cells and activation of eosinophilic granulocytes and B-lymphocytes, accompanied by the production of immunoglobulin-E antibodies. Th-2-type responses are believed to downregulate Th-1 responses and to protect against the development of cell-mediated immunity. Although possibly beneficial with regard to Th-1 mediated autoimmunity, unbalanced Th-2 immune reactivity may also lead to autoimmune disorders, such as allergic asthma, atopic eczema or systemic lupus erythematosus (SLE) [54-56].

1.2.2.1 Different cytokines with variable effects on IDDM

T1D is considered a Th-1 mediated disease, related to the type of immune cells that infiltrate the pancreatic islets. Cytokines may play a crucial role in the induction and progression of the pathogenic process. Typical Th1 cytokines were expected to accelerate disease, while Th2 cytokines were studied for their capacity to improve or prevent T1D. The role of cytokines in T1D development is still incompletely understood given the diverse outcome of these studies.

IL-12 is regarded a typical Th-1 cytokine. Systemic administration of IL-12 accelerated the progression of diabetes in NOD mice, while treatment with an IL-12 antagonist prevented the onset of disease [57, 58]. IL-4, IL-10 and TGF-β are typical Th2-cytokines, known for their capacity to downregulate Th-1 responses. Acting as such, IL-4 or TGF-β, transgenically expressed in the pancreatic islets of NOD mice prevented

the development of diabetes [59-60]. By contrast, islet-specific overexpression of IL-10 accelerated diabetes in NOD mice, whereas systemically delivered IL-10 did prevent the development of disease [61, 62]. Furthermore, systemic administration of the Th1 cytokine IFNγ, or proinflammatory cytokine TNF-α, reduced the incidence of diabetes and the destruction of β-cells in NOD mice, as did treatment with low levels of IL-1 [63, 64]. Additional complexity of cytokine function is shown by the redundancy of above-mentioned cytokines in the unaffected progression of diabetes in NOD mice that lack functional IL-12, IFNγ, IL-4 or IL-10 [65-68].

Thus, whether autoimmunity is suppressed by certain cytokines or accelerated by others depends on the capacity of the immune system to retain a homeostatic balance, preventing a shift towards a typical Th1-response or an evident Th2-response. The mode of action of cytokines and their capacity to steer the autoimmune process leading to T1D is both time and place dependent. This is clearly exemplified by the pro-inflammatory effect of TNF-α which accelerates diabetes when given in the early phases of the immune response, while late administration of this cytokine protects NOD mice from developing IDDM [64, 69]. The discrepancy in IL-10 functionality appeared to be place-dependent. Locally expressed IL-10 dramatically increased the expression of intracellular adhesion molecule-1 (ICAM-1) in the islets [70, 71]. Anti-ICAM-1 treatment in combination with monoclonal antibodies (mAbs) against its counterreceptor lymphocyte function-associated antigen-1 (LFA-1), abolished the CD8+ T-cell mediated lysis of β-cells *in vitro* and *in vivo*, and significantly prevented diabetes of NOD mice [72].

1.2.3. Progression towards diabetes III: selectin, integrin and chemokine receptors regulate leukocyte infiltration

Before lymphocytes can destroy the β-cells, they need to access the pancreas and extravasate from the circulation that they have entered after their initial priming in the lymph nodes. Endothelial transmigration of circulating leukocytes to enter peripheral tissues firstly requires movement arrest and tight adhesion to the endothelium. Leukocyte rolling and arrest is initiated by selectin-mediated binding to specific endothelial receptors (addressins) followed by integrin-regulated adherence (figure 1.1). Firm adhesion is initiated by the binding of chemokine receptors to specific chemokines that are bound to negatively charged proteoglycans on the endothelial surface. Circulating leukocytes are only triggered to extravasate the blood stream if signaled by chemokine gradients displayed locally. Cell trafficking to inflammatory sites will thus be determined by the selective expression pattern of selectin-, integrin- and chemokine-receptors on leukocytes and differential expression of chemokines within the target tissue. Their unique combination will permit the specific recruitment of leukocyte subsets to the signaling tissue.

1.2.3.1 The chemokines IP10, CCL2, CCL3 and CCL5 are involved in the late recruitment of leukocytes to the inflamed pancreas

More than 15 chemokine receptors and over 50 chemokines have been identified thusfar [73]. Several of these chemokine-chemokine receptor pairs appear to be involved in the progression towards T1D.

The CXC-chemokine receptor-3 (CXCR3) is expressed on Th1 T-cells and recognizes the chemokine interferon-inducible protein-10 (IP-10). IP-10 is constitutively expressed in pancreatic nerve-tissue of both control and NOD mice [74], and an age-related increase in the expression of IP-10 has been observed in NOD islets [75] that possibly correlates to the ongoing inflammatory process. Moreover, IP10 expression was induced in islets when exposed to a mixture of IL-1β, TNFα and IFNγ *in vitro* [76]. Importantly, mice deficient for CXCR3 show a delayed onset of diabetes [77] indicating the involvement of IP10-CXCR3 interaction in IDDM.

CCL2, also known as macrophage chemoattractant protein-1 (MCP-1), is recognized by the common corresponding receptor-2 (CCR2) expressed on monocytes and Th1 T-cells. Transgenically-induced islet-specific expression of CCL2 resulted in chronic insulitis of mainly F4/80$^+$ macrophages, but the sole expression of islet CCL2 was not sufficient to induce diabetes in these transgenic mice [78]. This study suggests that CCL2 can be involved in the pancreas-specific recruitment of leukocytes. Interestigly, NOD islets show an age-related expression of CCL2 with maximal expression levels at 8 wks of age [79] i.e. when the lymphocytic infiltrates have accumulated in the pancreas. Furthermore, CCL3 (macrophage inflammatory protein-1α (MIP-1α)) might be involved in T1D pathology in NOD mice as well, since NOD mice deficient in CCL3 show a reduced incidence of destructive insulitis [80].

The chemokine receptor CCR5 is expressed by monocytes, macrophages, immature DC and also by Th1 T-cells. Treatment with a neutralizing antibody against CCL5 (RANTES, the ligand for CCR5), did not alter early insulitis, but did prevent β-cell destruction and diabetes in NOD mice [81]. Thus like CCL3, CCL5 is most probably involved in the intra-islet invasion and β-cell destruction rather than the early recruitment of leukocytes to the pancreas.

1.2.3.2 Leukocytes infiltrate the pancreas via normal endothelium or HEV-like vessels

Due to the chronic exposure to inflammatory mediators, the endothelium of the pancreas changes its morphology into structures that resemble high endothelial venules (HEV) [82]. HEV are distinct from normal endothelium by their cuboidal appearance and the expression of specific vascular addressins that further facilitate leukocyte binding to the endothelial surface. Under normal conditions HEVs are particularly found in lymphocyte-enriched areas such as secondary lymphoid organs (with the exception of the spleen). Furthermore, structures resembling HEVs are often observed in peripheral tissues during chronic inflammation. The HEV-specific addressins MAdCAM-1 or PNAd, were detected in the islets and/or pancreas after the infiltration of lymphocytes [82, 83] and also the expression of ICAM-1 was observed exclusively in inflamed islets. In addition to the expression of MAdCAM-1 and ICAM-1 in the inflamed islets, the expression of the receptors L-selectin, α4β7 and LFA-1 were detected on lymphocytes within the islet-infiltrates. Importantly, the treatment of young or adult NOD mice with mAbs against α4β7, LFA-1 or MAdCAM-1, preventing their receptor-counterreceptor interaction, resulted in longstanding protection against diabetes [84, 85], underlining the importance of these adhesion pathways in the recruitment of leukocytes to the pancreas.

Additionally, the interference of very late antigen-4 (VLA-4)-mediated interaction also prevented diabetes in NOD mice [86]. VLA-4 ($\alpha4\beta1$) is an integrin that recognizes the matrix protein fibronectin (FN) in addition to recognition of the endothelial adhesion molecule, vascular adhesion molecule-1 (VCAM-1) [87, 88]. This adhesion molecule is expressed by endothelium and its expression is increased by inflammation.

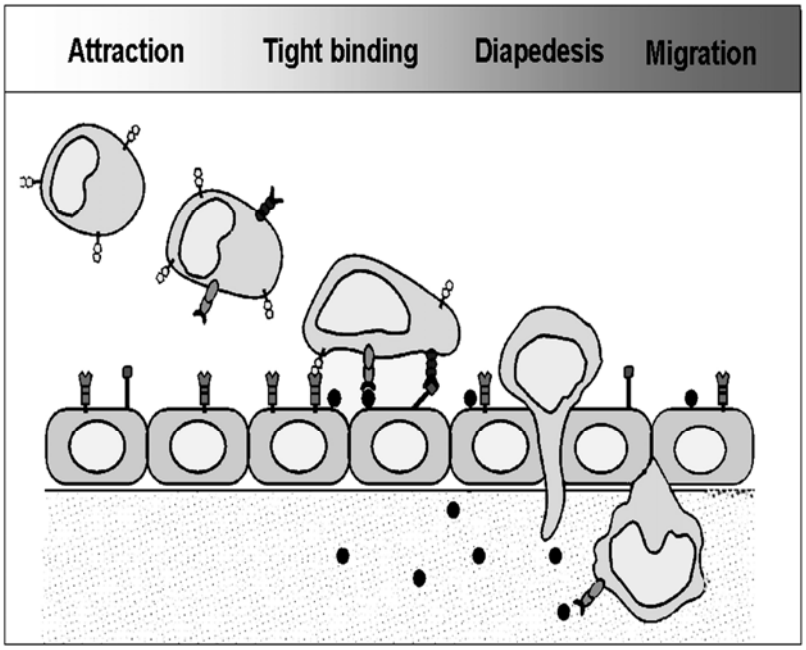

Figure 1.1
Peripheral blood leukocytes are attracted by chemokines released by the endothelium and inflamed tissue. The initial rolling and attachment is regulated by binding of selectins (orange) expressed by the endothelium. This binding is strengthened by the binding of leukocyte adhesion molecules (red) like LFA-1 or CD49d to ligands on the endothelial surface and enforced by the binding of chemokines (blue) with chemokine receptors (green). The tight binding will allow leukocytes to transmigrate through the endothelium (diapedesis) and migrate through the underlying basement membrane and matrix towards the chemokine gradient [89].

1.2.4 Progression towards diabetes: Leukocytes mediate the final destructive phase

The early phases of immune infiltration of the pancreas progress asymptomatically and the immune response has already reached its final destructive phase at clinical onset: over 80% of the islets have been destroyed when exogenous insulin treatment is required. Most of the destructed islets are devoid of insulin-producing β-cells and merely consist of glucagon- and somatostatin-producing cells [90, 92]. In man, the remaining insulin-containing islets show infiltrates of mononuclear cells that are of a comparable composition as the immune cell infiltrates in islets of NOD mice and BB-rats [93-95].

1.2.4.1 Immune cells induce apoptosis in β-cells

Programmed cell death (apoptosis), rather than necrosis was identified as the main form of immune-mediated β-cell death [96, 97]. Toxic mediators or cell-cell contact-dependent mechanisms activate intracellular death-pathways in the β-cell. Following prior stimulation of naïve T-cells, most probably in the peripheral lymph nodes, $CD4^+$ and $CD8^+$ T-cells will destroy the β-cells upon re-encounter with β-cell antigens in the pancreas. Cytotoxic $CD8^+$ T-cells infiltrate the pancreas and can kill β-cells upon recognition of the corresponding peptide-MHC class I complex. Insertion of the tubular perforin in the β-cell-membranes and subsequent release of lytic enzymes or downstream activation of Fas via Fas/FasL interaction both result in apoptosis of β-cells.

However, puzzling results have been found in studies examining the role of Fas/FasL activation in T1D, since β-cells themselves do not constitutively express Fas [98]. Yet, Fas expression can be induced in β-cells via stimulation with IL-1 [99]. Moreover, as a consequence of β-cell destruction, blood glucose levels will rise, exposing the remaining β-cells to high glucose concentrations. These high glucose levels will subsequently induce the production of IL-1β in the β-cells, followed by endogenous expression of Fas and finally apoptosis [100]. Interestingly, NOD mice carrying a defective mutated form of Fas do not develop diabetes [101], similar to NOD mice that carry a mutated form of FasL [102]. If the protection against IDDM in these mice is ascribed to a reduction in Fas/FasL-mediated β-cell lysis, then the induction of Fas expression in β-cells must precede Fas-mediated β-cell destruction. The early pancreatic infiltration and activation of leukocytes might be a possible trigger for Fas expression in β-cells in accordance to the strict correlation of Fas expression in β-cells with the presence of insulitis in T1D patients [103]. However, one has to take the contribution of Fas-mediated apoptosis into account or that of other Fas expressing cells involved in the process of T1D, like autoreactive T-cells or per-islet neuronal tissue [74]. Islets are heavily innervated and the islets' endocrine function is under strict neuronal regulation. Noteworthy, it was shown very recently that the destruction of glial fibrillary acidic protein-positive peri-islet Schwann cells preceded β-cell death in NOD mice [104]. Thus, the early apoptosis of pancreatic neuronal tissue might be of particular interest considering the neuronal regulation of the islets endocrine function.

Since β-cells do not readily express MHC class II molecules, reactivation of $CD4^+$ T-cells will depend on the presence of APC such as DC and macrophages. Activation can result in the release of nitric oxide (NO) or various cytokines like IL-6, IL-1β and TNF-α, either directly by the T-cell, or via the activation of macrophages that are in close vicinity. Of these inflammatory mediators NO, TNF-α and IL-1β were shown to be directly toxic for β-cells [105-107]. Correspondingly, staining patterns of TNF-α in the NOD pancreas strongly resembled the distribution of DC and macrophages at the first stage of insulitis [108]. Activated macrophages additionally secrete IL-12 that stimulates $CD4^+$ T cells to secrete IFN-γ and IL-2. IFN-γ in turn, activates other macrophages to release more IL-1β, TNF-α, and free radicals [109]. Macrophage-derived cytokines that are locally produced in the pancreas will, in addition to their cytotoxicity, increase the infiltration of lymphocytes by endothelial activation, attracting more effector cells to the site of inflammation [110]. In support of the β-cell cytotoxicity via locally released cytokines, it was shown very recently that a reduced incidence of diabetes in NOD mice correlated to the expression of suppressor of cytokine signalling-1 (SOCS-1) in β-cells. SOCS-1 suppressed the phosphorylation of cytokine-induced signal transducer and activator of transcription (STAT) in β-cells [111], reducing cytokine-mediated activation.

1.3 Autoreactive T-cell priming and the process preceding

1.3.1 Insulitis: identification of step 1?

Although insulitis, i.e. the islet infiltration of T and B-lymphocytes, is the first pancreas-specific damage mediated by the adaptive immune system, it merely reflects the re-encounter of autoreactive effector T-cells with their antigens after initial priming. Therefore, the actual initiation of islet autoreactivity takes place prior to pancreatic insulitis. Since naïve T-cells normally do not traffic through peripheral tissues, the priming and first encounter with their antigen probably takes place in the tissue-draining lymph-nodes. Here antigen will be presented by professional APC in the appropriate environment to prime naïve T-cells.

1.3.1.1 Antigen presentation takes place in the pancreas-draining lymph nodes in a restricted time-window

Peripheral tissue APC will mature and subsequently migrate to the tissue draining lymph nodes as a result of pathogenic invasion or influenced by inflammatory stimuli. APC that have sampled tissue-specific antigen will also migrate to the draining lymph nodes under steady state conditions, although at lower levels [112]. Local induction of inflammation in the pancreas will be reflected best in the pancreas-draining lymph nodes (PLNs). T-cells of prediabetic NOD mice that had acquired an activated phenotype were relocated in the PLNs upon transfer into recipient animals [113]. In transgenic BDC2.5-TCR NOD congenic mice proliferation of transferred T-cells was also observed in the PLNs. This proliferation, however, was dependent on the age of the recipients and only observed in recipients older than 10 days. Proliferating T-cells were no longer observed when cells were transferred after the age of 3 weeks old [114].

A restricted time-window for the initiation of autoreactivity became evident from experiments performed by Gagnerault *et.al*. [115]. Surgical removal of the PLNs at 3 weeks of age protected NOD mice from lymphocytic insulitis and IDDM. This protection was incomplete when PLNs were excised at 4 wks of age and removal of PLNs at 10 wks of age or the removal of the spleen in young or adult mice was not protective at all. This strongly indicates that the initial priming does take place in the PLNs at 2-3 wks of age, when insulitis is not yet observed.

1.3.2 Macrophages and DC accumulate around islets prior to lymphocytic insulitis

The process preceding insulitis can only be examined in pancreata of animals that spontaneously develop IDDM. In both NOD mice as well as in BB-rats features of tissue-specific inflammation are first noted in the pancreas as compared to other tissues. The first pancreatic accumulations of leukocytes in NOD mice are seen in the peri-vascular areas, as early as 3 wks of age (denoted stage I) [116]. These early infiltrates in majority exist of cells that belong to the non-specific innate immune system. Acid phosphatase negative, sialoadhesin$^+$ (MOMA-1$^+$) and MMGL$^+$ (ER-MP23$^+$) macrophage/DC-like APC rapidly surround the islet-periphery, while lymphocytes were

still absent. Acid phosphatase positive, F4/80$^+$ (BM8$^+$) macrophages and CD11c$^+$ DC accompanied the sialoadhesin$^+$ and MMGL$^+$ macrophages (stage II). When lymphocytes enter the pancreas, around 7-10 wks of age, F4/80$^+$ macrophages and CD11$^+$ DC are observed in intra-islet-infiltrates (stage III). Strong peri-islet accumulations of lymphocytes with various subsets of macrophages and DC were observed completely surrounding the islets at approximately 15-17 wks of age (stage IV). Most of the islets were massively infiltrated by T- and B-lymphocytes, CD11c$^+$ DC and BM8$^+$ and ER-MP23$^+$ macrophages around 17-22 wks of age (stage V) that rapidly led to complete destruction of the islets (stage VI).

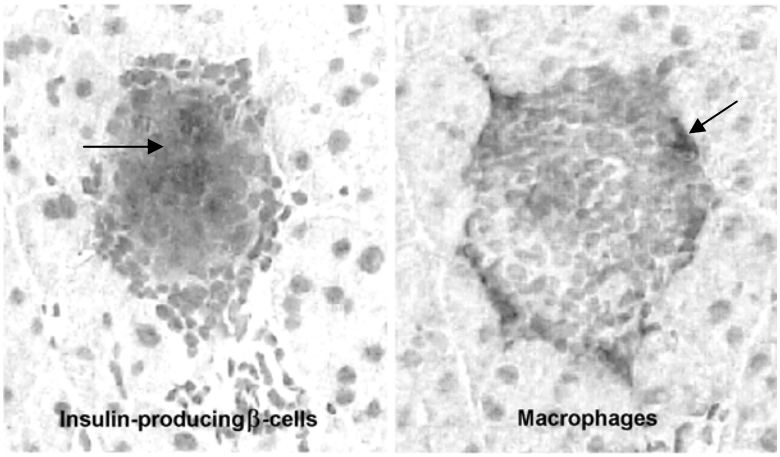

Figure 1.2
Macrophages accumulate at the periphery of the insulin-producing islets, before other signs of inflammation are evident. Original magnification 400x.

1.3.3 Macrophages and DC are involved in the initiation and progression of IDDM

Bone marrow (BM)-derived monocytes are the blood-born precursors of macrophages and of several subtypes of DC. These myeloid macrophages and DC belong to the mononuclear phagocyte system and comprise a diverse population of cells. Macrophages are generally characterized by a large horseshoe-shaped nucleus, prominent rough surfaced endoplasmic reticulum, and large numbers of mitochondria and cytoplasmic vacuoles. Their considerable heterogeneity is simplified by the subset classification based on their function, localization or phenotypic appearance (table 1). Macrophages are highly phagocytic and potential producers of a wide-range of cytokines and growth factors. Macrophages play a major role in T1D pathogenesis. The injection of macrophages that display a typical Th-1 profile severely aggravated diabetes in NOD mice [117]. The preferential killing of macrophages via treatment with toxic silica in very young NOD mice, i.e. before effector T-cells were activated, protected against diabetes [118]. Such protection was also obtained by macrophage depletion in BB-rats [119] Furthermore, blocking CD11b-mediated adhesion of myeloid leukocytes in an experimentally accelerated T-cell transfer model of diabetes, prevented the intra-islet infiltration of macrophages and subsequently diabetes in NOD mice [120].

DC are regarded as a separate subset of APC and can be myeloid-derived like macrophages, [121] or generated from lymphoid-committed precursors [122]. The DC compartment comprises Langherhans cells in epithelial tissues, interdigitating cells in lymphoid organs, veiled cells in the lymph and interstitial DC in connective tissues of non-lymphoid organs [123]. DC have a broad overlap with macrophages regarding their

function, surface marker expression and distribution in lymphoid tissues (see table 1). Their appearance is comparable to that of macrophages and morphologically they are difficult to distinguish. However, compared to macrophages, DC show a marked increase in MHC II and adhesion molecule expression upon stimulation. Therefore, they are regarded as more efficient APC, profoundly activating naïve T-cells. In addition to the increased expression of MHC class II molecules, DC maturation also leads to the enhanced numbers of fine cytoplasmic protrusions and increased cell mobility. Mature DC are highly motile, which enables them to migrate to the draining lymph nodes to present antigen to naïve T-cells upon activation.

Considering their unique capacity to initiate specific immune responses, the DC compartment of NOD mice has been extensively studied. Surprisingly, instead of displaying a highly mature phenotype, both splenic and BM-derived $CD11c^+$ NOD DC have defective MHC class II expression and low expression of CD80 costimulatory molecules, even upon strong stimulation with lipopolysaccharide (LPS). [124, 125]. This suggests that NOD DC are poor T-cell stimulators and not well-equiped to readily induce T-cell activation. Accordingly, NOD APC were shown deficient in the induction of T-cell proliferation [126]. Importantly, decreased maturation and efficiency in antigen presentation of blood monocytes was observed in T1D patients as well [127]. By contrast, NOD DC exhibit elevated levels of the transcription factor (TF) nuclear factor kappaB (NF-κB). This NF-κB-hyperresponsiveness is associated with enhanced production of IL-12p70 [128-130] and may promote the induction of a Th1-response.

The functional involvement of the aberrant NOD DC in T1D development has been studied mainly via DC transfer studies. Interestingly, the transfer of NOD-derived DC that were manipulated to express a reduced activity of NF-κB [131] or of phenotypically “normal” mature NOD-DC that were obtained by using specific culture conditions [132-134], protected euglycemic NOD mice from developing diabetes. This DC-mediated protection was associated with reduced insulitis and increased levels of IL-4 and IL-10 in the spleen or in the islets of treated mice, suggesting a shift towards a Th2-profile. Furthermore, Shinomiya et al. [135] used IFNγ-stimulated DC to prevent diabetes and showed that the transferred DC had migrated into the pancreas and pLNS, suggesting that the anti-diabetogenic effects are exerted locally in the target-organ and its draining lymph nodes.

Next to DC, macrophages are possibly involved in the initiation of IDDM as well, since depletion of macrophages after the onset of insulitis was less effective than early depletion [136]. Early macrophage depletion decreased the level of macrophage-derived IL-12 and induced a shift in the immune balance towards a local suppressing Th2 environment associated with IL-4, IL-10 and TGF-β-1 expression. In this respect it is noteworthy that pancreatic macrophages with a phenotype similar to those observed in the early peri-islet accumulations, are already present in NOD and normal mice from birth on. These macrophages are randomly distributed in the connective tissue of the pancreas and probably represent resident macrophage populations. Their origins as well as their normal functions are, thus far, unknown. Interestingly, increased numbers of macrophages and DC were observed in the pancreas of NOD mice from birth up to 3 weeks of life, as compared to different control strains [137]. Although macrophages are

potentially capable of producing a wide array of different molecules, their actual functional state will largely depend on their state of maturation and differentiation and that will be directed by the local microenvironment. The ontogeny and normal function of pancreatic macrophages is unknown. It is possible that they arise from precursors that proliferate locally in the pancreas, similar to what has been described for Kupffer cells in the liver. Elucidating their origin will improve our insight in their trafficking pattern to the pancreas.

1.4 Macrophage ontogeny and differentiation

1.4.1 Hematopoietic progenitors give rise to macrophages and DC

Hematopoietic stem cells (HSC) are first observed in the murine yolk sac at embryonic age 7.5 (E7.5). These early hematopoietic cells can be discriminated from other cell types and endothelial precursors by the expression of the tyrosine kinase CD45, a general leukocyte-specific marker. HSC are direct descendents of multipotent stem cells that posses full capacity of lineage differentiation. HSC develop in parallel with the angioblasts that eventually will form the vasculature. Interestingly, endothelial cells and leukocytes are closely related and believed to arise from a common precursor called the hemangioblast.

The only mature hematopoietic cells that are present within the yolk sac are the primitive (or embryonic) erythrocytes and macrophages. The origin of the latter is uncertain and they may represent a distinct primitive population of macrophages as they show rapid maturation that bypass the normal stages of monocytic development [138, 139]. At this early point in ontogeny, no hematopoietic precursors or mature cells are found in the mouse embryo itself. HSC from the yolk sac seed the para-aortic splanchnopleura (P-Sp) at E8.5-E9.5 and the aorta-gonad-mesonephros (AGM) region at E10.5-E11.5, where they expand in parallel with a decline of hematopoiesis in the yolk sac at E10-E11 and prior to the re-localization of hematopoieis to the liver at E12 [140]. The maturation of the distinct hematopoietic lineages, however, does not take place in the yolk sac, but in the embryo itself.

During the course of maturation in the fetal liver, HSC obtain lineage commitment by the acquisition of selective sensitivity for specific growth factors that restricts their developmental potential to differentiation into either lymphocytic lineages (like B-, T or NK cells), or myeloid lineages (like granulocytes, and monocyte/macrophages) [85]. The restricted expression of various lineage specific transcription factors (TFs), like SLC, GATA-1, NF-E2, GATA-2, c-Myb, C/EBPa and PU.1 [141] was observed in lymphoid and myeloid progenitors. The kinetics of TF expression as well as their respective expression levels, determine which lineages eventually develop, influenced by the extracellullar microenvironment (a.o. cytokines) and subsequent signaling cascades activated. Interestingly, TF expression levels are controlled by the direct interaction between TFs that attenuate their mutual activity. For instance, GATA-1 inhibits PU.1-mediated transcription, whereas PU.1 in turn interferes with the DNA-binding activity of GATA-1 [142]. Additionally, physical interaction of the transcription factor CCAAT-enhancer binding protein C/EBPa with PU.1, inhibits the function of PU.1 and favors the development of granulocytes versus macrophages.

1.4.2 The expression levels of PU.1 dictate the differentiation of macrophages versus granulocytes and B-cells

Expression of the transcription factor PU.1, encoded by the Spi gene, appears particularly important to the development of macrophages. Loss of PU.1 expression results in embryonic lethality at E16-E18 and the total absence of both lymphoid and

myeloid cells in the fetal liver. PU.1-/- progenitors show proliferation, albeit reduced, in response to the general growth factors IL-3, IL-6 and stem cell factor (SCF) [143]. Importantly, they are completely unresponsive towards the myeloid specific growth factors granulocyte/macrophage-colony stimulating factor (GM-CSF), macrophage-CSF (M-CSF), and granulocyte-CSF (G-CSF), although responsiveness to the latter could be rescued by the addition of IL-3. Thus, PU.1 expression is not essential for the survival of lymphoid or granulocyte progenitors, but is required for their further differentiation. Interestingly, the enforced expression of PU.1 in these progenitors induced myeloid-committed development [144]. Moreover, the presence of high concentrations of PU.1 resulted in the differentiation of solely macrophages, while low PU.1 expression levels favor the differentiation of B-lymphocytes [145].

The expression of PU.1 does appear vitally important for the survival and differentiation of macrophage precursors and PU.1-/- mice do not possess mature F4/80$^+$ macrophages in tissues where F4/80$^+$ cells are normally present [146]. The promotor of the gene encoding the myeloid specific cytokine receptor (CSF-R), c-fms, contains several PU.1 binding sites, suggesting that PU.1 exerts its role via the regulation of expression of the receptor for the key macrophage growth factor M-CSF.

M-CSF is involved in the proliferation, differentiation and activation of cells of the monocyte/ macrophage lineage. Mice that carry a recessive mutation in the gene for CSF-1 (op/op mice) fail to produce M-CSF and completely lack various mature macrophage populations, while several other populations are present in reduced numbers. These populations probably represent M-CSF-independent macrophages derived from earlier macrophage-granulocyte committed progenitors [147-149]. Although PU.1-/- mice have been reported to lack c-fms gene transcription, others have demonstrated the presence of c-fms expressing cells in PU.1-/- embryos [150]. This suggests that PU.1 is not an absolute requirement for c-fms transcription. In consistence with these observations, Henkel and coauthors showed the presence of cells with monocytic characteristics in fetal and neonatal livers of PU.1-/- mice that committed to the monocytic lineage upon addition of GM-CSF *in vitro* [151]. Thus, similar to the presence of M-CSF-independent macrophages in the op/op mice, PU.1-/- independent macrophages exist as well, indicating that macrophage development is not exclusively regulated by M-CSF or PU.1.

Macrophages develop from monocytes that have differentiated from promonocytes preceded by a monoblastic stage of appearance (Figure 1.3). Each stage of cell differentiation is characterized by a distinct profile of cell surface receptors, according to the cell-specific needs at that stage, and regulated by the cellular microenvironment and growth factors like M-CSF. The Mab F4/80, an antibody that recognizes the G-protein coupled receptor EMR-1 [152], recognizes most mature macrophage populations. Several other markers have also been identified and are expressed on macrophages at different stages of maturation or by specific tissue resident populations (histiocytes) (table 1). In the mouse embryo, myeloid precursors are found among the first blood cells in the vascular lumen of the yolk sac, already early in ontogeny at E8 [153, 154]. At E9, F4/80$^+$ fetal macrophages are found in the mesenchymal layers of the yolk sac, but not yet in any of the peripheral tissues. When

the vitelline veins connect with the cardiovascular system, at E10, these macrophages leave the yolk sac and colonize the various embryonic tissues. Expanding macrophage populations were observed in the liver and mesenchyme at E12 [155, 156], in parallel with the initiation of hematopoiesis in the spleen at E15 and finally at E17 in the bone marrow, as bone marrow formation does not start before E16 [157] .

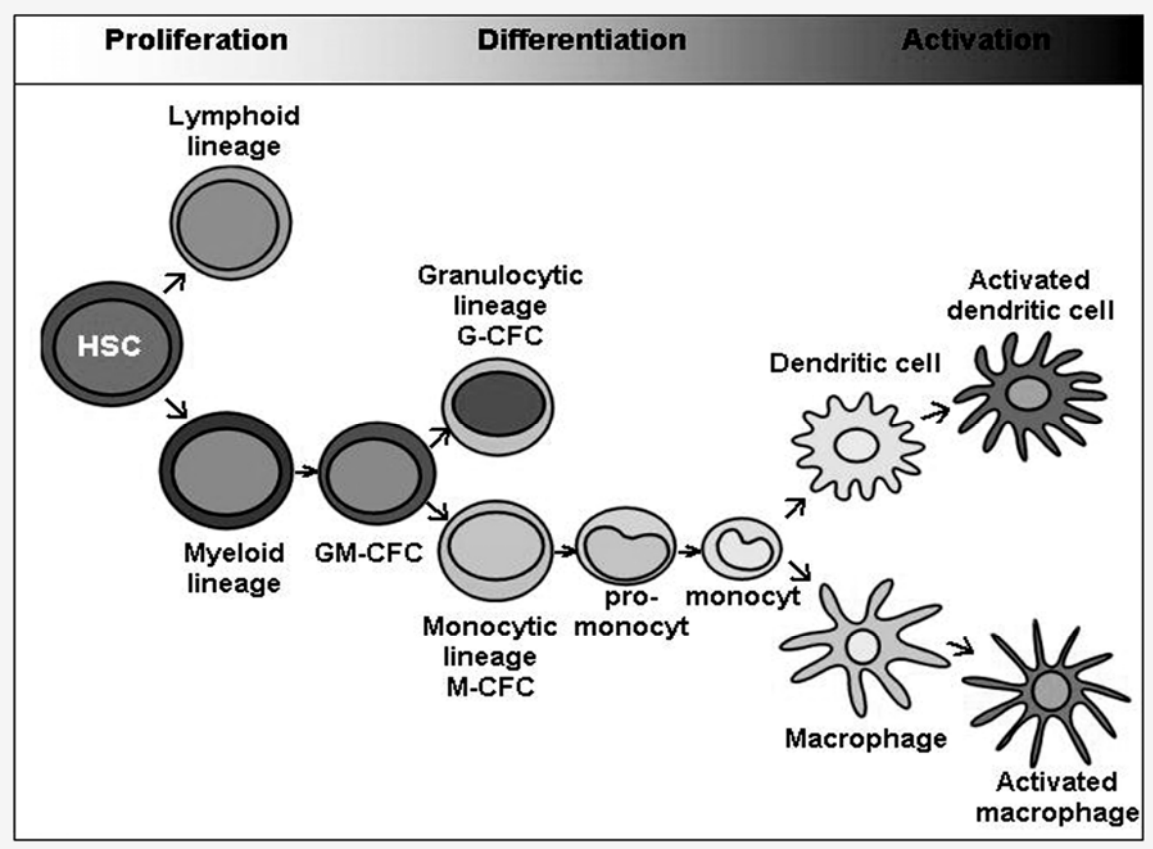

Figure 1.3
Hematopoietic stem cells (HSC) posess multipotential differentiation capacity and give rise to lymphoid and myeloid progenitors. The myeloid progenitors in turn differentiate into granulocyte-macrophage colony-forming cells (GM-CFC) that have the capacity to develop into either granulocyte precursors (G-CFC) or macrophage precursors (M-CFC), the latter is represented by the monocytic lineage. Monoblasts retain the capacity of proliferation and lineage-specific differentiation. They will differentiate into pro-monocytes, followed by the differentiation into monocytes. Monocytes have lost the capacity of proliferation and will either differentiate into dendritic cells or macrophages. Like monocytes, dendritic cells and macrophages do not proliferate. They are in the last stage of differentiation and will adapt to the micro-environmental needs by the activation of their final maturation. Fully matured macrophages and dendritic cells will be able to perform various specialized functions, depending on the stimulus received.

In the adult mouse, myeloid cells are replenished by hematopoietic stem cells in the bone-marrow. Multipotent hematopoietic stem cells give rise to bipotent granulocytic/macrophage colony forming cells (GM-CFC) that further differentiate into granulocyte-colony-forming-cells (G-CFC) that become granulocytes, and macrophage-colony-forming-cells (M-CFC) that become monocytes. The monocytes finally mature to either macrophages or DC. BM precursors committed to the myeloid lineage can be separated from other progenitors by their expression-pattern of PECAM-1 (CD31) and Ly-6C. The earliest M-CSF and GM-CSF-responsive cells are $CD31^{+}Ly6C^{-}$ and still contain progenitors from all hematopoietic lineages (figure 1.3). The myeloid-committed progenitors are within this population, and are recognized by the mAb ER-MP58 [158].

BM-derived monocytes are blood-born precursors of macrophages and DC, but the ontogeny of tissue resident macrophages is poorly understood. Some tissue resident macrophage populations have been reported to arise independently from circulating monocytes [159]. Fetal macrophages are possibly responsible for the first seeding of resident macrophage-subsets, like Kupffer cells in the liver, microglia in the central nervous system and alveolar macrophages in the lungs [160-162]. Under steady state conditions, the trafficking of resident macrophages will be restricted, in contrast to the motile exudate macrophages that descend from blood monocytes, infiltrating the peripheral tissues in response to an acute or chronic inflammatory stimulus. It is uncertain whether the different tissue macrophage populations are replenished by the blood monocyte or by BM-independent precursor pools that proliferate locally. Both processes will probably proceed in common [163, 164]. The ontogeny of pancreatic macrophages is unknown. In humans the presence of macrophages and that of T- and B-lymphocytes has been described in the embryonic pancreas, found randomly scattered within the exocrine and connective tissue [165]. Unfortunately, the presence of macrophages in the fetal mouse pancreas has not been studied yet.

Table 1. Monoclonal antibodies recognizing antigens of mouse macrophages and DC.

mAb	Antigen	Ag description	Tissue localization	Reference
Immature macrophages:				
ER-MP12	CD31 (PECAM-1)	single chain glycoprotein	subpopulation of myeloid precursor cells and endothelial cells, low on lymphocytes	1
ER-MP20	Ly6C	glycoprotein	macrophage precursors (late CFU-M), monocytes and endothelial cells	1, 2
ER-MP58	unidentified		myeloid precursors	3
Mature macrophage markers:				
F4/80	EMR1	glycoprotein	mature macrophages, low on monocytes, splenic DC and eosinophils	4
BM8	*see F4/80*			
M1/70	CD11b (Mac-1α)	C3bi integrin, CR3	myeloid cells	5
ER-BMDM1	CD13	Aminopeptidase N	macrophages. interdigitating cells and DC	6
Macrophage subset-specific markers:				
ER-HR-3	unidentified	two distinct single chain glycoproteins	macrophages related to hematopoietic islands	7
ER-TR9	mSIGN-R		splenic marginal zone macrophages	8
M1/22.25	Forssman Ag	Glycosphingolipid	subset of macrophages in lymphoid organs	9
Monts-4	unidentified	Glycoprotein	resident tissue macrophages	10
SER-4	SER, sialoadhesin	Sialylated glyco-conjugate R		11
MOMA-1	sialoadhesin		metallophilic macrophages tissue macrophages and Kupffer cells	12
MOMA-2				13
ER-MP23	MMGL	galactose-specific lectin	histiocyte-like macrophages	14
Dendritic cell-specific markers:				
N418	CD11c	Ax integrin	DC, low on monocytes	15
NLDC 145	DEC 205	glycoprotein	High on DC, veiled cells, Langerhans cells, Ia positive Interdigitating cells and thymic epithelial cells	16

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1.4.3 Macrophage differentiation: macrophages need a matrix

The final phenotype and function of fully differentiated macrophages will be strongly influenced by their local microenvironment. Properly directed macrophage differentiation will enable them to adapt functionally to the tissue-specific needs. Interactions of macrophages with the extracellular matrix (ECM) therefore play a critical role in macrophage function and differentiation. For example, macrophage phagocytosis was enhanced by binding to collagen [166], while the lack of contact with ECM components may even induce macrophage apoptosis [167].

1.4.3.1 ECM drives monocyte differentiation

ECM plays a significant role in the cross talk of immune cells with the tissue. ECM forms a physical barrier and acts as a selective filter to soluble molecules. It also forms a local store of different growth factors that are bound to distinctive ECM proteins, like TGF-β-1 that is bound to decorin and released via diffusion of (macrophage-mediated) proteoglycan proteolysis. Monocytes, macrophages and DC all adhere to the ECM by the expression of specific sets of adhesion molecules (integrins) and are simultaneously attracted by the growth factors that are released from the ECM. Intriguingly, the sequential interaction of monocytes with different ECM components and other complexed molecules drives their differentiation into either macrophages or DC [168]. Macrophage and DC precursors will first *proliferate*, then *mature* and simultaneously *differentiate* (see also figure 1.3). Maturation and differentiation thus broadly overlap and are characterized by the irreversible loss and gain of cell type specific-features like the expression of specific cell surface receptors. Interestingly, integrin expression is strictly regulated during macrophage and DC maturation.

Integrins belong to a large family of heterodimeric membrane receptors comprised of α- and β-subunits. Integrins specifically recognize ECM components or other cell surface receptors as counter ligands, and are divided in subgroups based on a β-subunit that polymerizes with different α-subunits. Additional complexity of integrins is provided by the mRNA splice variants for the cytoplasmic domains of the α- and β-subunits. Monocytes and mature macrophages have been reported to constitutively express the β-integrin chains β1 (CD29) and β2 (CD18) [169, 170] that form, among others, the integrins α4β1 (CD49d/CD29), α5β1 (CD49e/CD29) and α6β1 (CD49f/CD29) respectively LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18). The integrin αxβ2 (CD11c/CD18) is mainly detected on mature DC.

The maturation of BM-precursors into macrophages is stimulated by M-CSF and accompanied by an increase in the expression of the α-integrins CD49d and CD49e. This additionally enhances their capacity to interact with the Arg-Gly-Asp (RGD) and CS1 sequences of FN respectively [171]. CD49d was correspondingly identified as a maturation marker for human monocyte-derived DC [172]. Importantly, the developmentally regulated adaptation of the integrin expression profile will allow monocyte extravasation through the endothelium or facilitate the tissue trafficking of resident macrophages and thus determine their mobility and tissue localization. Additionally, macrophages can create new integrin binding sites in their direct

surroundings by the release of ECM-degrading agents, facilitating their own migration [173, 174].

1.4.3.2 ECM engagement controls cellular activation in addition to the regulation of cell mobility

Activation of macrophages and DC can induce specific functions in these cells or boost functions acquired earlier. Integrin-mediated adhesion of macrophages and DC to ECM components activates different signal transduction pathways leading to the phosphorylation of a diversity of signaling molecules downstream of the intracellular part of the integrin (outside-in-signaling) that can result in the expression of various proteins. For instance, CD49e-mediated interaction of peritoneal macrophages to FN resulted in the specific release of IL-6 [175, 176]. The adherence of BM-precursors to FN, laminin, collagen I and collagen IV induced the secretion of both IL-6 and TNF-α in a dose-dependent manner [177]. Furthermore, leukocytes responded exclusively to TNF-α when adherent to matrix components, but not when they adhere to uncoated plastic surfaces [178]. Additionally, it was reported recently that the engagement of monocytes with FN resulted in the activation of a wide array of different genes ranging from transcription factors relevant for myeloid development (a.o. PU.1, STAT5a, IRF-1), molecules involved in the JAK/STAT signaling pathway or NF-κB signaling cascade (a.o. IRAK, MyD88) and also that of a variety of cytokines (IL-1β, IL-8, TNF-α) and chemokines (a.o. CCL2, CCL3 and CCL5) [179]. Thus, macrophages are not solely attracted to chemokines, but also capable of producing them while in contact with the ECM. Interestingly, not only chemokines, but also increasing gradients of tissue-complexed ECM proteins show chemotactic activity (haptotaxis) for leukocytes [180]. Hence, ECM coordinates APC differentiation, migration and also the magnitude and specificity of APC activation.

Higher numbers of NOD macrophages and DC were observed in the NOD pancreas from birth till weaning, i.e. when the second stage of (postnatal) pancreas development takes place in rodents [137]. Considering the atypical peri-islet accumulation of resident macrophages in NOD mice, these macrophages possibly receive attracting stimuli from the islets or structures in the islet-vicinity. Macrophages are involved in tissue morphogenesis in various tissues, indicating a certain level of direct cross-talk with their microenvironment, even under non-inflammatory conditions. The processes that take place during fetal and early postnatal pancreas development might give insight in the causes of the peri-islet accumulation of NOD macrophages.

1.5 The target-organ: an innocent bystander?

The pancreas is an epithelial organ comprising two different cell typres: the exocrine cells that secrete enzymes into the intestine and the endocrine cells that secrete hormones into the bloodstream. Morphologically the exocrine tissue is a lobulated and branched acinar gland, with exocrine cells grouped in acini in a typical pyramidal shape. Exocrine cells are easily identified by their basal nuclei and numerous secretory granules containing digestive enzymes like proteases, amylases, lipases and nucleases. The endocrine tissue is grouped in islets that are randomly scattered in the exocrine tissue. The islet consists of 4 different types of endocrine cells: the β–cells secreting insulin and the insulin antagonist amylin, α-cells (A or A2-cells) secreting glucagon, δ-cells (D or A1-cells) secreting somatostatin and the PP-cells (or F-cells) secreting pancreatic polypeptide.

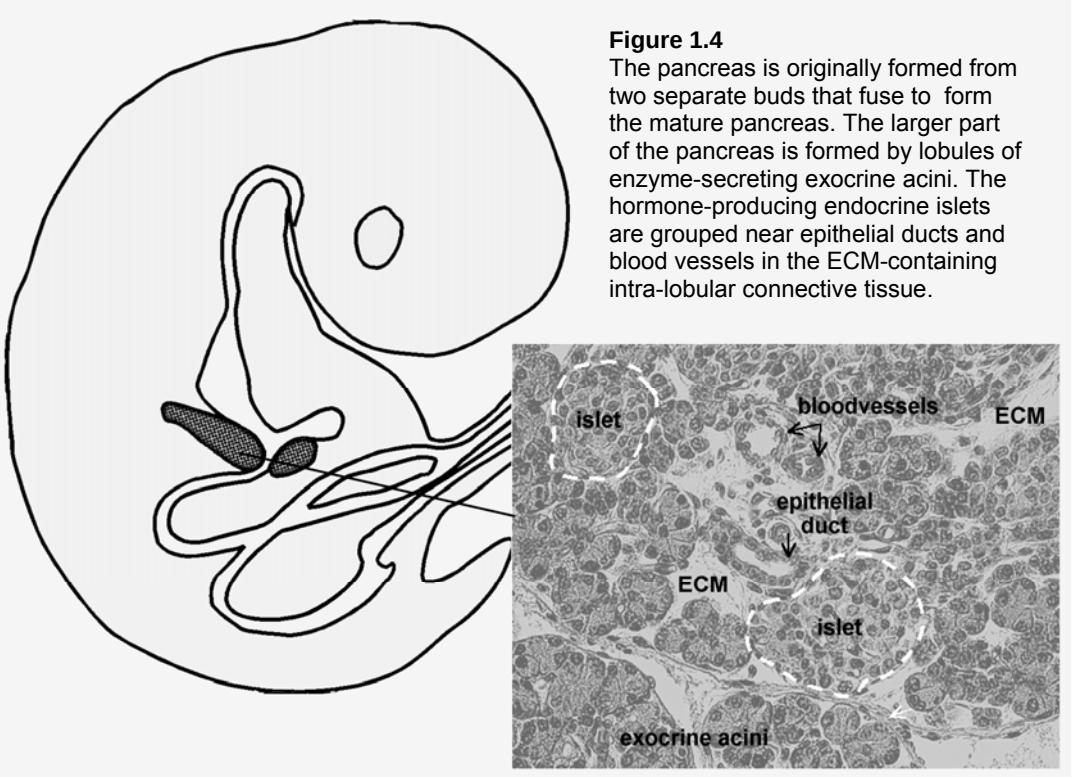

Figure 1.4
The pancreas is originally formed from two separate buds that fuse to form the mature pancreas. The larger part of the pancreas is formed by lobules of enzyme-secreting exocrine acini. The hormone-producing endocrine islets are grouped near epithelial ducts and blood vessels in the ECM-containing intra-lobular connective tissue.

1.5.1 Fetal and postnatal pancreas development

The development of the individual pancreatic structures is dependent on the interplay of a diversity of growth factors and the strictly time-regulated expression of various transcription factors. Since the regulation of pancreas development is beyond the scope of this thesis and very accurately reviewed by others [181] [182], only the most relevant aspects are discussed here.

The initial pancreas anlage is formed during embryogenesis. The fetal pancreas arises from the foregut endoderm, from two independently developing epithelial buds on the dorsal and the ventral side of the duodenum. When the stomach and duodenum rotate, the ventral bud and hepatopancreatic orifice move around to contact and fuse with

the dorsal bud. By then, epithelial ducts have formed independently in both pancreatic buds and these will fuse to become the main pancreatic duct of Wirsung. The proximal part of the dorsal duct becomes a small accessory duct (duct of Santorini) usually with an opening into the duodenum. Insulin expression will be initiated upon the induction of the expression of the pancreas duodenum homeobox gene-1 (pdx-1), when the ventral and dorsal buds contact the endothelial walls of the adjacent lying vitelline veins respectively dorsal aorta, around E9.5 in the mouse embryo [183]. The primary differentiated endocrine cells will contain both insulin and glucagon. The endocrine cells are fully differentiated at E15.5 and will be restricted to the expression of a single hormone. At this stage, the endocrine tissue makes up about 10% of the embryonic pancreas, gradually decreasing due to the expansion of the exocrine tissue. Acini and ducts become clearly visible as differentiated structures by 14.5 days of gestation, when the exocrine enzyme amylase is detectable as well. Importantly, grouped islets of endocrine cells are only observed just before the end of gestation, around E18.5 in the mouse.

1.5.2 Islet aggregation is guided by extracellular matrix

The epithelial cells of the fetal pancreas migrate into a defined stromal matrix. This ECM mainly consists of glycoproteins, proteoglycans and glycosaminoglycans, and is already present in the embryo from the bicellular stage on [184]. The composition of the ECM differs between tissues. In human fetal pancreas the ECM consists of type IV collagen, FN (mainly in the basal membrane of platelet endothelial cell adhesion molecule- (PECAM-1)-positive bloodvessels) and vitronectin in the basal membranes of ductal epithelium. Importantly, ECM forms the template for newly developing islets. An endocrine islet is not formed by clonal proliferation, but arises via cell aggregation of epithelial ductal cells that are believed to be the early progenitors of endocrine cells. Once these immature ductal epithelial cells have differentiated to mature endocrine cells, they migrate into the surrounding ECM and associate into islets. The ECM components laminin and FN have additionally been described to affect β-cell differentiation, proliferation, and even insulin secretion [185, 186]. Thus, next to physically shaping endocrine tissue, ECM acts as a regulator of endocrine development and function, as it does for macrophages. Likewise, interaction of β-cells with the ECM is also coordinated by integrins. Human pancreatic ductal cells and undifferentiated precursors express high levels of the integrins αVβ3 and αVβ5 to emerge from the ductal epithelium, infiltrating the surrounding ECM [187]. The pancreas structure additionally depends on the activation of matrix metalloproteinases (MMPs) that specifically degrade different ECM components [188], and on chemokine-signaling which regulates cell migration. The chemokine stromal cell derived factor-1 (SDF-1), originally identified as an important regulator of secondary lymphoid organ development, stimulates $CXCR4^+$ ductal cell migration in the fetal pancreas [189].

1.5.3 Postnatal pancreas development: a trigger for the diabetes-related pathogenesis?

In rodents pancreas neogenesis can be divided into two different stages, with the second stage taking place after birth. The rodent pancreas is an immature organ at

birth, and considerable developmental changes will take place from birth till the end of the weaning period. Changes within these first 3 weeks of life are crucial in the initiation of IDDM. In various organs, resident macrophages are involved in the maintenance of tissue homeostasis. Macrophages are, nonetheless, excellent APC. Considering the atypical peri-islet accumulation of resident macrophages in NOD mice, before any signs of tissue injury or inflammation are observed, it is conceivable that initial triggers inducing the preferential peri-islet localization of macrophages are evoked by the target-organ itself. Postnatal islet neogenesis is accompanied by a wave of apoptosis of β-cells around the second week after birth, in normal as well as in diabetes-prone animals. [190]. This neonatal apoptosis of β-cells has been suggested as an initial trigger for the development of IDDM [191-193]. The number of apoptotic cells is increased in NOD mice as compared to control BALB/c strains during preweaning [194] and this might be associated with the presence of increased numbers of resident APC in preweaning NOD mice. However, the uptake of apoptotic cells is believed to lead to immune silencing, rather than to activation [195].

1.5.3.1 Diabetes-prone mouse strains display islet abnormalities preceding inflammation

The functional state of β-cells is possibly associated to IDDM pathogenesis, yet fluctuations in β-cell metabolism and turnover are rather believed to appear after the initial inflammatory insult. However, there are indications that endocrine abnormalities preceed the onset of insulitis in diabetes-prone strains.

Islets from NOD male and female mice show an increased release of insulin in response to glucose stimulation, especially at 4-6 wks of age before the onset of insulitis. This transient hyperinsulinemia was accompanied by mild hyperglucagonemia between 2 and 4 weeks of age [196-198]. At 4 weeks of age, higher numbers of SOM/PDX-1 endocrine precursor cells are also present in the NOD pancreas [199], followed by the appearance of significantly enlarged islets designated “mega-islets” around 6-8 weeks of age [200]. Mega-islet formation was also observed in NOD*scid* mice and thus arises independently from the presence of T- and B-lymphocytes. These observations are suggestive for early α- and β-cell hyperactivity in NOD mice followed by exhaustion of the islets possibly reflected by the enlargement and faint insulin staining of the islets and the subsequent induction of islet neogenesis (increase in SOM/PDX-1^+ cells).

Despite the compensatory β-cell proliferation, insulin secretion steadily falls in NOD mice after insulitis onset at a rate disproportionate compared to the progressive reduction in β-cell mass [201]. This further suggests early β-cell dysfunction. Whether the hyper responsiveness of islets is intrinsically regulated in β-cells themselves, or the result of abnormal exogenous (possibly neuronal) stimulation of the islets in NOD strains is currently not clear.

1.5.3.2 Early endocrine abnormalities are accompanied by macrophage accumulation

Treatment with exogenous insulin reduces the demand for endogenous insulin, resulting in decreased β-cell activity. Intriguingly, exogenous insulin treatment also reduced the incidence of diabetes in NOD mice [202, 203], possibly reducing the vulnerability of the islets to direct cytokine-mediated cytotoxicity, or resulting in a decrease in availability of islet-antigens. Importantly, this protective effect of insulin was also observed in man [204]. Furthermore, the development of spontaneous diabetes in BB-rats was prevented by treatment with the mitochondrial K-(ATP) channel opener diazoxide that inhibits the secretion of endogenous insulin [205]. Interestingly, prophylactic insulin treatment not only reduced the incidence of diabetes in NOD mice, but also the number of pancreatic ER-MP23^{+} macrophages and the severity of insulitis [203, 206]. The maternal environment influences fetal islet development and glucose homeostasis of neonatal islets. Prophylactic insulin treatment of pregnant prediabetic NOD mothers decreased the number of mega-islets in pancreases of their offspring and, surprisingly, also the influx of pancreatic macrophages [206]. Thus, reduced mega-islet formation is associated with reduced β-cell activity and reduced accumulation of pancreatic macrophages. Whether the presence of macrophages directly affects the size and activity of the β-cells unfortunately remains unclear.

1.5.4 Cross-talk between macrophages and islets

Considering the correlation of β-cell activity with the presence of macrophages it seems plausible that insulin acts as a chemo-attractant to attract macrophages. This might explain the accumulation of macrophages in early NOD pancreases simultaneously with the appearance of β-cel hyperactivity. Correspondingly, Leiter *et al.* showed that *ex vivo* isolated murine islets of normal mice or commercially obtained bovine insulin induced chemotaxis of macrophages, while no macrophage migration was seen towards glucagon or somatostatin [207]. Macrophages were also attracted to islets in rats, but their migration appeared to be independent of insulin and was lost upon disaggregation of the islets [208]. No insulin-mediated chemotaxis of macrophages was observed in T1D patients or in healthy controls [209]. It is therefore uncertain whether insulin-mediated chemotaxis is a specific trait of murine macrophages or whether other macrophage attractants are the major mediators involved in islet-induced chemotaxis of macrophages.

A potential candidate might be the pituitary hormone migration inhibiting factor (MIF) that inhibits random macrophage migration. Interestingly, MIF is secreted by glucose-stimulated β-cells in a dose-dependent manner. Importantly, MIF has been shown to regulate the release of insulin in a stimulatory autocrine fashion [210] [211]. The role of pancreatic MIF in the peri-islet retention of macrophages has not been studied in NOD mice thus far. Increased MIF-release by (aberrant) glucose-mediated stimulation of NOD-islets may, hypothetically, result in the enhanced production of insulin that in synergy with MIF can result in the arrest and accumulation of resident macrophages residing proximal to the islet-periphery. Their inappropriate retention may lead to their activation and possibly damage to the adjacent β-cells.

1.5.5 Are macrophages involved in pancreas morphogenesis?

The early presence of pancreatic macrophages, already during neonatal pancreas development, is suggestive for their involvement in normal tissue turnover. Such a role for macrophages is well recognized and described for the postnatal development of mammary gland tissue and limb development during embryogenesis. In adult life, mature macrophages produce matrix proteins like laminin and FN [212, 213] and also various MMPs that facilitate macrophage, endocrine and exocrine precursor migration [214-216]. Additionally, macrophages can contribute to tissue turnover and neogenesis by the production of relevant growth factors like IL-6 and TGFβ1.

TGFβ1 is an important signaling molecule, not only in the communication between immune cells, but in pancreas development as well. TGFβ-1 specifically promotes the development of the endocrine compartment *in vitro* and interferes with normal islet morphology by the activation of MMP-2 [217]. Overexpression of TGFβ-1 in the pancreas in different transgenic mouse models is followed by the deposition of ECM proteins like collagens type I and type III, FN and laminin, and is often accompanied by the infiltration of macrophages and proliferation of fibroblasts [218, 219]

Another typical macrophage-derived growth factor is IL-6. IL-6 can affect the normal pancreas structure and significant changes in the pancreas morphology and islet architecture, like islet hyperplasia, neo-ductular formation and fibrosis, were observed in BALB/c mice with overexpression of IL-6 in their islets. These symptoms coincided with mononuclear cell infiltration, although diabetes did not develop in these mice [220]. IL-6 additionally increased the expression of preproinsulin mRNA and is thus potentially involved in endocrine metabolism [221]. IL-6 mRNA expression was detected in murine and rat islets and is expressed by human pancreatic endocrine cells as well [222]. Endogenous expression of IL-6 in resident pancreatic macrophages has not been reported thus far.

1.6 Does macrophage malfunction lead to diabetes?

Due to their numerous functions, defects in macrophages often lead to severe pathologies. Despite the significant role they play in the development and remodeling of tissues, it is their involvement in immune regulation that has revealed the effects of various defects in macrophage function. The abnormal Fcγ-receptor expression of synovial macrophages correlated to the severity of synovial inflammation and cartilage destruction during experimentally induced arthritis in a model for inflammatory disease of the main joints [223, 224]. Tissue resident macrophages of rheumatoid arthritis patients show defects in the function of their complement receptor [225], while macrophages from patients with autoimmune systemic lupus erythematosus exhibited impaired interactions with iC3b-opsonized apoptotic cells [226].

1.6.1 Macrophages of NOD mice and T1D patients show various defects that may promote the progression of diabetes development

Several defects have been detected in the myeloid compartment of NOD mice and T1D patients and these abnormalities are possibly related to the initiation and progression of IDDM. NOD BM-precursors show defective development of macrophages due to the inefficient priming of BM-precursors by M-CSF and defective regulation of protein kinase C in HSC of NOD mice [227]. Defective macrophage maturation resulted in the failure of efficient antigen presentation, preventing T-cells from the NOD mouse to proliferate properly [228]. Both macrophages and DC of NOD mice express lower basal levels of the costimulatory molecule CD86 as compared to C57BL/6 and BALB/c mice [126]. This defective CD86 expression by macrophages further reduces efficient antigen presentation, possibly contributing to the defective tolerance induction in NOD mice. The reduced antigen presentation by APC in the thymus might result in defective negative selection and escape of autoreactive T-cells. However, reduced antigen presentation does not explain the activation of these self-reactive T-cells in the periphery. Enhanced APC function rather than defective antigen presentation would be expected to initiate autoreactive T-cell activation. So, other functions of NOD APC are possibly affected as well.

Intrinsic abnormalities of the macrophage may promote the progression of IDDM development in NOD mice. The adoptive transfer of NOD BM-cells to the diabetes-resistant F1 progeny of nonobese normal (NON)xNOD mice, resulted in autoimmune destruction of the islets in these F1 mice [229]. Additionally, the transfer of peritoneal macrophages of diabetic NOD mice to naïve NOD mice accelerated disease, while neonatal transfer of “normal” allogeneic thymic BALB/c macrophages to NOD mice protected against diabetes [230]. An unequivocal explanation of these results is not provided, but these might relate to the following findings.

Peritoneal macrophages of NOD mice express substantially higher levels of the pro-inflammatory cytokine IL-12, and slightly enhanced levels of IFNγ following stimulation with various stimuli [130]. Enhanced metabolism of arachidonic acids was evident after zymosan A stimulation [231]. Interestingly, the constitutive expression of the

enzyme prostaglandin synthase E2 (COX-2) was reported for monocytes of T1D patients [232, 233]. Overexpression of COX-2 may lead to increased production of prostaglandin E2 that in turn enhances the expression of IL-6 in macrophages [234, 235]. Hyper-responsiveness was additionally observed in macrophages of T1D patients following stimulation with LPS, resulting in increased expression of PGE2 and the pro-inflammatory cytokines TNF-α, IL-1β and IL-6. [236]. Thus, macrophages of both NOD mice and T1D patients exhibit a pro-inflammatory profile that may influence the local microenvironment, possibly reducing the threshold for T-cell activation.

1.7 Aim of this thesis

The delicate balance that is required for a properly acting immune system may be disturbed by malfunction of macrophages in NOD mice and T1D patients. The abnormal early peri-islet macrophage accumulation is still unexplained and might be related to the initiation of insulitis. Furthermore, the ontogeny of the pancreatic macrophage compartment is unknown and probably gives rise to different macrophage subsets with possibly even different origins. If they indeed arise from precursors that proliferate locally, then their differentiation will additionally be determined by the local microenvironment. Defective macrophage maturation may lead to changes in their receptor expression profile and impair the capacity to efficiently interact with the ECM.

Abnormalities of the endocrine tissue in diabetes-prone animals or individuals susceptible for diabetes may be genetically determined and expressed independently from aberrancies in the immune system. Abnormal islet behavior may lead to the release of attractive agents and the recruitment of macrophages. Since macrophages of the NOD mice are defective in several ways, this early macrophage accumulation may accidentally create a local pro-inflammatory microenvironment and damage, initiating insulitis and finally diabetes.

The objective of the research presented in this thesis was to gain more insight in the abnormal peri-islet accumulation of NOD macrophages. Therefore, we have examined the target tissue as well as the capacity of NOD macrophages to interact with relevant components of their pancreatic microenvironment. Since the ontogeny of the pancreatic macrophage is largely unknown, we additionally characterized the macrophage compartment of the fetal and adult pancreas and the possible involvement of these macrophages in islet development.

We first studied the involvement of pancreatic macrophages and DC in T1D pathogenesis by inducing the depletion of macrophages and DC by using clodronate-loaded liposomes. This treatment induced the temporary depletion of macrophages and DC in the pancreas. Moreover, it induced a significant delay in the onset of diabetes and strongly decreased the diabetes incidence by 35 wks of age. Interestingly, the disappearance of pancreatic macrophages and DC resulted in the complete disappearance of lymphocytes from the pancreas, despite the presence of established insulitis when the treatment was initiated. These results are presented in chapter 2.

The characterization of different APC-subsets in adult and fetal pancreases is described in chapter 3. Mature macrophages are not present in fetal pancreases before E14.5. By using fetal pancreatic explant cultures at E12.5, with or without the addition of M-CSF, we were able to study the presence of macrophage precursors and their involvement in endocrine cell growth.

In the fourth chapter, we have focused our studies on the tissue microenvironment of the pancreatic macrophages during the preweaning period. Increased numbers of APC were observed in NOD mice during this period. We show that the resident pancreatic macrophages mainly reside in the fibronectin-containing ECM of

the connective tissue. Interestingly, higher levels of fibronectin are present in early postnatal pancreases of NOD mice concomitantly with an increase in endocrine tissue and enlarged and irregular-shaped islets.

Considering the co-localization of the macrophages with fibronectin in the pancreas, we have studied more closely the expression and function of the fibronectin integrin-type receptors CD49d/CD29 and CD49e/CD29, in NOD and control macrophages. Intriguingly, NOD mice show decreased levels of the integrin α-chain CD49d and reduced adhesion and migration to fibronectin. We show that ERK-1/2 negatively regulates CD49d expression in macrophages. Increased levels of the mitogen activated protein kinase (MAPK) extracellular-regulated kinase (ERK)-1/2 may suppress CD49d expression in NOD macrophages upon stimulation with LPS. This work is presented in the fifth chapter

Multiple hematopoïetic lineages in NOD mice display hampered fibronectin receptor expression. Like NOD macrophages, NOD thymocytes and subsets of peripheral T-cells also show reduced fibronectin-mediated migration. Interestingly, these cells show defective expression of CD49e/CD29 instead of CD49d. The implication of such integrin-defects for the development of diabetes in NOD mice is discussed in the sixth chapter. The final conclusions of the presented work, the general discussion and the future directions are given in chapter 7.

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Chapter 2

Dendritic cells and Macrophages are essential for the retention of Lymphocytes in (peri)-Insulitis of the Non-Obese diabetic Mouse: a phagocyte depletion study

Submitted for publication

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Abstract

Dendritic cells (DC) and macrophages (Mϕ) are present in higher numbers in the pancreas of the NOD mouse during the diabetogenic process from very early stages onwards. In this study we used clodronate-loaded liposomes to mediate the temporary systemic depletion of these phagocytic cells and monocytic precursors in order to modulate the pancreatic inflammation. Two intra-peritoneal injections given with a 2-day interval to 8-week old NOD mice depleted monocytes from the circulation and monocytes, DC and Mph from the spleen within the first days after the injections. Monocytes, DC and Mph re-appeared in the circulation and the spleen within one week and had an unchanged phenotype and antigen presenting function. Interestingly, this treatment caused a delayed, long lasting disappearance (7–21 day post-injection) of DC and Mϕ from the endocrine pancreas at a time when monocytes, DC and Mϕ had already repopulated the circulation and spleen. The depletion of DC and Mϕ from the endocrine pancreas was accompanied by a total disappearance of lymphocytes from the pancreas. DC, Mϕ and lymphocytes re-appeared in the pancreatic inflammatory infiltrates in treated mice from 28 days post depletion onwards. Importantly, the treatment significantly postponed the onset of diabetes, leading to a strongly decreased incidence by 35 weeks of age.

Taken together, our data show an essential role of phagocytic cells, i.e. DC and Mϕ, in the recruitment of lymphocytes to the pancreatic islets in NOD mice.

Abbreviations: APC, antigen-presenting cells; DC, dendritic cells; Lip-CL_2MDP, clodronate-loaded liposomes ; i.p., intra-peritoneal; Mϕ, macrophages, NOD, nonobese diabetic; STZ, streptozotocin.

2.1 Introduction

Type I diabetes is an autoimmune disease in which a self-destructive immune process against the pancreatic β-cells leads to insulin deficiency. In the non-obese diabetic (NOD) mouse, a widely used animal model for autoimmune diabetes, dendritic cells (DC) and macrophages (Mϕ) have been proposed to be important for the initiation, progression and final destruction of the β-cells [1]. Histological studies show slightly raised numbers of DC and Mϕ already in the pancreas of neonatal NOD mice [2]. A further accumulation of DC and Mϕ around islets at 4-5 weeks of age precedes the peri-insular infiltration of lymphocytes. A relocation of lymphocytes and Mϕ into the islets characterizes the initiation of the final β-cell destruction [3, 4]. The continuous presence of elevated numbers of DC and Mϕ in the NOD pancreas from birth onwards strongly suggests an important role of these cells in the diabetogenic process. Yet, these histological studies are descriptive and therefore inherently inconclusive regarding the functional role of these cells.

Functional approaches have involved the blocking of the intra-islet infiltration of inflammatory cells by an intervention of leukocyte-adhesion to the pancreas endothelium [5-7] or by inducing a long-term depletion of phagocytic cells [8-10]. These methods protected mice from diabetes, further strengthening the concept of an essential role of Mϕ and DC in the development of autoimmune diabetes in the NOD mouse. However, previous studies mainly focused on the depletion of Mϕ and did not consider the effects of DC depletion. We have previously demonstrated that clodronate-loaded liposomes (lip-CL_2MDP) not only deplete Mϕ, but also DC in liposome targeted-tissues [11].

We here report an investigation on the intra-pancreatic and peripheral changes in the monocyte, DC and Mϕ compartment after a short-term lip-CL_2MDP treatment of NOD mice. We show that two intra-peritoneal (i.p.) injections of lip-CL_2MDP given with a 2-day interval in 8-week-old pre-diabetic NOD mice significantly delayed diabetes onset for more than 20 weeks in the majority of treated mice. Considering the short-term disappearance of monocytes, Mϕ and DC from the circulation and the spleen, and the rapidly regenerated normal function of the newly recruited spleen DC, the beneficial effects could not be attributed to a modulation of cells from this compartment. However, the same treatment induced a delayed and prolonged disappearance of DC and Mϕ from the endocrine pancreas. The absence of DC and Mϕ directly influenced the presence of lymphocytes and lymphocytic (peri)-insulitis resolved completely. Our observations point to an essential role of DC and Mϕ in the continued persistence of lymphocytic infiltrates the pancreas and subsequent β-cell destruction.

2.2 Materials and methods

2.2.1 Animals

Female NOD/Ltj and C3HeB/FeJ mice were bred at the animal facility of the ErasmusMC, Rotterdam, The Netherlands. C57BL/6j, BALB/c and NOR female mice were obtained from Harlan (Horst, The Netherlands). All strains were kept under specific pathogen-free conditions and fed *ad libitum*. By 30wks of age, the incidence of diabetes in our NOD colony is 90% for females and 60% for males. For all experiments, mice were sacrificed at postnatal ages of 7-12wks or at 35wks of age. Glycosuria was tested with the Gluketur test (Roche Diagnostics GmbH, Mannheim, Germany). A minimum of 3 animals per age/strain/time point was used for all studies. Animal handling followed the ethical rules of the European Union and was approved by the Erasmus University Animal Welfare Committee.

2.2.2 Liposome preparation and in vivo application

Multilamellar liposomes containing clodronate (dichloromethylene bisphosphonate, a gift from Roche Diagnostics GmbH) (lip-CL_2MDP) in the aqueous phase were prepared as described previously [12, 13]. Liposomes consisted of phosphatidyl choline and cholesterol in 6:1 molar ratio. After washing, the liposomes were resuspended in PBS. A volume of 0.2 ml, containing about 2mg of liposome-entrapped clodronate was injected twice intraperitoneally (i.p.) with a two-day interval between two consecutive injections into 8-week old mice (in a stage of well advanced peri-insulitis). This approach was chosen in order to obtain a complete depletion of phagocytic cells in the spleen. Mice were sacrificed (by CO_2 exposure) at various time points after the second injection (days 2, 4, 7, 14, 21, 28) and indicated organs were removed for analysis.

2.2.3 Diabetes incidence after treatment with clodronate-loaded liposomes

Mice were followed until 35wks of age and the presence of glucose and ketones in the urine was measured weekly. A group of non-injected mice of the same age were used as control. At 35wks of age, mice were sacrificed and organs removed. Insulitis was characterized by analyzing the composition of islets with respect to the presence of insulin- and glucagon positive cells, T cells, DC or Mϕ in histological examination of sequential sections stained for each cell type, according to the staging established previously [3]. In addition, a numerical score was ascribed to the four stages as follows: stage 0 (unaffected islets)–score 0, stage I+II (early para-insular accumulation of DC and Mϕ, no T cells present, insulin$^+$)–score 1, stage III+IV (para- and peri-insular accumulation of DC, Mϕ and T cells, insulin$^+$)–score 2, stage V (infiltration of T cells into islets, insulin$^+$)–score 3, stage VI (end stage, insulin$^-$)–score 4. In the latter case, islet remnants were identified by glucagon-labeling. A score was assigned to each counted islet and an average of all scores per 100 counted islets was calculated.

2.2.4 Preparation of single cell suspension of circulating leukocytes and spleen

Blood was obtained by heart puncture after exposing the organ, and collected in heparin-coated tubes or in syringes containing 1ml PBS with 8mM EDTA. Erythrocytes were eliminated using lysing solution (BD Biosciences, San Diego, CA, USA) and leukocytes were washed twice by centrifugation at 400g in phosphate-buffered saline (PBS) supplemented with 0.5% BSA and 20nM NaN_3 (further referred to as the FACS buffer).
The spleen was cut into two parts. The larger part was used for histological analysis. The smaller part of the spleen was cut into small pieces and incubated for 1h at 37°C with collagenase-D (Roche Diagnostics GmbH; 0.27 U/mg) at a final concentration of 1mg/ml in RPMI-1640 medium supplemented with 60μg/ml penicillin and 100μg/ml streptomycin. The resulting digested tissue suspension was pushed through a 100μm cell strainer using a rubber-end of a 5ml syringe plunger and centrifuged. Subsequently, the cell suspension was washed with RPMI-1640 medium supplemented with antibiotics and 10% fetal calf serum (FCS).

2.2.5 Spleen DC isolation by MACS procedure

DC were sorted from the spleen cell suspension by magnetic enrichment using auto-MACS. Cells were centrifuged, resuspended to obtain a concentration of 10^8 cells in 400μl MACS buffer (PBS supplemented with 1% FCS and 2mM EDTA) and incubated with 100μl CD11c microbeads (Miltenyi Biotec GmbH, Germany) for 30min on ice. Subsequently, cells were washed twice and then resuspended in MACS buffer to obtain a concentration of 10^8 cells/ml. The program for high purity of cells with low frequency was used on the autoMACS machine (Miltenyi Biotec GmbH) and a positive fraction was collected. In all experiments, the purity was higher than 95% as determined by subsequent flow-cytometric analysis.

2.2.6 Phenotypic analysis

The list of antibodies used in this study is given in the Table 1. Aliquots of $2x10^6$ cells were incubated with the prepared mix of monoclonal antibodies. Each incubation step was performed at room temperature for 10min. All biotinylated antibodies were detected by streptavidin-APC (BD Biosciences). Anti-CD71 was detected by R-PE labeled goat-anti-rat IgG (mouse-absorbed; GαRa-PE) purchased from Caltag Laboratories, San Francisco, CA.

2.2.7 Mixed leukocyte reaction

The capacity of sorted spleen DC to activate allogeneic lymph node (LN) cells was measured as previously described (14). Briefly, responder T cells, isolated from LN of C3H mice, and stimulator cells (DC) were resuspended in RPMI-1640 (with Hepes), 10% FCS (heat inactivated; 0.2 μm filtered), 60 μg/ml penicillin, 100 μg/ml streptomycin, 20 mg/l sodium pyruvate and 50 μM 2-ME (further referred to as MLR medium). T cells were resuspended at a concentration of 10^6/ml. The concentration of stimulator cells varied depending on the desired stimulator: responder cell ratio. The cells (100μl of each cell suspension) were incubated in round-bottom 96-well plates for 4 days at 37°C in 7%

CO_2. Stimulator and responder cells, incubated separately in MLR medium, were used as negative controls. Mitogenic stimulation of T cells by concanavalin A (final concentration 1.25 μg/ml) (Sigma Chemical Co., St. Louis, MO, USA) was used as a positive control for cell proliferation.

After 4 days of co-culture, cells were harvested and analyzed by flow cytometry. Triple labeling of cells with antibodies against CD4, CD8 and CD71 (anti-transferrin receptor) was performed and 1.5 x 10^4 events were acquired within the live cell gate. Data analysis was performed using Cell Quest Pro analysis software. The percentage of CD71-positive T cells, as a measure of proliferating cells, was determined within the $CD4^+$ and $CD8^+$ population.

2.2.8 Immunohistochemistry

Pancreases were embedded in Tissue-Tek (Miles, Elkart, IN, USA) and frozen in liquid nitrogen. Cryostat sections (5μm thick) were cut at 100 or 200μm intervals. Series of 5 or 6 pancreas sections cut at different levels were fixed for 10min in acetone containing 0.03% hydroxyperoxide to block endogenous peroxidases. PBS with 0.05% Tween-20 was used for the washing steps. Unconjugated primary antibodies specific for insulin, glucagon, T-cells (CD3), dendritic cells (CD11c) or subsets of Mϕ (ER-MP23 or BM8 [15]) were subsequently detected with appropriate peroxidase-coupled- (DAKO, Glosstrup, Denmark) secondary antibodies in the presence of 1.5% normal mouse serum. The 3-amino-9-ethylcarbazole substrate (Sigma Co., St.Quentin Fallavier, France) dissolved in 50mM sodium acetate/0.02% hydroxyperoxide was used to detect peroxidase activity. The resulting labeling was examined by light microscopy.

2.3 Results

2.3.1 Phagocyte depletion with lip-Cl_2MDP in 8 week-old pre-diabetic NOD mice, significantly delays the onset of diabetes.

To investigate the modulating effects of temporary phagocyte depletion on pancreatic inflammation and diabetogenesis, we treated NOD mice with two i.p. injections of lip-Cl_2MDP. This treatment caused a significantly delayed onset of diabetes ($p<0.01$) (Figure 2.1A). At the endpoint of our studies (35 weeks of age) the majority of treated NOD mice (73%) was still normo-glycemic. However, immuno-histological examination of the pancreases of these mice showed that none of the mice had been permanently protected from insulitis. Leukocyte accumulations in the endocrine pancreas were present in all treated mice at 35 weeks of age. The infiltrates comprised DC, Mϕ and T cells and the infiltrates composition varied per individual islet, depending on the stage of the destructive process, as has been described before for non-treated mice [3]. The insulitis severity score (Figure 2.1B) of the non-diabetic 35wk old treated NOD mice was comparable either to that of non-treated pre-diabetic (11wks old) or to the diabetic NOD mice. Hence, a two-fold injection of lip-Cl_2MDP strongly reduced the progression of the destructive process and significantly delayed the diabetes onset in majority of treated mice for 18-24 weeks.

2.3.2 Monocytes, DC and Mϕ return rapidly to the spleen of NOD mice after the depletion.

We examined the effects of lip-Cl_2MDP on the disappearance of monocytes, DC and Mϕ from the circulation and the spleen of NOD mice as well as the kinetics of their repopulation and compared these parameters to those of identically treated C57BL mice. Blood monocytes were quantified using a recently established analysis [16] in which monocytes are gated as $SSC^{lo}CD11b^{hi}$ cells. Blood monocytes consist of an immature pool (Ly-$6C^{hi}$), readily recruited into inflammations, and mature monocytes (Ly-$6C^{lo}$), a resident DC and Mϕ precursor pool [17]. For the quantification of DC and Mϕ in the spleen by a flow-cytometric analysis, monoclonal antibodies against CD11c, CD8α, CD11b and F4/80 were applied simultaneously. This enabled definition of four distinct cell populations (Figure 2.2A). $CD11c^{+}$ cells (gate: R1) were subdivided into $CD11c^{+}CD11b^{hi}CD8^{-}$ (myeloid DC) and $CD11c^{+}CD11b^{lo}CD8^{+}$ cells ($CD8^{+}$ DC). A third population, $CD11c^{-}CD11b^{hi}F4/80^{+}$ (gate: R2), represent the red pulp Mϕ as verified by immunohistochemistry. The F4/80-negative fraction in the gate R2 were granulocytes. Finally, the $CD11c^{lo}CD11b^{hi}$ cells (gate: R3) formed a fourth population; these cells were $F4/80^{lo}$, Ly-$6C^{lo/med}$, $CD43^{+}$, $CD31^{+}$ and correspond to the mature blood monocytes as we have recently defined [16].

Treatment with lip-Cl_2MDP (i.v.) causes the virtually complete depletion of circulating monocytes during the first hours, followed by a rapid return [16]. Starting from the day 2 after the i.p. treatment, the pool of Ly-$6C^{hi}$ monocytes did not change. In contrast, the Ly-$6C^{lo}$ monocyte population was almost completely depleted at day 2 and

significantly reduced until the end of the observation period (day 28) (Table 2.2). Similar kinetics were found for the C57BL control mice (not shown).

Mature monocytes, DC and Mϕ in the spleen were clearly depleted at day 2 after the last injection of lip-Cl_2MDP (Figure 2.2B). All four investigated cell groups were present in the spleen of the treated mice, starting from day 4 in both mouse strains. Myeloid DC reached steady state levels in the second week post-treatment, while $CD8^+$ DC needed more than two weeks to normalize (Figure 2.2B, upper row). Similar kinetics was found for the NOD and C57BL mice. Red pulp Mϕ were restored almost instantly: despite a short initial drop, the rate of NOD red pulp Mϕ remained stable at the steady state level with even a slight increase at day 11. However, in C57BL mice, the frequency of red pulp Mϕ remained significantly higher till the end of the observation period. Splenic mature monocytes were also depleted at day 2, but returned to normal levels shortly thereafter and remained stable from day 4 onwards in both mouse strains (Figure 2.2B - lower row).

2.3.3 Observations in flow-cytometric analysis are confirmed by the immuno-histological analysis

Immunohistological analysis of $CD11c^+$ (myeloid DC and $CD8^+$ DC) and $F4/80^+$ cells (mature monocytes and Mϕ) in the spleen indicated the same kinetics as the flow-cytometric analysis. As shown in Figure 2.3, treatment with lip-Cl_2MDP induced a complete depletion of both Mϕ and DC from the spleen of NOD mice: 2 days after the second injection, the red pulp was devoid of viable F4/80 or CD11c positive cells (Figure 2.3C and 2.3D). The remaining F4/80 and CD11c positive labeling in the white pulp at day-2 resembled non-viable pycnotic cells that lacked a typical membrane staining as present in the spleen of non-treated control mice (Figure 2.3A and 2.3B).

$F4/80^+$ cells started to return to the red pulp from day 4 post-injection onwards (Figure 2.3F) and at day 7 (Figure 2.3H) the number and the distribution of the F4/80 positive population were comparable to non-depleted control mice. The repopulation of the spleen by $CD11c^+$ cells also started at day 4 (Figure 2.3E) and at day 7 $CD11c^+$ cells in NOD mice had regained their normal staining pattern as compared to the non-treated controls (Figure 2.3G). In addition, other Mϕ populations like metalophillic (MOMA-1^+) and marginal zone (ER-$TR9^+$) Mϕ were depleted in NOD mice by lip-Cl_2MDP in the same fashion as has been reported for other mouse strains [18]. These cells, however, only reappeared several weeks after depletion (data not shown).

Taken together, the kinetics of depletion and repopulation by different populations of splenic DC and Mϕ in NOD mice is fully comparable with the repopulation in C57BL mice.

2.3.4 A normal phenotype and function of re-populated spleen DC in NOD mice after the depletion

Despite the rapid return, we argued that the treatment with lip-Cl_2MDP may have caused a modulation of the function of the returning phagocytes, contributing to an altered role in diabetogenesis. Therefore, we compared the phenotype and the function of spleen $CD11c^+$ cells, isolated by an autoMACS procedure, from treated (day 7 post-

injection) and from non-treated NOD and C57BL mice. Although we utilized a high purity separation program, both $CD11c^{hi}$ and $CD11c^{lo}$ cells were isolated, based on the phenotypic analysis (Figure 2.4A). We found no difference in the cell yield between NOD and C57BL mice from both untreated and treated mice. Also, the isolated $CD11c^{+}$ cells from treated mice displayed the same expression of MHC class II and of the co-stimulatory molecules CD80 and CD86 in comparison to such cells isolated from non-treated mice (Figure 2.4A). In addition, the T-cell stimulating capacity of the isolated DC co-cultured with allogeneic LN cells was similar before and after the treatment, in both mouse strains (Figure 2.4B). Similar proportions of allogeneic $CD4^{+}$ or $CD8^{+}$ T cells had up-regulated CD71 (transferring-receptor), irrespective of the fact that the cells isolated from treated mice had a higher proportion of mature monocytes than before treatment.

2.3.5 Lip-Cl_2MDP treatment leads to a late disappearance of DC and Mϕ from the NOD pancreas

To examine the effect of lip-Cl_2MDP treatment on the target organ inflammation, pancreases were removed before and after the injections (at days 0, 2, 4, 7, 14 and 28 post-treatment), and the presence of the different cell populations was examined by immuno-histochemical staining.

Untreated (8wk old) NOD mice had a considerable lymphocytic peri- and intra-islet infiltration. $BM8^{+}$ and ER-$MP23^{+}$ Mϕ (Figure 2.5) were found scattered throughout the pancreas and most likely represent resident Mϕ. Unlike Mϕ, DC ($CD11c^{+}$) were localized only in close proximity to the islets and not in the intra- and interlobular septa of the exocrine pancreas (Figure 2.7A).

The treatment with lip-Cl_2MDP had no immediate effect on pancreas Mϕ and DC, unlike hitherto observed in the spleen and the circulation. However, starting from day 4 after the treatment, we observed reduced numbers of $BM8^{+}$ and ER-$MP23^{+}$ Mϕ in both exocrine and endocrine pancreas. The decline in the Mϕ number further continued and many islets were clear from Mϕ (Figure 2.5C and 2.5D). Quantification of Mϕ-containing islet-inflammations at day-7 post-treatment showed that about 95% of islets were free of Mϕ (Fig. 2.6A) and the frequency of islets that contained Mϕ was almost as low as in C57BL mice of the same age. Additionally, at day 14 after injection, many islets and exocrine lobes were devoid of Mϕ (Figure 2.5E, 2.5F and 2.6A). The distribution of the few remaining Mϕ had also drastically changed as they were found only in confined areas such as the inter-lobular connective tissue, but not associated with endocrine or exocrine cells. At 28 days after treatment Mϕ had reappeared again at the islet periphery (Figure 2.5G and 2.5H).

Similar to Mϕ, the number of pancreatic DC declined from day 4 after treatment onwards (Fig. 2.6B). At days 7 and 14, the short-term injection of lip-Cl_2MDP had almost completely depleted DC from NOD pancreases. When quantified, the number of DC-free islets was similar to the number of Mϕ-free islets (Figure 2.6B). Also like Mϕ, DC were again present in islets at day 28 after lip-Cl_2MDP injection (Fig. 2.7).

2.3.6 The loss of Mϕ and DC from NOD pancreas leads to a prolonged disappearance of lymphocytes

In parallel with the loss of Mϕ and DC, we observed a disappearance of lymphocytes from the pancreases of treated mice (Figure 2.6B). The decline in DC and Mϕ numbers at day 4 already led to the loss of lymphocytes from the pancreatic infiltrates (not shown). This phenomenon was more prominent at days 7 and 14 after treatment and observed in parallel with the absence of DC (Figure 2.7C-F). At day-28, when a substantial number of DC and Mϕ had returned to the pancreas, T cells also re-appeared. B cells demonstrated a similar kinetics as T cells. At day-4, the numbers of B cells started to decline and the cells had completely disappeared by day 7. Interestingly, B cells seemed to require other conditions to infiltrate the pancreas again, as they did not reappear by day-28 but returned to the pancreas much later, i.e. at day-49 post-treatment (data not shown).

2.4 Discussion

In this study we bring to light three important phenomena related to the role of dendritic cells (DC) and macrophages (Mϕ) in the development of insulitis and diabetes in the NOD mouse model.

First, a short-term treatment with lip-Cl_2MDP given in the stage of a progressing peri-insulitis (8wks of age) was sufficient to induce a significant decline in the number of Mϕ and DC in the islet vicinity for a period of at least three weeks in NOD mice. Second, the lasting loss of DC and Mϕ from the pancreas revealed an essential role of these cells in the local recruitment and retention of lymphocytes. Lymphocyte infiltrations dissolved completely when the DC/Mϕ pool was absent and reappeared only upon return of DC and Mϕ in close proximity to the endocrine tissue. Third, the depletion of the DC and Mϕ did not happen concomitantly in the pancreas and in the circulatory compartment. Monocytes, DC and Mϕ were depleted and rapidly returned to the circulation and the spleen within one week with similar kinetics in NOD and control mice, whereas in the pancreas their disappearance started at the end of the first week. Furthermore, in line with the observed changes in the pancreas, the diabetes onset and incidence were strongly delayed and decreased, respectively, after short-term lip-Cl_2MDP-treatment of NOD mice at 8 weeks of age.

It is well known that an i.p. injection of lip-Cl_2MDP depletes Mϕ from the spleen, peritoneal cavity and the omentum [19], but the pancreas has not been included in such previous depletion studies. Our study shows that i.p. injections of lip-Cl_2MDP also influence the accumulation of monocytes, Mϕ and DC in the pancreas. Remarkably, however, when we compared the effects of depletion in the pancreas with that of the spleen/circulation, we observed different kinetics of disappearance and return of the phagocytes: DC and Mϕ took a week longer to disappear from the pancreas. Unfortunately, we cannot readily explain these differential disappearance patterns.

The return of mature monocytes to the circulation and the spleen, preceded the return of DC and Mϕ. This supports the view that spleen APC originate from blood monocytes. Treatment with lip-CL_2MDP ineffectively deplete phagocytes in the connective tissue [20]. Indeed, the delayed disappearance of pancreatic DC and Mϕ upon treatment strongly suggests that liposomes do not reach these phagocytes directly. Instead, it is more likely that the natural turnover of the cells, under conditions when the precursors might be lacking, causes their decline. It is however difficult to understand why monocytes did not rapidly replenish DC and Mϕ in the pancreas, like they do in other target organs such as spleen and liver, since the disappearance of pancreas DC and Mϕ started when monocytes were back in the circulation.

One possible explanation is that pancreas DC and Mϕ might originate from other precursors than those found in the circulation. The origin of pancreas resident Mϕ and DC is not known. They might develop from intra-organ precursors, like in the skin [21], or from precursors that reside in the vicinity of the pancreas. In this context it is noteworthy that i.p. application of lip-Cl_2MDP to rats induced a depletion of Mϕ in the peritoneum and omentum within two days, but the repopulation of the omental Mϕ was not seen within

the next 23 days [22]. This “postponed” pattern of re-appearance overlaps with the here described slow pattern of re-appearance of Mϕ and DC in the pancreas and this might either point to the omentum as a source of precursors for pancreas DC and Mϕ, or to a shared precursor of omental and pancreas DC and Mϕ. Further investigation is needed to evaluate whether such peripheral precursors might be affected by the i.p. lip-Cl_2MDP treatment more extensively than the circulating monocytes. Alternatively, the lip-Cl_2MDP treatment does interrupt the continuous influx of monocytes into the pancreas for several days and thereby also the production of appropriate chemoattractants for other inflammatory cells [23, 24]. In this case, the dynamic inflammatory process might be transiently interrupted and subsequently slowly restored, similarly to what has been demonstrated previously [25].

A role for DC in the NOD lymphocyte accumulation has been suggested previously. The administration of streptozotocin (STZ) to C57BL/6 mice, an induced diabetes model, led to an accumulation of DC in the pancreas, followed by an enhanced expression of adhesion molecules on the pancreatic endothelium and an increased adherence and infiltration of lymphocytes [26]. Furthermore, lymphocyte adhesion and infiltration decreased upon silica-mediated depletion of the Mϕ additionally suggesting that the Mϕ were involved in the recruitment of lymphocytes (27, 28). The DC and Mϕ possibly act by inducing the addressins VCAM-1, MAdCAM-1 and ICAM-1 on the pancreatic endothelium [5, 29, 30]. The dependence of lymphocyte infiltration on Mϕ and DC has also been shown in other situations. Elimination of phagocytic cells by lip-Cl_2MDP reduced the lymphocytic infiltration of transplanted fetal pig pancreas xenografts in NOD mice [31]. In addition, the loss of marginal zone Mϕ as the result of chronic *Leishmania* infection severely abrogated normal trafficking of lymphocytes into the white pulp of the spleen [32].

The responsible Mϕ or DC-derived factor that initiates the infiltration and maintenance of lymphocytes in the pancreas has not been identified thus far. Several chemokines like IP-10, MCP-1 and RANTES have been implicated in the attraction of monocytes and Th1-cells to the pancreatic islets during the progression of autoimmune diabetes [33-36]. Although the expression of some of these chemokines was found to be islet-specific, DC and Mϕ are also able to make other suitable chemokines that possibly attract and retain lymphocytes to the pancreas.

In conclusion, a depletion of DC and Mϕ via a regimen of two injections of lip-Cl_2MDP in the stage of a progressing peri-insulitis (at 8 weeks of age) was sufficient to clear DC and Mϕ almost completely from the pancreas, and to delay the re-appearance of these cells considerably. The presence of DC and Mϕ in the pancreas dictated the presence of lymphocytes, which should be taken in account for developing possible new treatments for diabetes.

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2.5 References

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Table 2.1 Monoclonal antibodies used in this study

Marker	**monoclonal antibody**	**Conjugate**	**origin**
Ly-6G	1A8	PE	BD Biosciences[a]
Ly-6C	ER-MP20	FITC	own laboratory
CD11b / Mac-1	M1/70	PerCP	BD Biosciences
CD11c	N418; HL3	∅[b]; FITC, PE	own laboratory; BD Biosciences
CD43	S7	FITC, PE	BD Biosciences
CD8α	53-6.7	FITC, APC	BD Biosciences
F4/80	F4/80	FITC	Caltag Lab, San Francisco, CA, USA
CD31	ER-MP12	bio	own laboratory
CD45RB	16A	FITC	BD Biosciences
MHC class II (I-A^b)	ER-TR3	bio	BMA Biomedicals AG, Switzerland
MHC class II (I-A^{g7})	10.2.16	bio	own laboratory
CD80	16-10A1	PE	BD Biosciences
CD86	GL1	FITC	BD Biosciences
CD4	RM4-5	APC	BD Biosciences
Insulin		∅	DAKO,Carpinteria, USA
Glucagon		∅	DAKO,Carpinteria, USA
CD3	KT3	∅	own laboratory
ER-MP23	ER-MP23	∅	own laboratory
BM8	BM8	∅	BMA Biomedicals AG, Switzerland
CD71	ER-MP21	∅	own laboratory

[a]San Diego, CA, USA; [b]unconjugated

Table 2.2 Kinetics of the blood monocytes repopulation in NOD mice after lip-Cl_2MDP treatment

Time point (days)	All monocytes	Inflammatory Ly-6C^{hi} monocytes	Mature Ly-6C^{lo} monocytes
0	13.03 ± 1.36 [a]	4.47 ± 0.33	8.56 ± 1.05
2	5.83 ± 0.57*	5.28 ± 0.61	0.55 ± 1.07**
4	9.74 ± 0.81	5.87 ± 1.72	3.87 ± 0.04*
7	10.87 ± 1.49	5.97 ± 1.04	4.90 ± 0.74*
14	14.04 ± 3.51	9.73 ± 2.68	4.31 ± 0.93*
28	12.26 ± 1.59	7.37 ± 1.57	4.89 ± 0.83*

[a] data represent mean ± SEM

*p<0.05; **p<0.01 vs. 0 time point

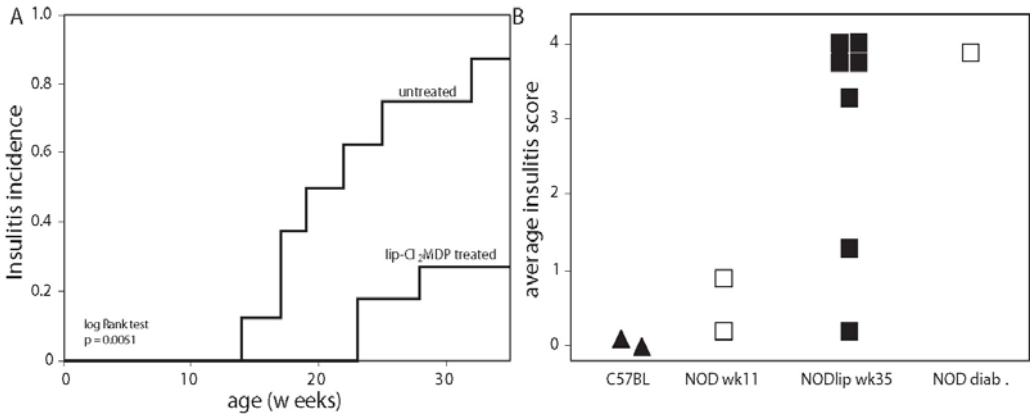

Figure 2.1 A short-term treatment of 8 week old NOD mice with lip-Cl_2MDP induced a significant delay of diabetes onset, resulting in strongly reduced incidence at 35 weeks of age.

A. Kaplan-Meier analysis of the diabetes incidence in treated and untreated mice. The Log Rank statistical test was performed to compare the Kaplan-Meier curves from treated and untreated mice ($p<0.01$).

B. Insulitis score of control untreated C57BL mice, untreated NOD mice, NOD treated with lip-Cl_2MDP at 35 wks of age and untreated 17wks old diabetic mice, respectively. Symbols represent the values of the average score of mice, individually. For a description of scoring, see the Material and Methods section.

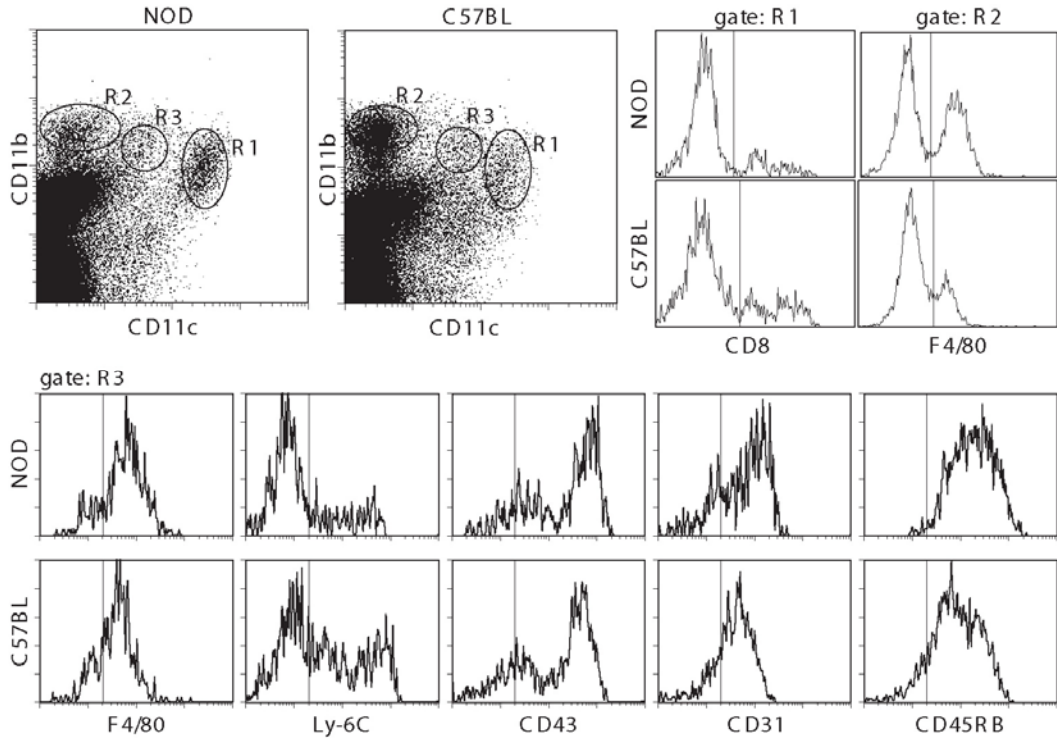

Figure 2.2A Normal maturation kinetics of the NOD DC and Mph compartment after lip-Cl_2MDP in the spleen.

A. Phenotypic definition of spleen DC and Mph populations according to CD11b and CD11c expression. Histograms represent the expression profile for a given marker of cells that belong to the indicated gate. $CD11c^{hi}$ cells (Gate:R1) can be divided into $CD8^+$ DC and myeloid DC ($CD8^-$). The $F4/80^+$ population within $CD11c^-CD11b^{hi}$ cells (Gate:R2) represent the red pulp Mϕ (verified by immuno-histochemistry). The $CD11c^{lo}CD11b^{hi}$ (gate: R3) cells correspond to mature blood monocytes and are $F4/80^{lo}$, Ly-$6C^{lo/med}$, $CD43^+$, $CD31^+$ and $CD45RB^+$. The vertical line represents a negative control limit.

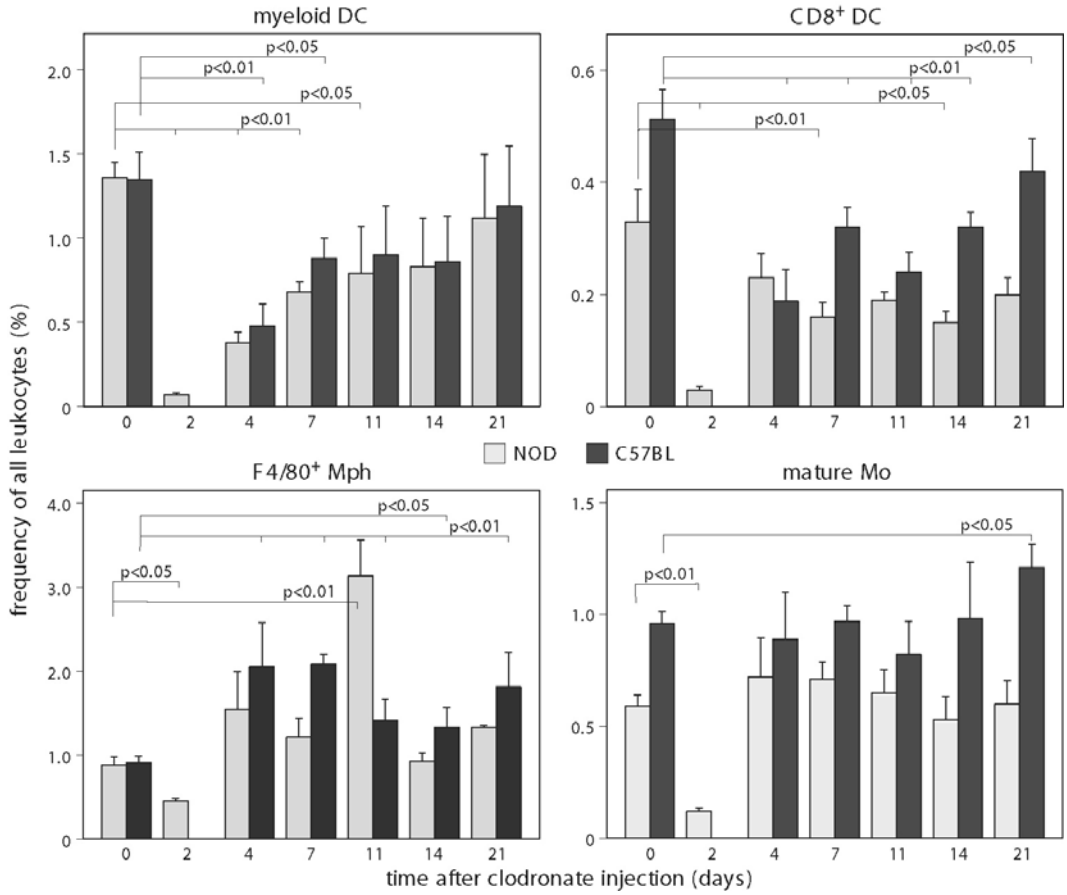

Figure 2.2B Normal maturation kinetics of the NOD DC and Mph compartment after lip-Cl_2MDP in the spleen.

B. Time course of the depletion and repopulation of the myeloid cells in the spleen as defined by phenotype in A. Each bar represents an average value of a minimum of 3 mice per mouse strain per time point. P-values indicated graphs represent the statistical significance (Student's t-test) of the indicated time point vs. the point 0 in a given mouse strain.

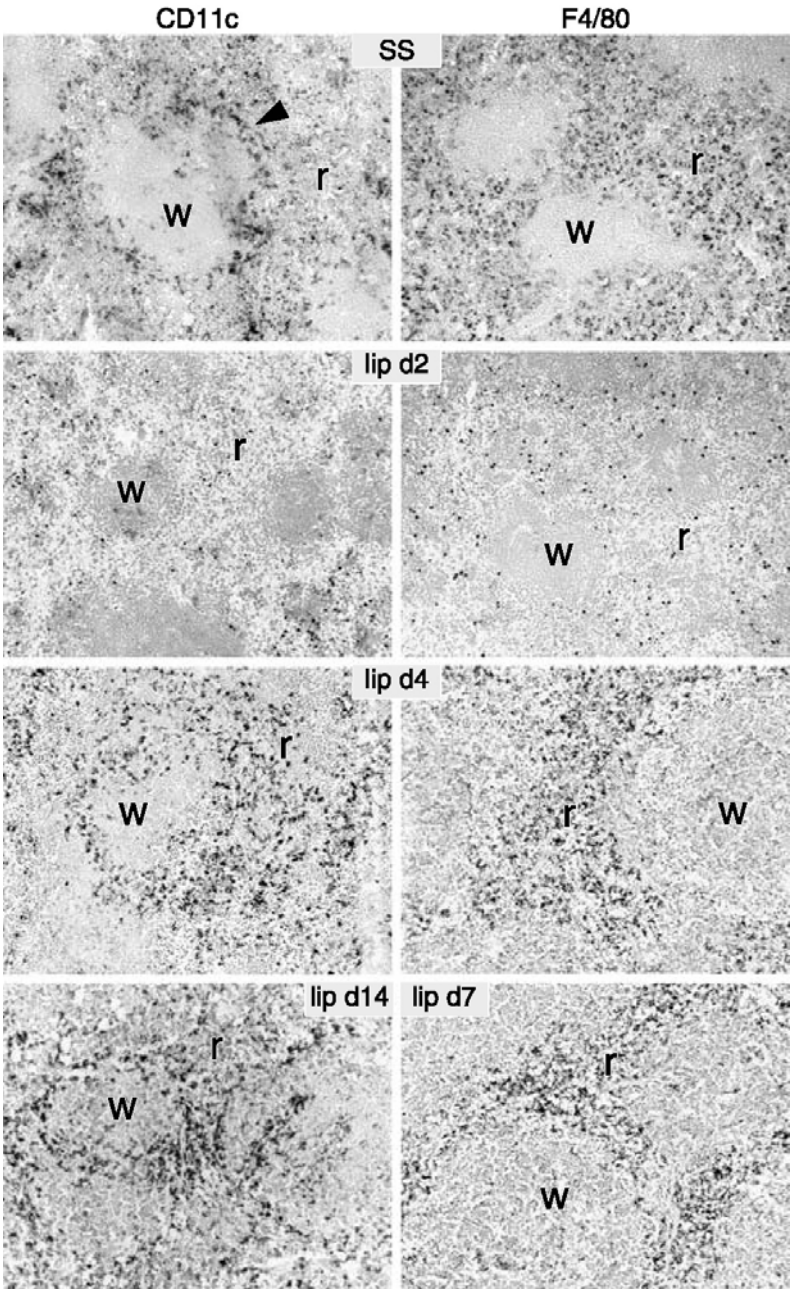

Figure 2.3 Immuno-histochemical analysis of the distribution of CD11c$^+$ and F4/80$^+$cells in the NOD spleen at different time points after lip-Cl_2MDP treatment.

A-B. Distribution of cells before the treatment (SS, steady state). **C-D**. At day 2 after injection (lip d2) DC and Mϕ were completely depleted from the spleen red pulp. **E-F**. The return of F4/80$^+$ cells in particular, is evident already at day 4 after injection (lip d4). **G-H**. Normal distribution of CD11c$^+$ and F4/80$^+$ cells was observed from days 14 (lip d14) and 7 (lip d7) after depletion, respectively. Symbols depicted indicate: r: red pulp, w: white pulp.

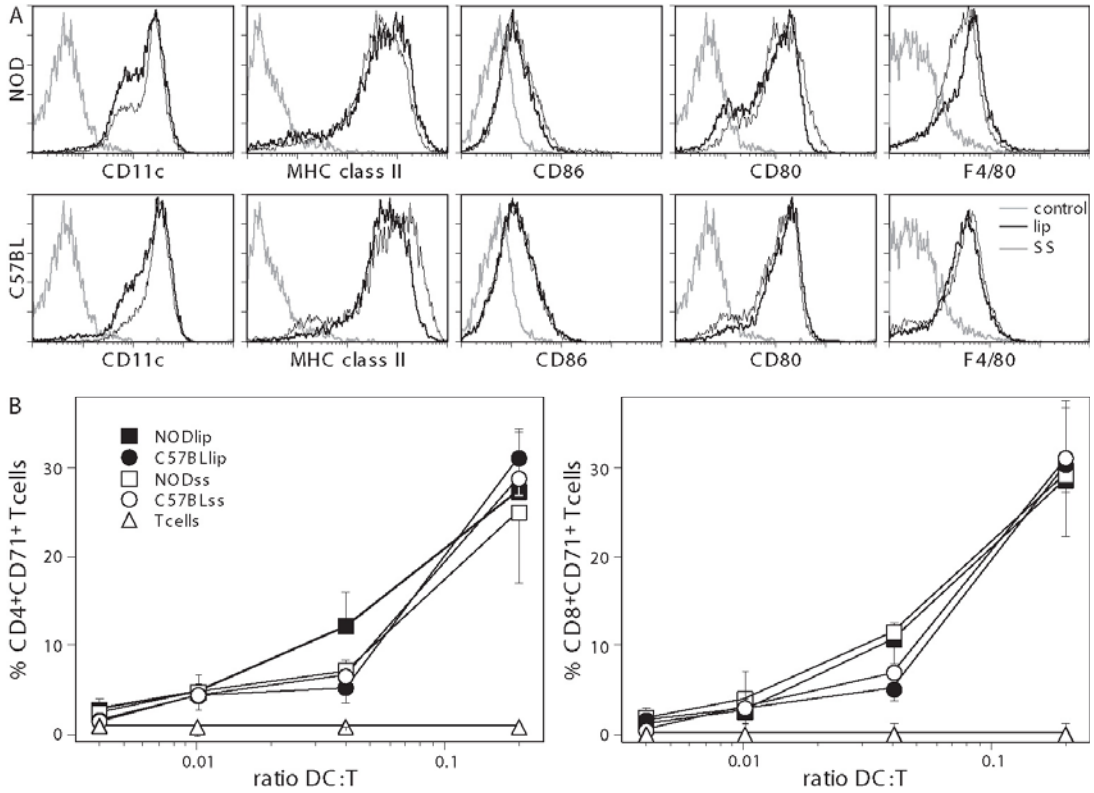

Figure 2.4 Phenotype and T cell stimulatory capacity of DC isolated from spleens from treated or untreated NOD and C57BL mice.

A. Isolated CD11c+ cells from the spleens of treated or untreated NOD and C57BL mice showed a normal mature DC phenotype. Histograms display the marker expression in the autoMACS-isolated population. The grey line represents the negative isotype control, the thin-black line the marker expression by cells isolated from untreated mice and the thick-black line the expression pattern of cells isolated from treated mice at day 7 after treatment.

B. The stimulation capacity of isolated DC was the same in NOD and C57BL mice irrespective of whether DC were derived from treated or untreated mice. Stimulation of $CD4^+$ and $CD8^+$ cells was measured as a percentage of cells that have upregulated expression of transferrin-receptor (CD71) on their surface. Representative histograms (in A.) and average values ± SEM (in B.) of three separate pools from two independent experiments for each group are shown in the figure.

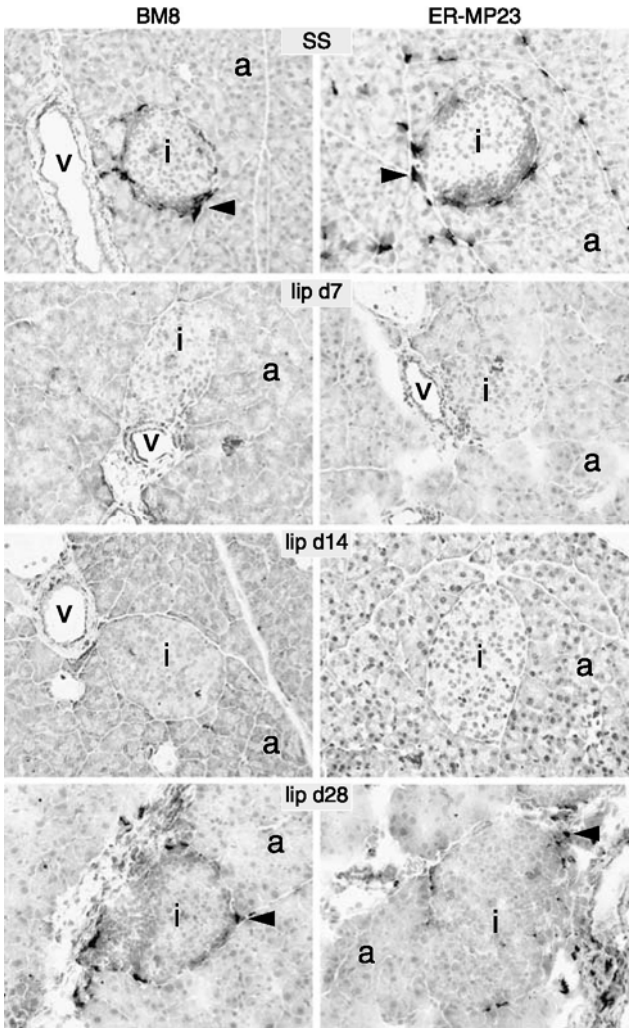

Figure 2.5 Prolonged disappearance of Mϕ from the pancreas of NOD mice after lip-Cl_2MDP treatment.

A-B Peri- and intra-islet infiltrates from untreated 8wk old NOD mice contain $BM8^+$ and $ER\text{-}MP23^+$ Mϕ. Both subsets of Mϕ were also found scattered throughout the connective tissue of the exocrine pancreas. C-D. From day 7 after lip-Cl_2MDP treatment a considerable amount of resident Mph had disappeared from the endocrine pancreas. E-F. At day 14, the pancreas is found similarly devoid of Mϕ as at day 7. G-H. Mϕ were again detectable at day 28 after the lip-Cl_2MDP treatment and present in the close proximity of pancreatic islets. Symbols depicted indicate: a: exocrine tissue, i: islet, v: vessel.

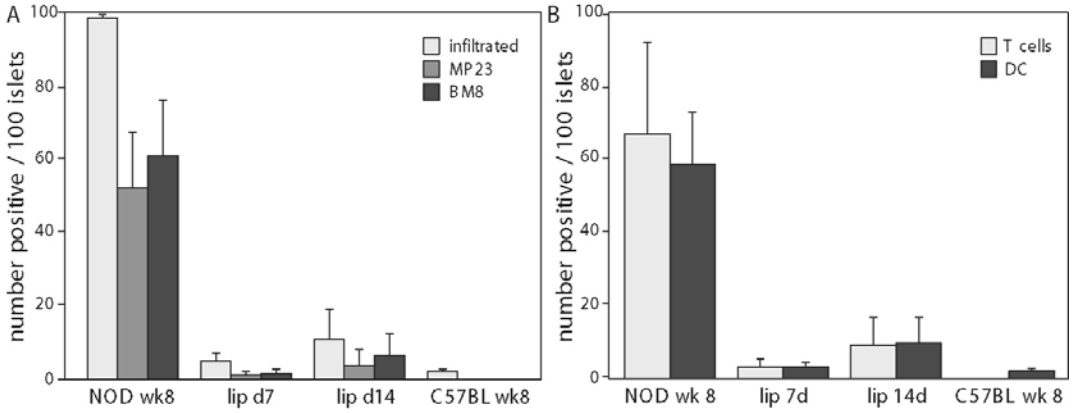

Figure 2.6 Differential analysis of the islets with inflammatory infiltrations before and after lip-Cl_2MDP treatment.

The treatment significantly decreased the frequency of islets infiltrated with Mϕ and DC, which directly correlated to the disappearance of lymphocytes from the pancreas.

A. Correlation of the Mϕ presence with islet infiltration, at different time points. Light-grey bars represent the percentage of infiltrated islets, determined by morphological examination of glucagon-positive islets. Dark-grey bars show the frequency of islets associated with ER-MP23$^+$ Mϕ and black bars represent values for the BM8$^+$ Mϕ; before the treatment, at different post-treatment time points and in untreated C57BL mice.

B. Correlation of a DC loss with the departure of T cells, in the pancreas of treated mice. Black bars represent the percentage of islets that contained CD11c$^+$ DC and light-grey bars the percentage of islets that contained T cells in NOD mice, like in A. All bars show the average value (SEM) calculated from 3 or 4 mice per group for each strain and each time point.

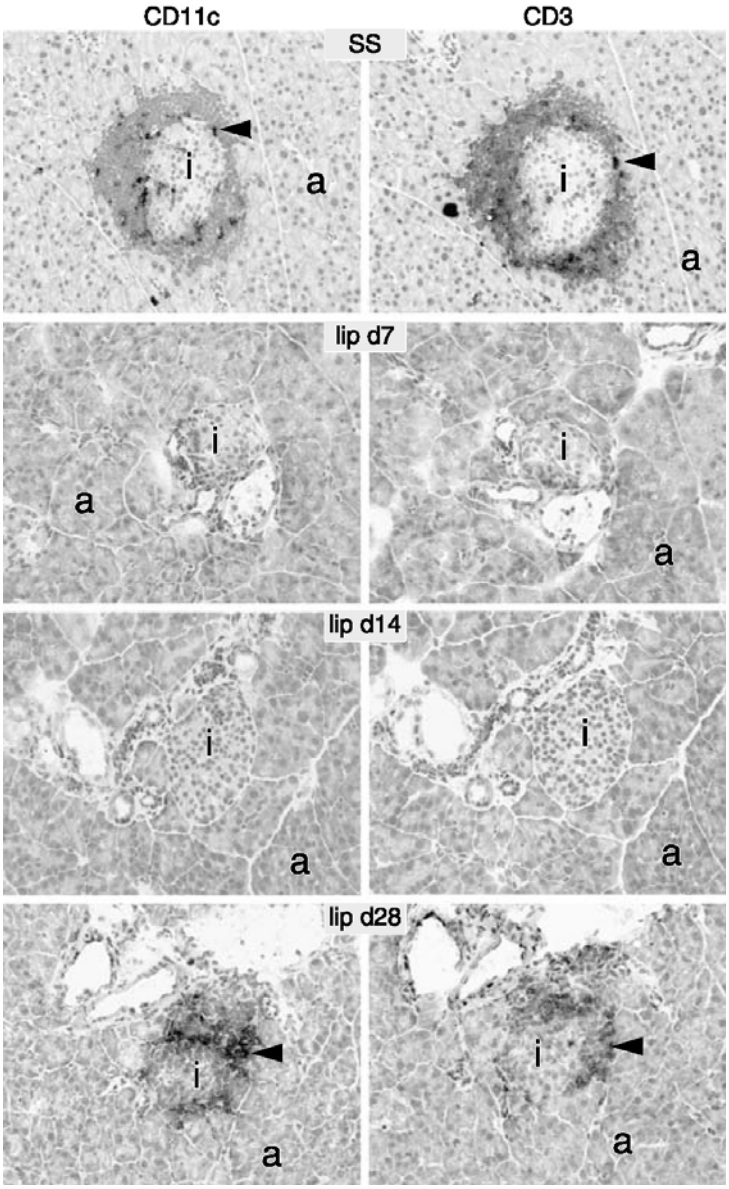

Figure 2.7 The presence of T cells correlated directly with that of DC in the pancreas of NOD mice.

A-B. Before the treatment with lip-Cl_2MDP, DC were found in the close proximity of T cells as judged by the immuno-histochemical analysis of serial sections of the NOD mouse pancreas. **C-D.** At day 7 after the treatment, DC had disappeared from the pancreas and T cells as well. **E-F.** Islets that were free of DC were also free of lymphocytic accumulations in the pancreas, at day 14 after the treatment. **G-H**. At 28 days after the treatment, return of DC was found in a few islets, paralleled by the return of T cells. Symbols depicted indicate: a: exocrine tissue, i: islet.

Chapter 3

Macrophages in the Murine Pancreas and their involvement in Fetal Endocrine Development *in vitro*

Submitted for publication

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Abstract

Macrophages are a heterogeneous population of cells that belong to the mononuclear phagocyte system. In addition to their role in tissue homeostasis and remodeling, they are also potent immune regulators. Pancreatic macrophages are critically involved in the development and pathogenesis of autoimmune diabetes. Yet, the ontogeny of pancreatic macrophages is not known. Therefore, we characterized in this study the macrophages present in the adult and developing mouse fetal pancreas of normal mice. We additionally examined the presence of macrophage precursors and the involvement of macrophages in the growth of endocrine tissue in the fetal pancreas.

We have identified two phenotypically distinct macrophage subsets in the adult pancreas. The majority of macrophages were characterized by the expression of murine macrophage galactose-type C-type lectin and sialoadhesin. Under non-inflammatory conditions only a minority (approximately 5 percent) of the pancreatic macrophages additionally expressed the macrophage marker F4/80. In contrast, in the fetal pancreas phenotypically mature macrophages were exclusively identified by their expression of F4/80 and they lacked the expression of murine macrophage galactose-type C-type lectin and sialoadhesin. Furthermore, we show that macrophages develop from pre-existing precursors that are present in the fetal pancreas at E12.5. The number of macrophages significantly increased in fetal pancreas explants cultured with M-CSF. Importantly, this increase of F4/80-positive cells was paralleled by an increase in the number of insulin-producing cells, suggesting that macrophages support the growth of insulin-producing cells.

Abbreviations used:

Bone marrow, BM; dendritic cell, DC; macrophage-colony stimulating factor, M-CSF; normal mouse serum, NMS; tyramide signal amplification^{TM}, TSA; nonobese diabetic mouse, NOD.

3.1 Introduction

Macrophages belong to the mononuclear phagocyte system and, regarding their function and phenotype, comprise a heterogeneous population of cells. Next to their important immune-regulatory role they are also involved in the remodeling of various tissues during embryonic development and adult life [1]. Unfortunately, macrophage activity does not always have a beneficial outcome and macrophages are believed to play key roles in the autoimmune process leading to type 1 diabetes [2-5]. In non-obese diabetic (NOD) mice that develop diabetes spontaneously, macrophages are among the first leukocytes that accumulate around the insulin-producing islets, before lymphocyte-reactivity against islet-cells is evident. The early intra-islet infiltration of macrophages is associated with the progression of insulitis and finally the development of diabetes. Yet, the ontogeny and primary function of pancreatic macrophages under non-inflammatory conditions is not known.

It is generally accepted that bone marrow (BM)-derived monocytes are the blood-borne precursors of macrophages [6], but the ontogeny of resident tissue macrophages overall is poorly understood. Under inflammatory conditions, monocytes will infiltrate affected peripheral tissues in response to acute or chronic stimuli and differentiate into either dendritic cells (DCs) or exudate macrophages [7]. Peripheral blood monocytes express differential levels of Ly-6C, uniform levels of the early myeloid marker ER-MP58, high levels of CD11b, low levels of F4/80, and insignificant levels of markers specific for mature macrophages like CD16/CD32 [8,9]. BM-derived monocytes rapidly loose the expression of Ly-6C upon differentiation into macrophages *in vivo* [10] or *in vitro*, when stimulated with macrophage-colony stimulating factor (M-CSF) [11].

It is not certain whether tissue macrophages are replenished by blood monocytes under non-inflammatory conditions. Adult tissue-resident macrophage populations that are maintained independently from circulating monocytes have been identified in different organs, and they possibly derive from a BM-independent precursor pool that proliferates locally [12]. In conjunction, populations of resident macrophage-subsets, like Kupffer cells in the liver and microglia in the central nervous system, may derive from primary seedings of fetal macrophages [13-16].

Mature F4/80$^+$ (BM8$^+$), murine macrophage galactose-type C-type lectin-positive (MMGL$^+$/ER-MP23$^+$) and sialoadhesin$^+$ (MOMA-1$^+$) macrophages [17-19] and CD11c$^+$ DCs have been detected in the early postnatal pancreas of various mouse strains from birth on [3, 20]. Their frequency and in particular that of the F4/80$^+$ macrophages significantly decreases from birth to weaning as the result of exocrine expansion during the postnatal development of the pancreas.

In spite of the important role in diabetogenesis that was suggested for pancreatic macrophages, they have not been fully characterized in the non-inflamed pancreas. The distinctive tissue localization and age-related changes in the frequency of cells labeled positively for the different macrophage markers suggest that different macrophage subsets are present in the mouse pancreas. The examination of the pancreatic macrophage compartment under non-inflammatory conditions may give more insight into their ontogeny and normal function.

Therefore, we characterized in this study the macrophages present in the adult and developing fetal pancreas under non-inflammatory conditions by using different combinations of antibodies (Table 1) against antigens expressed by mature resident macrophages like F4/80, sialoadhesin (MOMA-1), MMGL (ER-MP23), M-CSFR and CD11b, and antibodies against macrophage-precursors like CD31 (ER-MP12), Ly-6C (ER-MP20) and ER-MP58. Additionally, we have examined the presence of early macrophage precursors in fetal pancreas explants by culturing these explants with or without M-CSF and analyzing the presence of macrophages thereafter. Since macrophages are involved in tissue development, we have studied in parallel the development of insulin- and glucagon-producing cells in these cultured fetal pancreas explants.

3.2 Material and Methods

3.2.1 Animals.

Pregnant C57BL/6 female mice were bred at the animal facility of BIOMEDICUM, Helsinki, Finland. Mothers were sacrificed and embryos removed at embryonic ages E12.5, E14.5 and E17.5. Adult 7-wk old female C57BL/6 and BALB/c mice were obtained from Harlan (Horst, the Netherlands) and kept at the animal facility of the Erasmus MC. Adult mice were sacrificed at 11 or 35 wks of age. All strains were kept under specific pathogen-free conditions and fed *ad libitum*. A minimum of 5 animals per age/strain/group was used for all studies. Animal use and handling followed the ethical guidelines provided by the European Union.

3.2.2 Pancreatic explant culture.

Pancreatic explant cultures of C57BL/6 mice of embryonic age 12.5 (E12.5) were used to study the presence of early macrophage precursors. The appearance of the vaginal plug was noted as E0.5. The embryonic duodenal loop along with the dorsal and ventral pancreatic bud and stomach were microdissected and cultured according to methods designed for embryonic kidney culture [21, 22]. Briefly, the explants were placed on Nucleopore filters (1.0 μm pore size, Costar, Corning, NY) on metal grids and cultured at the air-liquid interphase in serum-free improved MEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with transferrin (30 μg/ ml), penicillin (100 IU/ml), streptomycin (100 μg/ml) and with or without 50 ng/ml recombinant murine M-CSF (R&D Systems, Abingdon, UK). After 5 days of culture the explants were embedded in Tissue-Tek (Miles, Elkart, IN, USA), frozen in dry-ice chilled isopentane and stored at -80 °C or fixed for 4h at room temperature in Bouin's fixative, rinsed with 50% alcohol and stored in 70% alcohol before dehydration and paraffin embedding.

3.2.3 Immunohistochemistry and immunofluorescence.

Adult pancreases were embedded in Tissue-Tek (Miles, Elkart, IN, USA) and frozen in liquid nitrogen. Five μm thick cryostat sections were cut at 100-200 μm intervals in series of 5 or 6 sections per pancreas, dried and stored at -80°C. Fetal pancreases or cultured explants were entirely cross-sectioned in 5 μm thick cryostat sections or 2 μm thick paraffin sections. Cryosections were fixed for 10 min in acetone containing 0.03% hydroxyperoxide and subsequently air-dried. Paraffin-embedded sections were deparaffinized, fixed for 30 min in methanol containing 0.03% hydroxyperoxide and rehydrated. Further processing followed similar proceedings as the cryosections.

Sections were incubated with 2% normal mouse serum (NMS) in PBS for 1h at RT to block non-specific binding sites. If stainings were amplified with Tyramide Signal Amplification™, NMS was diluted in 0.1 M Tris-Cl, 0.15M NaCl, 0.5% blocking reagent (Perkin Elmer Life Sciences, Boston, USA). If biotinylated antibodies were used an additional blocking step against endogenous avidin and biotin was applied according to the manufacturer's protocol (Vector Laboratories, Burlingame, USA). Slides were washed

in phosphate-buffered saline with 0.05% Tween-20 and incubated overnight at 4°C with primary antibodies specific for different macrophage, endothelial or endocrine markers (Table 1). Sections were washed with PBS-Tween-20 and incubated with appropriate secondary antibodies in the presence of 1.5% normal mouse serum (peroxidase-conjugated: swine-anti-guinea pig (PO141), swine-anti-rabbit-peroxidase (PO399) and goat-anti-rat (PO162), (all DAKO, Carpinteria, USA), alkaline phosphatase-conjugated: swine-antirabbit (DO306), (DAKO) or fluorochrome-conjugated: $F(ab')_2$ goat-anti-rat-FITC, (Serotec GmbH, Düsseldorf, Germany) and goat-anti-rabbit-TRITC (BI 2207), (Biosys S.A.,Compiègne, France). Biotin-conjugated antibodies were detected with streptavidin-texas-red (Caltag, Burlingame, USA). The 3-amino-9-ethylcarbazole substrate (Sigma, Louisville, USA) was used for detecting specific peroxidase activity in 50 mM sodium acetate/0.02% hydroxyperoxide. Fast-blue BB base was used to detect AP-activity.

Immunohistochemical stainings were counterstained with Mayers hemalum solution (Merck, Darmstadt, Germany) and embedded in Kaisers glycerol gelatin (Merck). Immunofluorescence stainings were embedded in DAPI-containing Vectashield (Vector Laboratories). The resulting labeling was examined by normal light microscopy or fluorescence microscopy. Adult spleen and liver served as positive control tissue for all stainings.

3.2.4 Cell quantification.

To quantify macrophages and insulin- and glucagon-expressing cell numbers after explant culture, every fourth section of the cross-sectioned explants was successively stained for F4/80 (cryostat sections), insulin or glucagon (paraffin sections). For each antibody a minimum of 16 sections per explant, each section cut at a different level, was stained. Positively labeled cells with visible nuclei were counted by light microscopy at an optical magnification of 400x. The corresponding surfaces of the pancreas explants were measured by marking the border with a computer-interfaced freehand tool at an optical magnification of 8x. Data were analyzed with the VIDAS-RT software (Kontron Elektronik GmbH/Carl Zeiss, Weesp, The Netherlands).

3.2.5 Statistical analysis.

Data were expressed as mean ±SD or ±SEM; *P* values were calculated with the Man-Whitney *U* test.

Table 3.1 Description of antibodies used for immunohistochemical or immunofluorescent staining

MAb (host species)	Ag recognized	Ag description	Tissue localization	company
Endocrine cell markers:				
insulin (guinea pig)	insulin	hormone	β-cells in pancreas	Dako
glucagon (rabbit)	glucagon	hormone	α-cells in pancreas	Dako
Endothelial cell marker:				
von Willebrand factor (rabbit)	FVIII	clotting factor	endothelial cells	Dako
General leukocyte marker:				
30G12 (rat)	T-200/CD45	protein tyrosine phophatase	hematopoietic cells	hybridoma ownculture
Precursor macrophages:				
ER-MP12 (rat)	CD31	single chain glycoprotein	subpopulations of myeloid precursor cells, endothelial cells, low on lymphocytes	ownculture
ER-MP20 (rat)	Ly-6C	glycoprotein	macrophage precursors, monocytes, endothelial cells	ownculture
ER-MP58 (rat)	unidentified		myeloid precursors, granulocytes	ownculture
Mature macrophages or DC:				
F4/80 (rat)	homologue of human EMR1	glycoprotein	macrophages, low on monocytes and eosinophils	Caltag
BM8 (rat)	F4/80	glycoprotein	see F4/80	BMA,Augst Switzerland
M1/70 (rat)	CD11b (Mac-1)	C3bi integrin, CR3	myeloid cells	ownculture
AFS-598 (rat)	CD115	M-CSF receptor	monocytes and macrophages	ownculture
2.4G2 (rat)	CD16/32	FcRγ receptor II/III	myeloid cells, B-cells	ownculture
MOMA-1 (rat)	sialoadhesin	sialic acid-specific lectin	metallophilic macrophages and Kupffer cells	ownculture
ER-MP23 (rat)	MMGL/ DC-asialoglycoproteinR	galactose-specific lectin	Histiocyte-like macrophages	ownculture
N418 (hamster)	CD11c	Ax integrin	DC, low on monocytes	ownculture

3.3 Results

3.3.1 ER-MP23$^+$F4/80$^-$ macrophages represent the largest resident population in the adult pancreas.

High numbers of MMGL$^+$ macrophages were found randomly scattered in the connective tissue of inter- and intralobular septa and perivascular areas of the adult pancreas (Fig.3.1A). A similar distribution pattern was observed for the labeling of sialoadhesin (MOMA-1) (Fig.3.1B), a marker also expressed by metallophilic macrophages in the spleen and lymph nodes, and by Kupffer cells in the liver. As compared to MMGL expression, cells positive for the M-CSF receptor, CD115 (Fig. 3.1C), the general macrophage marker F4/80 (Fig. 3.1D) or the β2 integrins CD11b (Fig.3.1E) or CD11c (not shown) were rarely observed (table 3.2) and only occasionally found in the outer pancreatic capsule.

Different distribution patterns of labeling were observed for precursor macrophage markers. Cells positively labeled with the myeloid precursor specific antibody ER-MP58 were observed (Fig. 3.1F), though rarely, whereas individual cells expressing the myeloid precursor markers CD31 (ER-MP12) (Fig. 3.1G) and Ly-6C (ER-MP20) (Fig. 3.1H) were widely expressed throughout the exocrine tissue. These latter two antibodies additionally labeled vascular structures in the pancreas.

To assess the hematopoietic origin of the myeloid marker expressing cells we used fluorescent double labeling with the pan-leukocyte marker CD45. Labeling for CD45 was observed on all MMGL$^+$ cells as well as on all F4/80$^+$ and CD11b$^+$ cells (not shown). MMGL$^+$ cells were also labeled by the sialoadhesin-specific antibody MOMA-1 (Fig. 3.2A) and occasionally expression of CD115 was detected (Fig. 3.2B). F4/80$^+$ cells expressed MMGL (Fig. 3.2B), however consistent with the lower number of F4/80$^+$ cells present, the larger part of MMGL$^+$ cells were not labeled by F4/80 or BM8 antibodies

Labeling for ER-MP58$^+$ colocalized with CD45$^+$ (Fig. 3.2D), but not with Ly-6C (Fig. 3.2E). Most, if not all, Ly-6C$^+$ cells did express CD31 (Fig 3.2F). Notably, neither labeling of CD31 (Fig. 3.2G) nor that of Ly-6C (not shown) co-localized with that of CD45. Both markers co-localized with the endothelial marker Von Willebrand factor on endothelial cells of the vascular structures of the pancreas (Fig. 3.2H).

Table 3.2 Number of macrophages in the adult murine pancreas relative to the total pancreas surface area (mm^2)

Strain	antigen	cells/mm^2	n
C57BL/6	MMGL	17.98±7.28	5
BALB/c	MMGL	20.42±0.61	2
C57BL/6	F4/80	0.89±0.28	4
BALB/c	F4/80	0.35±0.31	2
C57BL/6	CD11b	ND	
BALB/c	CD11b	1.14±0.19	2
C57BL/6	CD11c	0.43±0.14	2
BALB/c	CD11c	0.20±0.03	2

3.3.2 Mature F4/80$^+$/CD45$^+$macrophages are first observed at E14.5 in the fetal pancreas.

By using immunofluorescence on fetal sections, CD45$^+$ cells were readily detected in the pancreatic buds, duodenal loop and stomach at E12.5 (Fig. 3.3A), the earliest embryonic age examined. However, labeling for markers of mature macrophages was not observed at this age. CD45$^+$F4/80$^+$ cells were first observed in the pancreas at E14.5 (Fig. 3.3B) in parallel with the appearance of F4/80, CD11b- and CD115-expressing cells in the fetal liver (not shown). Labeling for other markers of mature macrophages, even that of CD11b, was absent in the pancreas at E14.5. Pancreatic F4/80$^+$CD45$^+$ macrophages were still present in the fetal pancreas at E17.5 (Fig. 3.3C) and at this age often expressed CD11b (Fig.3.3D). Few MMGL$^+$ cells were present in the pancreas from E17.5 onwards (not shown), whereas expression of sialoadhesin was not observed at any age, in none of the pancreases examined. Interestingly, F4/80$^+$ cells were frequently found in close apposition to insulin-producing cells (Fig. 3.3E).

Cells positively labeled for the early myeloid precursor markers CD31, Ly-6C and ER-MP58 were present in the fetal pancreas from E12.5 onwards (data not shown). In the fetal liver CD31 and Ly-6C were expressed by both CD45$^+$ and CD45$^-$ cells at E14.5 (not shown). Similar to the adult pancreas, labeling of CD31 and Ly-6C did not co-localize with CD45 suggesting these markers were expressed by endothelial structures in the fetal pancreas as well. ER-MP58$^+$ cells did express CD45 in the fetal pancreas at E17.5, although labeling for ER-MP58 was rarely observed (not shown).

3.3.3 Macrophage precursors are present in the fetal pancreas at E12.5.

To examine the presence of macrophage precursors before any labeling of mature macrophage markers was detected in the fetal pancreas, pancreatic buds along with the stomach and duodenal loops were excised at E12.5 and cultured for 5 days with or without M-CSF. Interestingly, after 5 days of culture F4/80$^+$ cells were indeed observed in the explants, even without the addition of exogenous M-CSF (Fig. 3.4A). In accordance with the labeling of fetal pancreases at E17.5, CD11b was the only other marker observed in the cultured explants (not shown). Labeling of other mature macrophage markers was not detected, not even after immunohistochemical amplification of the signal. Thus, intra-pancreatic precursors were present at E12.5 and capable to differentiate into mature macrophages independent of the presence of M-CSF. However, supplying M-CSF induced a 3-fold increase in the number of F4/80$^+$ cells present per total explant surface area (Fig. 3.4B, C).

3.4 Exogenous M-CSF affected endocrine cell growth in fetal explants *in vitro*.

Since macrophages are involved in tissue development, we investigated whether the addition of M-CSF affected the development of insulin- and glucagon-expressing cells in the cultured explants. Insulin-producing cells were readily detected in explants cultured with M-CSF (Fig.3.5A-B) or without M-CSF (not shown), similar to glucagon-producing cells (Fig. 3.5A-B) that were found adjacent to the insulin-producing cells. Interestingly, the number of insulin$^+$ cells increased more than 4-fold when explants were cultured with

M-CSF. An increase in the number of glucagon$^+$ cells was also observed, but to a much lower extent (Fig. 3.5C) and the difference did not reach statistical significance.

3.4 Discussion

By using different combinations of antibodies recognizing mature macrophages or macrophage precursors, we have determined the phenotype of resident macrophages in the non-inflamed adult pancreas as $CD45^+MMGL^+sialoadhesin^+$ cells with the occasional expression of M-CSF-R. These cells are randomly distributed in the connective tissue of the adult pancreas in high numbers. By contrast, in the adult non-inflamed pancreas only a small subset of these cells is positive for the F4/80 antigen, an antigen that is regarded as a general marker for macrophages. $F4/80^+$ cells mostly reside in the outer capsule of the pancreas and less frequently in the connective tissue septa. Interestingly, F4/80 cells are abundantly present in the NOD pancreas as has been described earlier [3]. In NOD mice their presence is correlated to islet destruction and they are virtually absent in BDC2.5 TCR-transgenic NOD mice that have a strongly reduced incidence of diabetes [23]. This suggests that the pancreatic $F4/80^+$ cells in the NOD mouse possibly represent a subset of inflammatory macrophages that is distinct from the resident macrophages in the non-inflamed pancreas and that may have been recruited recently to the pancreas in response to local inflammation.

In previous studies precursors of macrophages and DCs were successfully detected using the ER-MP58 antibody and antibodies recognizing CD31 and Ly-6C antigens [24-26]. Since CD31 and Ly-6C are also expressed by endothelial cells [27, 28], identification of tissue-resident macrophage precursors depended on the co-expression of CD45. In the pancreas CD31 and Ly-6C were clearly expressed by vascular structures and by single cells that lacked the expression of CD45. Therefore, it is not likely that these cells represent precursors of pancreatic macrophages. The ER-MP58 antibody labeled individually scattered cells expressing CD45. Importantly, labeling with ER-MP58 is not fully specific for macrophage precursors as ER-MP58 additionally recognizes granulocytes and their precursors. Cells expressing Gr-1 have been observed in the adult pancreas (unpublished observations). Since labeling of ER-MP58 was not accompanied by that of other markers characteristic for macrophage precursors, the observed ER-MP58 labeling might also identify eosinophilic or neutrophilic granulocyte developmental stages.

Phenotypically mature macrophages were not observed in the fetal pancreas until E14.5, although $CD45^+$ cells were already present in the pancreatic buds and surrounding tissues from E12.5 on. Interestingly, in contrast to the pancreatic macrophages of adult mice, mature macrophages in fetal pancreases were all characterized by the expression of F4/80 and at later stages a sub-population of these cells additionally expressed CD11b. $MMGL^+$ cells were not observed until E17.5, and other markers like sialoadhesin or CD11c were not observed at all. The order of appearance of the expression of these different macrophage markers in the pancreas followed the kinetics of appearance that was described for other embryonic tissues. F4/80 was the first marker observed in the liver, spleen, thymus, peritoneum and kidney from E12 on [29], followed by the expression of CD11b in most of these tissues [30]. Immunohistochemical expression of MMGL was first observed in the small intestine at E14 [31], and sialoadhesin expression was detected in the gut and spleen at E15 and

E17 respectively [32]. The early expression of MMGL and sialoadhesin in respectively the intestine and spleen could not be confirmed in the present study and this might be explained by the usage of different antibodies or sensitivity of the method used.

The F4/80$^+$ cells that were observed at E14.5 do not likely enter the pancreas via the blood circulation, since functional blood vessels containing a lumen are not observed until E15.5 in the murine pancreas [33]. At E12.5 the pancreatic buds exclusively contained CD45$^+$ cells, whereas F4/80$^+$CD11b$^+$ were observed when the excised E12.5 pancreatic anlage was further cultured for 5 days in serum-free medium. This indicates the presence of macrophage precursors at E12.5 that possess the capacity to differentiate into cells with a macrophage-like F4/80$^+$CD11b$^+$ phenotype. In accord with the *in vivo* situation, the expression of MMGL or other mature macrophage markers was not observed in the cultured explants. This may suggest that MMGL$^+$sialoadhesin$^+$ cells represent a distinct population of macrophages that is seeded at later stages of gestation, possibly via the vasculature, and their presence appears to be continued after birth.

In the cultured pancreas anlage the differentiation of the precursors did occur without the addition of exogenous M-CSF and was possibly induced by endogenous M-CSF or other growth factors present within the tissue during culture. Importantly, we showed that the addition of M-CSF to the fetal pancreas explant cultures resulted in a 3-fold increase in the number of F4/80$^+$ cells. The presence of M-CSF-dependent and -independent macrophage populations was recently shown in the pancreas of *op/op* mice [34]. These mice lack functional M-CSF and are devoid of several macrophage populations in different organs. F4/80$^+$ cells were detected in the pancreas of *op/op* mice at E18.5 (albeit at very low numbers), supporting the presence of two distinct pancreatic macrophage populations. Interestingly, these macrophage-deficient mice additionally show a severe decrease in insulin mass as a result of hypoplasia and decreased proliferation of insulin$^+$ cells. In accordance with those results we show here that the addition of M-CSF to the fetal explant cultures resulted in a 4-fold increase of the number of insulin$^+$ cells *in vitro*. The presence of F4/80$^+$ macrophages often correlates to tissue organogenesis [35]. Taking into account the absence of CD115 on the exocrine and endocrine cells [this paper and 34], the effect of M-CSF is likely mediated via the differentiation and activation of the macrophage precursors. In this respect, IL-6 is an interesting involved candidate mediator considering its involvement in tissue morphogenesis, and M-CSF increases the expression and production of IL-6 in macrophages [36]. Overexpression of IL-6 results in islet hyperplasia and neo-ductular formation [37] and additionally increases the expression of preproinsulin mRNA and thus, can be involved in islet metabolism and neogenesis [38].

The current phenotypic analysis merely allows speculation on the developmental relationship of the F4/80$^+$ fetal macrophages and F4/80$^+$ and F4/80$^-$ macrophages observed in the adult pancreas. F4/80 expression may be down-regulated during the retention of the macrophages in the pancreas paralleled by the acquisition of MMGL and sialoadhesin expression. Although F4/80 expression normally increases with cell maturation [39], migrating Langerhans cells have been reported to lose the expression of F4/80 upon their activation [40]. Alternatively, a rapid decline in the number of F4/80 cells

was observed in the pancreas from birth to weaning [20]. This decline may be explained by the expansion of the exocrine pancreas during this period, but can also be suggestive for the disappearance of the early F4/80$^+$ fetal macrophages.

In conclusion, we show that the resident pancreatic macrophage compartment in adult mice is not represented by a single phenotype, but comprises different phenotypes with a major population of MMGL$^+$sialoadhesin$^+$F4/80$^-$CD45$^+$ cells and a minor population of MMGL$^+$sialoadhesin$^+$F4/80$^+$CD45$^+$ cells. Fetal pancreatic macrophages are exclusively identified by the expression of F4/80 and, similar to what has been published recently *in vivo* [31], they appear to play an important role in the growth of endocrine tissue in the fetal pancreas *in vitro*.

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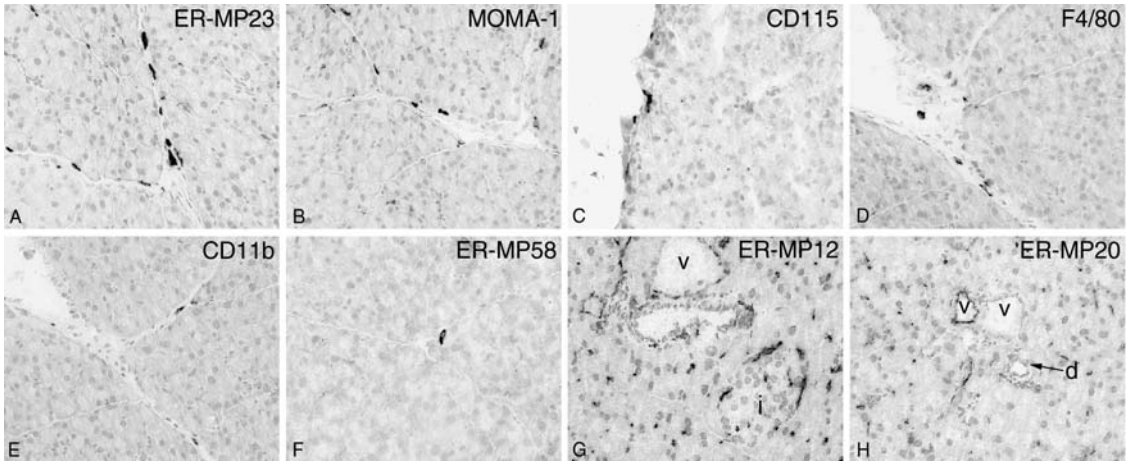

Figure 3.1. Differential labeling patterns of markers for mature macrophages and macrophage precursors in the adult murine pancreas.

Antibodies recognizing antigens expressed on mature macrophages or macrophage precursors showed a differential labeling pattern in the pancreas, here shown for C57BL/6 mice at 9 wks of age. Antibodies used are depicted in the figure. Symbols depicted indicate: **i**: islet, **d**: duct, **v**: vascular structures, original magnifications are 200x.

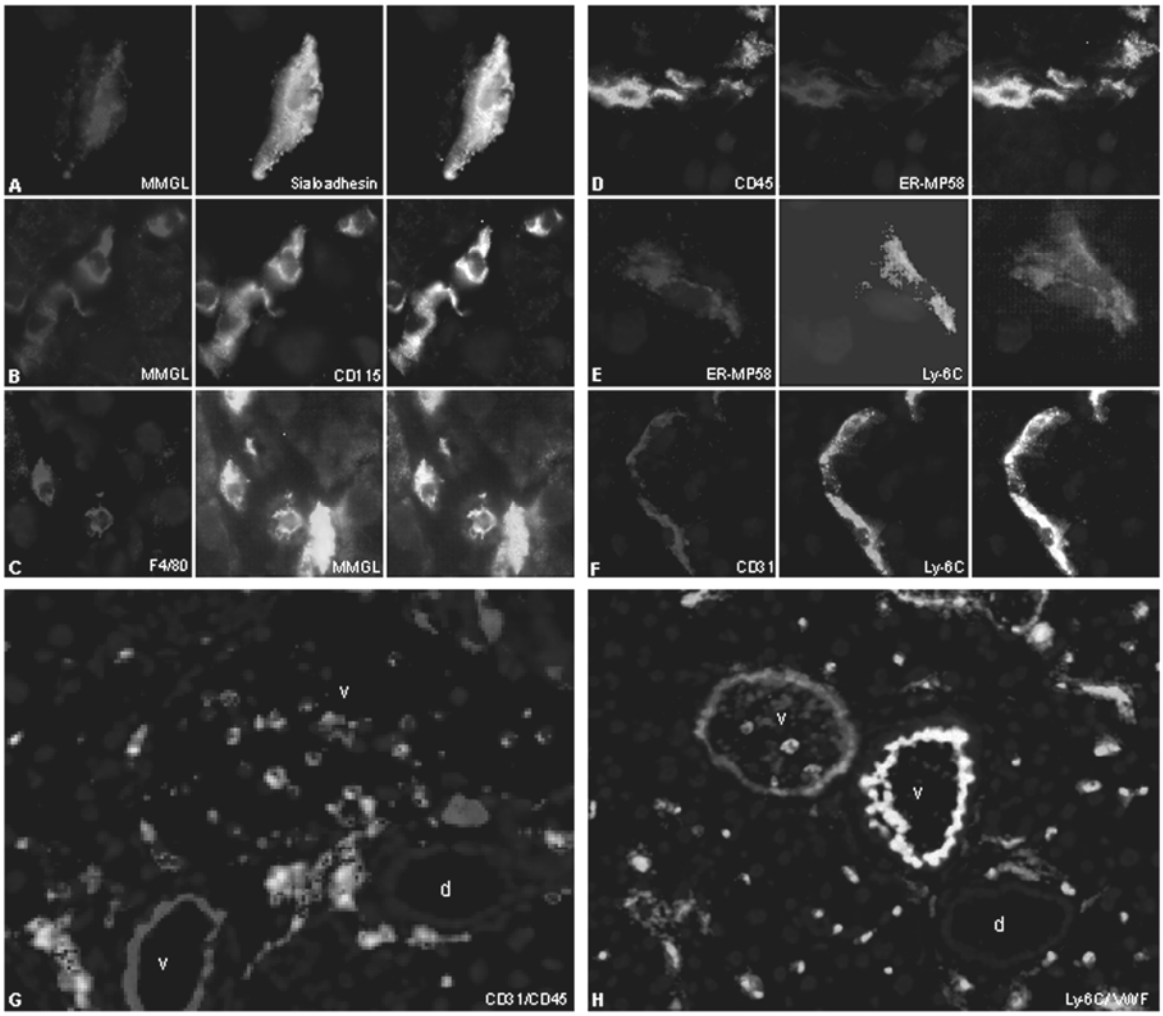

Figure 3.2 The adult pancreatic macrophage compartment is comprised of phenotypically distinct mature macrophage subsets.

Immunofluorescent labeling of frozen pancreas sections with combinations of different markers for mature macrophages (**A-C**) or macrophage precursors (**D-H**). Conjugates used: Texas red (red labeling) combined with FITC (green). **A-F**: Antibodies used are depicted. Co-localization of the labeling is depicted in yellow in the merged panels on the right. **G**. CD45-positive cells (green) do not colocalize with CD31-positive cells (red). **H**. Vascular structures are labeled with the endothelial marker van Willebrand factor (red) and Ly-6C (green). Original magnifications: **A and E**: 1000x; **B-D and F:** 600x; **G and H:**160x.

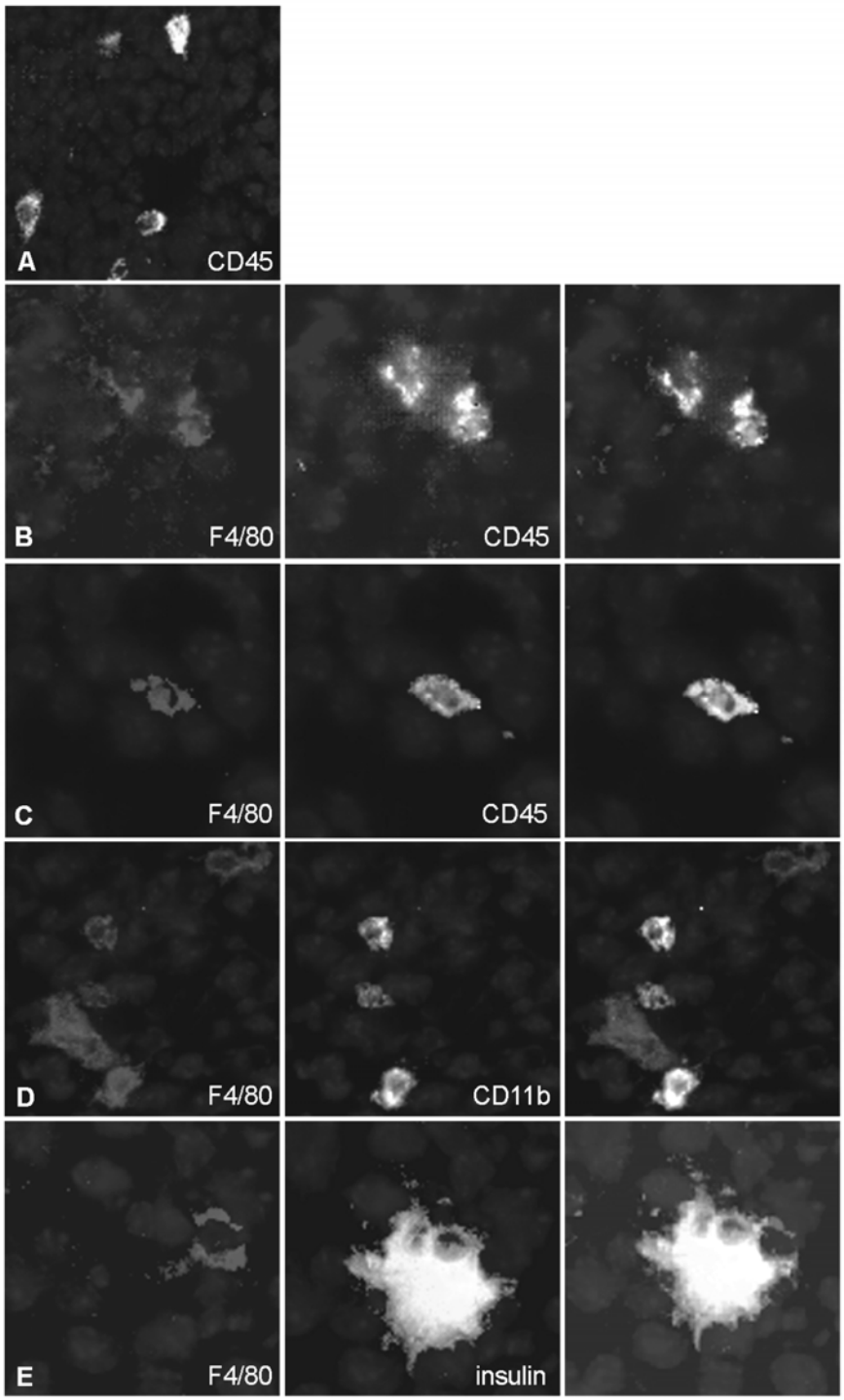

Figure 3.3 F4/80$^+$CD45$^+$ mature macrophages are present in the fetal pancreas. Immunofluorescent labeling of frozen fetal pancreas sections with combinations of different markers for mature macrophages. Conjugates used: Texas red (red labeling) combined with FITC (green). Antibodies used are depicted. Co-localization of the labeling is depicted in yellow in the merged panels on the right. **A**. Pancreatic bud of C57BL/6 mouse at E12.5. **B**. Pancreas of C57BL/6 mouse at E14.5. **C-E**. Pancreas of C57BL/6 mouse at E17.5. Original magnifications: **A**: 400x; **B-D**: 600x; E: 1000x.

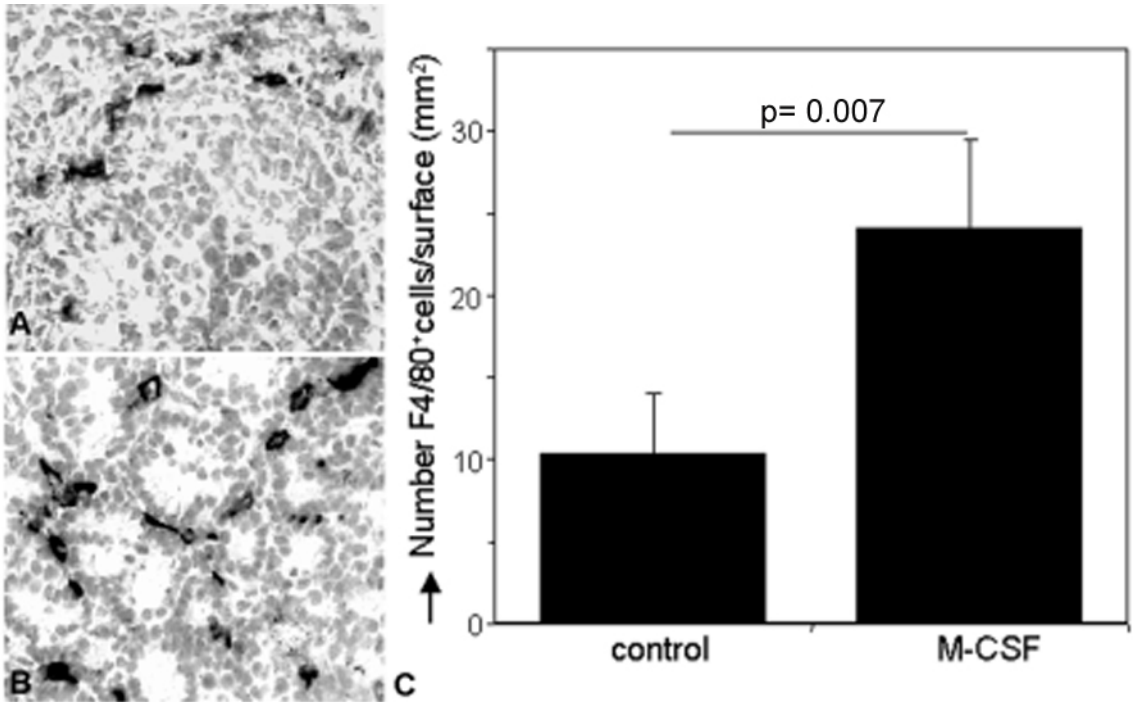

Figure 3.4 Exogenous M-CSF significantly increases the number of F4/80$^+$ cells in cultured E12.5 pancreas explants.

F4/80-positive cells are readily observed in pancreas explants cultured for 5 days without M-CSF (**A**) or with M-CSF (**B**). Original magnification 200x. The number of F4/80-positive cells relative to the total explant surface area/mm^2 ± SD is depicted for explants cultured without M-CSF (n=5) or with M-CSF (n=5).

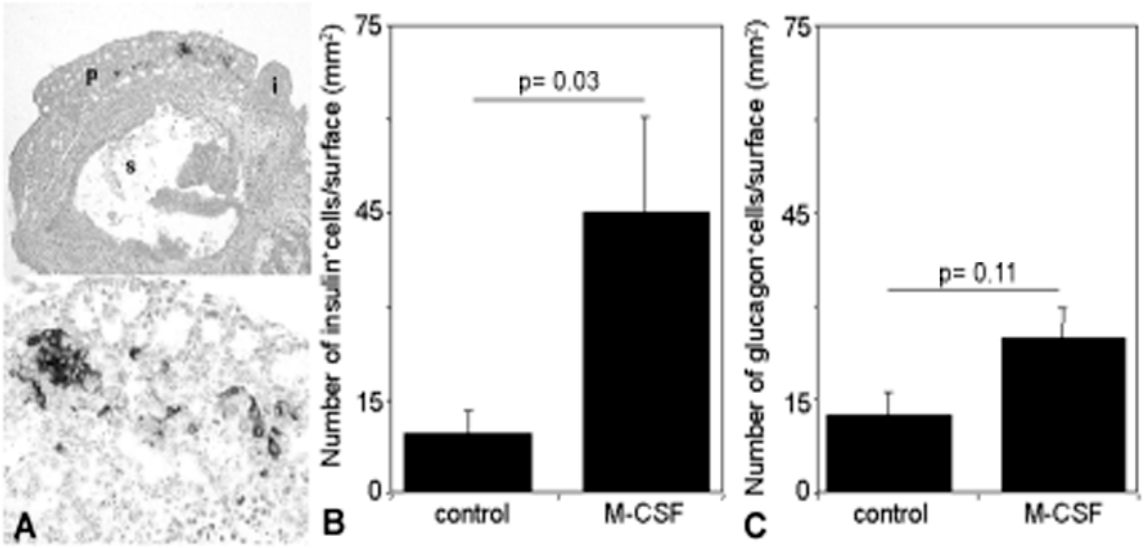

Figure 3.5 Pancreas explants cultured with M-CSF have increased numbers of insulin-producing cells.

A. Insulin (red) and glucagon (blue)-producing cells are observed in the explants after 5 days of culture. symbols depicted indicate **s**: lumen of stomach, **i**: intestine, **p**: pancreas tissue. Original magnifications of 40x and 200x respectively.

B-C. The number of insulin (**B)** and glucagon (**C**)-producing cells relative to the total explant surface area/mm^2±SEM are depicted for explants cultured without M-CSF (n=8) or with M-CSF (n=5).

Chapter 4

Extracellular Matrix distribution and Islet Morphology in the early Postnatal Pancreas: anomalies in the Nonobese Diabetic mouse

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Abstract

Previously we reported elevated numbers of macrophages in the pancreas of NOD mice, a spontaneous animal model for type 1 diabetes, during the early postnatal period. Extracellular matrix plays an important role in the tissue trafficking and retention of macrophages as well as in postnatal pancreas development. Therefore, we have examined the expression and distribution of laminin and fibronectin, two major extracellular matrix proteins and their corresponding integrin receptors, in the pre-weaning pancreases of NOD mice and control mouse strains. In addition, we have characterized the pancreas morphology during this period, since the morphology of the pre-weaning pancreas before the onset of lymphocytic peri-insulitis, when the pancreas is still subject to developmental changes, has been poorly documented.

We show that laminin labeling is mainly associated with exocrine tissue, whereas fibronectin labeling was mostly localized at the islet-ductal pole, islet periphery and in intralobular septa. Moreover, the protein expression level of fibronectin was increased in NOD pancreases at the early stage of postnatal development, as compared to pancreases of C57BL/6 and BALB/c mouse strains. Interestingly, pancreatic macrophages were essentially found at sites of intense fibronectin labeling. The increased fibronectin content in NOD neonatal pancreas coincided with altered islet morphology, histologically reflected by enlarged and irregular shaped islets and increased percentages of total endocrine area as compared to that of control strains.

In conclusion, increased levels of the extracellular matrix protein fibronectin were found in the early postnatal NOD pancreas, and this is associated with an enhanced accumulation of macrophages and altered islet morphology.

Abbreviations used:

BMDMs, bone marrow-derived macrophages; ECM, extracellular matrix; HE, hematoxylin-eosin; macrophage-colony stimulating factor, M-CSF; NOD, nonobese diabetic; T1D, type 1 diabetes.

4.1 Introduction

Abnormal immune regulation explains to some extent the autoimmune phenomena observed in diabetic patients and in spontaneous animal models for the disease [1,2]. Nevertheless, the precise reason for the immune system to target the pancreatic islets of Langerhans remains unclear.

Previously, we reported elevated numbers of macrophages in the pancreas of one of the spontaneous animal models of type 1 diabetes (T1D), the non-obese diabetic (NOD) mouse, as early as birth [3, 4]. Indeed, the initial triggering of the islet autoimmune response might take place locally at an early age, when the pancreas is still subject to developmental changes. The pancreas morphology of NOD mice has been poorly documented during this early pre-weaning period, before the first signs of a lymphocytic peri-insulitis (4-5 weeks of age) become evident.

The extracellular matrix (ECM) plays an important role in fetal and postnatal pancreas development. Once single immature endocrine cells have budded from the ductal epithelium [5], they migrate through the ECM and associate to form islets [6-8]. Laminin and fibronectin are major ECM constituents. Laminin is mainly present in basement membranes that form the interface between epithelia and connective tissues [9], whereas pancreatic fibronectin is observed underneath endothelial cells and epithelial ducts [10]. Both laminin and fibronectin affect β-cell differentiation, proliferation, and even their insulin secretion [11, 12].

ECM additionally plays an important role in the attraction, differentiation and retention of monocytes and macrophages that are able to interact with ECM proteins like laminin and fibronectin via the expression of specific integrin receptors [13, 14].

Integrin-mediated cell-matrix interactions are critical for a diversity of cellular processes, e.g. lack of contact with the matrix may induce apoptosis [15, 16]. Several integrins have also been identified on β-cells and their precursors. β-cells heterogeneously express the laminin receptor α6β1 on their surface and perturbation of the interaction with laminin influences their differentiation [17, 18]. Human pancreatic ductal cells and undifferentiated cells that emerge from the ductal epithelium are characterized by the expression of αVβ3 and αVβ5 integrins. These integrins mediate ductal cell adhesion and migration on ECM *in vitro* [10].

Thus, modifications in the ECM compartment or in the cellular integrin profile during the perinatal period, might affect both the normal differentiation of β cells as well as the migration, retention and differentiation of monocytes and macrophages in the developing pancreas.

In this study we have examined the distribution of laminin and fibronectin, as well as that of their corresponding integrin receptors, during the pre-weaning period in NOD mice and control mouse strains. Furthermore, we have studied the localization of resident pancreatic macrophages and the potential of peritoneal and bone marrow-derived macrophages (BMDMs) to adhere to fibronectin- and laminin-coated surfaces. Additionally, we have analyzed the pancreatic protein levels of fibronectin and investigated the kinetics of morphological changes of the endocrine tissue during pre-weaning.

4.2 Materials and methods

4.2.1 Animals

NOD, C57BL/6 and BALB/c female mice were bred at the animal facility of Necker Hospital, Paris, France. All strains were kept under specific pathogen-free conditions and fed *ad libitum*. By 28 wks of age, the incidence of diabetes in this NOD colony is 80% for females [19]. For all experiments, mice were sacrificed at postnatal ages of 3, 7 or 14 days. During gestation, NOD mothers (8-12 wks of age) were normoglycemic, as was tested with the Gluketur test (Roche Diagnostics GmbH, Mannheim, Germany). A minimum of 5 animals per age/strain/group was used for all studies. Animal handling followed the ethical rules provided by the European Union.

4.2.2 Reverse transcription polymerase chain reaction

Total RNA was extracted from mouse pancreases using RNAble reagent (Eurobio, Les Ulis, France) according to the manufacturer's instructions. Total mouse pancreatic RNA (2 µg) was reverse transcribed using a reverse transcription system (Promega) according to the manufacturer's instructions. Partial cDNAs for α-1 laminin chain, fibronectin and corresponding integrins were amplified by polymerase chain reaction using specific primer pairs (α-1 laminin chain sense: TCTCCGTGACTTCGACATGTG and antisense: CATTTTCCGATTGCCACCA, α6 integrin chain sense: GCCTTGCACGATGATATGGAG and antisense: ACCGATGAGCTGTCTGGAGAA, Fibronectin sense: ATGAAAGTCTCGTGCCCGTT and antisense: ACCACTGCCACATCATAGCCA, α4 integrin chain sense: TGGAGCTGGACATTTTCGAAG and antisense: ACTCCCCAAATCTTGCAGCA, α5 integrin chain sense: CTGTGATTTTCCCAGTCAGCC and antisense: TGCAGTTGCTGAGTCCTGTCA). Actin was used as internal reference. Amplification products were visualized with ethidiumbromide after agarose-gel electrophoresis to verify amplicon size.

4.2.3 Immunohistochemistry and confocal microscopy

Pancreases were embedded in Tissue-Tek (Miles, Elkart, IN, USA) and frozen in dry-ice chilled isopentane. Six µm thick cryostat sections were fixed for 10 min in acetone containing 0.03% hydroxyperoxide. Slides were washed in phosphate-buffered saline with 0.05% Tween-20 (Merck, Paris, France) and incubated with primary antibodies specific for rabbit anti-mouse laminin-1 or fibronectin, both used at the concentration of 25 µg/ml (Novotec, St. Martin La Garenne, France), α4 integrin chain (9C10, used at 5 µg/ml), α5 integrin chain (5H10-27, used at 5 µg/ml) and α6 integrin chain (GoH3, used at 7 µg/ml) (host species all rat, Becton Dickinson, Grenoble, France) or macrophages (clone ER-MP23, used as undiluted supernatant, a kind gift from Dr. P Leenen, Erasmus University, Rotterdam, The Netherlands). Subsequently, slides were washed with PBS-Tween-20 and incubated with appropriate peroxidase-coupled-secondary antibodies (Swine anti-rabbit at 2.5 µg/ml; Rabbit anti-rat at 7 µg/ml, DAKO, Trappes, France) or fluorescent-secondary antibodies (Goat anti-rabbit TRITC at 10 µg/ml, Biosys, Compiegne, France and Goat anti-rat FITC at 6.5 µg/ml, Jackson, West-Grove, USA) in

the presence of 1.5% normal mouse serum. The 3-amino-9-ethylcarbazole substrate (Sigma Co., St.Quentin Fallavier, France) was used for detecting peroxidase activity in 50 mM sodium acetate/0.02% hydroxyperoxide. For fluorescent double staining, primary as well as secondary antibodies were incubated simultaneously. The resulting labeling was examined by confocal microscopy.

4.2.4 *In vitro* adherence of macrophages to fibronectin- and laminin-coated surfaces

Six- or 24-well flat-bottom culture plates were coated overnight at 4°C with 10 µg/ml albumin, laminin-1 (both Sigma-Aldrich) or fibronectin (Chemicon International, Temecula, USA). Wells were washed 3 times with sterile PBS, and non-specific sites were blocked with 1% BSA for 1h at 37°C. Wells were washed twice with sterile PBS and once with sterile RPMI-1640-glutamax completed with 10% FCS (Invitrogen, Cergy Pontoise, France). Freshly harvested peritoneal macrophages or day 4-6 bone marrow-derived macrophages (BMDMs) were added in a concentration of $5x10^4$/ml in 500 µl RPMI-10% FCS and incubated overnight. Peritoneal macrophages were harvested by washing the peritoneal cavity with ice-cold PBS (Invitrogen, Cergy Pontoise, France). BMDMs were cultured from precursors of the bone marrow by stimulation with macrophage-colony stimulating factor (M-CSF). Bone marrow single-cell suspensions were prepared as described previously [20] and seeded in sterile culture dishes (Falcon) at $4x10^6$ cells/10 ml in RPMI-1640-glutamax, enriched with 10% FCS and 50 ng/ml recombinant murine M-CSF (R&D Systems, Abingdon, UK).

4.2.5 Immunoblotting

To evaluate laminin and fibronectin protein levels, we adapted an earlier described protocol [21]. Pancreases of individual animals were sonicated in 1% SDS lysis buffer containing EDTA-free protease-inhibitor cocktail (Complete mini, Roche, Mannheim, Germany) and centrifuged to discard cell debris. Total protein contents of each lysate was determined 4 times in duplicate with the bicinchoninic acid protein assay (Pierce, Rockford, USA). Three µg of individual lysates of 3 mice/strain/age were pooled in standard Laemli's loading buffer (100 mM Tris, 4% SDS, 30% glycerol and 2-mercaptoethanol) containing 0.2 M dithiothreitol and equal amounts of protein were resolved in duplicate on 3-8% gradient Precast Nupage Tris-acetate gels (Invitrogen, Paisley, USA). Thereafter, proteins were transferred using semi-dry blotting on Hybond C-extra nitrocellulose protein blots (Amersham, Buckinghamshire, England) and stained with Ponceau Red to control the blotting efficiency. Non-specific binding sites were blocked with Tris-buffered saline containing 5% powder milk, followed by an incubation of the first antibody, specific for rabbit anti-mouse laminin-1 or fibronectin, both used at 2.5 µg/ml (Novotec, St. Martin La Garenne, France). Blots were washed and incubated with the appropriate peroxidase-conjugated second antibody (Swine anti-rabbit, used at 80 ng/ml, DAKO, Trappes, France) Peroxidase-activity was visualized using the supersignal kit with ECL-hyperfilms (Amersham). Colored kaleidoscope broad-range markers (Bio-rad, Ivry, France) were used to indicate protein size, whereas purified laminin-1 (Sigma) and fibronectin (Chemicon, Euromedex, Souffelweyersheim, France) were included as

positive controls for antibody specificity. The chemiluminescence signal was exposed to Kodak films or counted directly by an electronically cooled LAS-1000 plus charge-coupled device camera system and analyzed with Image Gauche 4.0 software (Fuji Photo Film Co., Tokyo, Japan).

4.2.6 Morphometric analysis

Animals were sacrificed by elongation and their pancreases immediately fixed for 2h in Bouin's solution, followed by 4h incubation in absolute ethanol and 48h in 4% formaldehyde. Pancreases were paraffin-embedded and 5 µm thick sections were cut at 80 µm intervals. Total pancreas and endocrine surfaces of HE-stained sections were measured by marking the pancreas or islet border with a computer-interfaced freehand tool at an optical magnification of 4x and 8x respectively. Pancreases of 3-day-old mice weighed approximately 4 mg, those of 1-week-old mice 6 mg and pancreases of 2-week-old mice 8 mg. Three sections from each pancreas, taken at different levels, were evaluated and depending on age and pancreas surface, an average of 10-25 islets was measured for each section. Data were analyzed with the VIDAS-RT software (Kontron Elektronik GmbH/Carl Zeiss, Weesp, The Netherlands). Islets were denoted large when the islet area exceeded 7000 μm^2. Irregularity of the islet contour was defined as the deviation from a perfect circular shape with the value of 1. Islets with values < 0.6 were considered irregular.

4.2.7 Statistical analysis

Data were expressed as mean ± SD; *p*-values were calculated with the two-tailed Student's *t* test for unpaired values.

4.3 Results

4.3.1 Distribution of laminin and fibronectin and their corresponding integrin receptors in the developing postnatal pancreas.

Messenger RNAs for laminin and fibronectin and their corresponding integrin receptors were expressed in pre-weaning pancreases, as revealed by RT-PCR (data not shown). Since the exact distribution of these proteins in the early postnatal (pre-weaning) murine pancreas was not known, we examined their localization in pancreases of C57BL/6, BALB/c and NOD mice at 3 days, 1 week and 2 weeks of age.

Laminin-1 labeling was most evident at the basement membrane of the exocrine acini (Fig. 4.1A-B). In addition, labeling was detected in the basement membrane of epithelial ducts and in the matrix lining large blood vessels as well as intra-islet capillaries. Laminin distribution was similar for all pre-weaning ages irrespective of the strain examined, and resembled the distribution of laminin in the adult pancreas that has been described previously [9]. Staining for the α6 integrin chain of the α6β1 laminin receptor revealed a strong labeling of exocrine acini (Fig. 4.1C). Alpha6 co-localized with laminin in the endothelial layer of blood vessels and capillaries and at the pyramidal base of the exocrine cells (Fig. 4.1D), but was not detected on murine β-cells, in contrast to earlier reports [18].

Fibronectin was detected in the matrix surrounding the pancreas, and in the extracellular matrix of inter- and intra-lobular septa. Additionally, staining for fibronectin was particularly strong at the islet-ductal-pole, perivascular areas and at the islet periphery (Fig. 4.2A-B). For both NOD mice and control strains, labeling of the fibronectin receptor integrin α-chains α4 and α5 revealed positive cells in the fibronectin-positive extracellular matrix surrounding ducts and vessels (Fig. 4.2C-D). Alpha-4 was also detected at the surface of epithelial ductal cells, although faintly, and weakly on endocrine cells at the islet ductal pole (Fig. 4.2C-D). Strong staining of the α5-integrin α-chain was detected on endothelial cells (Fig. 4.2E). In addition, α5-positive cells were seen in the fibronectin-labeled perivascular and periductal areas, resembling the distribution of α4-positive cells in these areas (Fig. 4.2F).

4.3.2 Macrophages mainly localize at sites of fibronectin expression.

During the early postnatal period, pancreatic macrophages are mostly distributed in peri-islet, perivascular and periductular areas and interlobular septa [4], sites corresponding to fibronectin-labeled areas. Accordingly, resident ER-MP23^{+} pancreatic macrophages were essentially found at sites with intense fibronectin labeling (Fig. 4.3A-B), such as the peripheral- and interlobular connective tissue and perivascular areas.

Examination of freshly harvested resident peritoneal macrophages (not shown) or bone marrow-derived macrophages (BMDMs) showed that non-stimulated macrophages of C57BL/6 and NOD mice, attached and spread on fibronectin-coated (Fig. 4.3G-H) and albumin-coated surfaces (used here as an unrelated protein) (Fig. 4.3C-D), while laminin-coated surfaces inhibited the spreading of these macrophages *in*

vitro (Fig. 4.3E-F). This was confirmed by quantitative analysis showing an increase in the percentage of macrophages adherent to fibronectin after overnight incubation, as compared to the percentage of laminin-adherent macrophages, when related to the total number of cells applied (C57BL/6 mice: 1.95 ± 1.13% fibronectin-adherent as compared to 1.16 ± 0.08% laminin-adherent BMDMs (p=0.32, n=6). NOD mice: 0.5 ± 0.13% fibronectin-adherent as compared to 0.28 ± 0.13% laminin-adherent BMDMs (p=0.025, n=6).

4.3.3 Fibronectin protein levels are increased in early postnatal NOD pancreases.

Fibronectin supports cell migration and adhesion, and increasing fibronectin concentrations favors leukocyte adhesion *in vitro* [17, 22]. However, immunohistochemistry cannot reveal differences in the concentration of this protein. Therefore, we assessed fibronectin protein levels in whole pancreas lysates by using a quantitative immunoblotting method adapted from Stoffers and co-workers [21]. Interestingly, direct measurement of the chemiluminiscent signal intensity showed a significant increase in fibronectin-levels in pancreases of NOD mice, when compared to that of C57BL/6 and BALB/c mice at 3 days of age (Fig. 4.4 and table 4.1). At 7 days of age the fibronectin level still appeared higher in NOD mice, although differences were not statistically significant (table 4.1). The protein level of fibronectin was no longer increased in NOD pancreases at 2 weeks of age.

The levels of laminin-1 were also determined by this same method. NOD pancreases did show higher expression levels during pre-weaning at all ages examined when compared to C57BL/6 pancreases, but these differences were not significant and a high variability in the expression levels was observed (table 4.1).

Table 4.1 Direct quantification of chemiluminiscent signal of immunoblotted pancreas lysates*

Age (days)	**3**		**7**		**14**	
Fibronectin		***p* vs NOD**		***p* vs NOD**		***p* vs NOD**
C57BL/6	273560±44672	0.004	213766±90169	0.20	173587±37605	0.51
BALB/c	179327±55424	0.03	175859±34941	0.06	215660±17014	0.55
NOD	421155±45938		327419±125664		196362±53424	
Laminin						
C57BL/6	54223±35853	0.68	37842±41557	0.65	84472±97357	0.84
NOD	66688±33032		59027±74632		102263±106559	

* data are presented as mean counts ±SD of 3 or 4 individual experiments. ND: not done.

4.3.4 The pre-weaning NOD pancreas is characterized by higher percentages of endocrine area and irregularly shaped islets.

The fibronectin-containing pancreatic ECM forms the template for developing islets and fibronectin is involved in the adhesion and spread of fetal and adult islet cells [10]. Since we observed increased levels of fibronectin in the early postnatal NOD pancreases, we additionally investigated whether this was accompanied by aberrancies in islet morphology.

Analysis of hematoxylin-eosin (HE)-stained neonatal pancreas sections revealed a not fully developed organ, in both control and NOD mice. Lobules consisted of loose connective tissue with scattered exocrine acini that could be discriminated from endocrine islets by the localization of their nuclei at the basal pole. Islets were found lining the epithelial ducts, with artery and vein structures in close vicinity. At 3 days of age, the earliest age examined, islets were small and few in number. In pancreases of control strains, these islets were circular of shape (Fig. 4.5A-B), whereas in NOD mice islets appeared enlarged and irregular of shape (Fig. 4.5C), when compared to the circular islets of control strains.

In order to better characterize these observations, we quantified the percentage of endocrine tissue in pancreases of NOD and C57BL/6 mice, from 3 to 14 days of age. Significantly higher percentages of endocrine area (relative to total pancreas surfaces) were observed in NOD pancreases, when compared to those of C57BL/6, at all ages examined (Fig. 4.6A). Due to the linear expansion of the total pancreas surface between 3 and 14 days of age (not shown), the percentage of endocrine area significantly decreased with age in both strains (Fig. 4.6A). The expansion of pancreas surface is caused by the earlier described expansion of the exocrine tissue during the pre-weaning period [23].

Neither total pancreas surfaces nor relative numbers of islets differed between NOD and C57BL/6 mice, irrespective of the age examined. Thus, the higher percentage of endocrine area in NOD mice was most likely due to a larger islet size. Indeed, when we quantified islet surfaces of 3-day-old pancreases, the mean surface of islets in NOD pancreases was larger when compared to that of control mice (Fig. 4.6B). The mean islet surface did not change with age for either NOD or C57BL/6 strains and remained larger in NOD mice at 7 and 14 days of age, although these differences were not statistically significant (not shown).

The differences in endocrine area observed between NOD and C57BL/6 pancreases were most obvious at 3 days of age. In order to verify whether these differences reflected abnormal pancreas development in the NOD, rather than in the C57BL/6 strain, we compared the results obtained at 3 days of age with an additional control strain. The percentage of total endocrine area, relative to the pancreas surface at 3 days of age, was also significantly higher in NOD mice when compared to BALB/c mice (Fig. 4.6C), as was the mean islet surface (Fig. 4.6B). No differences were observed between pancreases of BALB/c and C57BL/6 mice.

Endocrine tissue in the pancreases of control mouse strains primarily consisted of circular-shaped islets, whereas in NOD mice islets appeared to be irregular of shape.

We therefore determined the form of the islets by defining their deviation from a circular-shape with morphometric analysis.

During the pre-weaning period, NOD mice exhibited a significantly increased percentage of irregular-shaped islets from 3 to 14 days of age, as compared to C57BL/6 mice (Fig. 4.7A). In both strains, percentages of irregular-shaped islets decreased with age. As for islet size, we compared, in an additional experiment, the form of NOD islets to that of BALB/c islets at 3 days of age. We confirmed that NOD islets were also more irregular than islets of BALB/c mice (40.83 ± 7.90 percent irregular islets in NOD mice compared to 12.35 ± 6.91 percent irregular islets in BALB/c mice; $p < 0.001$).

To further characterize the differences between NOD and control mouse islets, we grouped islets as a function of their shape and size in small/circular, large/circular, small/irregular and large/irregular-shaped islets. At 3 days of age, the circular islets in control strains were mostly small (< 7000 μm^2). When compared to NOD pancreases, control strains showed a higher percentage of small/circular-shaped islets, (Fig. 4.7B-C), whereas NOD mice exhibited an increased percentage of large islets that were irregular-shaped (Fig. 4.7D-E). Interestingly, when pancreases of 2-week-old animals were examined, NOD mice exhibited a higher percentage of small islets that were irregular-shaped when compared to C57BL/6 mice (Fig. 4.8A-C).

4.4 Discussion

In the NOD mouse model, various abnormalities within the immune and the endocrine system have been documented. Although morphological changes of the pancreas in early and advanced stages of diabetes have been extensively studied [24, 25], its morphology before the onset of lymphocytic peri-insulitis, when the pancreas is still subject to developmental changes, has been poorly documented. Here, we have studied the distribution of the ECM proteins laminin and fibronectin and their corresponding integrin receptors in addition to islet morphology, in pancreases of NOD and control mouse strains throughout the pre-weaning period.

During this early postnatal period, labeling of laminin and the integrin α6 chain of the laminin receptor, was mainly localized to the exocrine tissue and vessels, in agreement with laminin labeling in the adult pancreas [9]. The ECM protein fibronectin was predominantly present in the ECM of interlobular septa, perivascular areas, the islet periphery and at the islet-ductal pole. The fibronectin-specific integrin alpha-chains α4 and α5 were mainly localized to the pancreas endothelium, resembling the localization of the endothelial cell-specific adhesion molecule PECAM-1 (data not shown). Cells within the fibronectin-positive matrix of the perivascular area and islet-ductal pole also expressed the integrin α4-chain, although weakly. This localization suggests that in the early postnatal mouse pancreas α4-mediated interaction with fibronectin may be used by endothelial cells, epithelial cells, possibly fibroblasts, as well as by macrophages.

Previously, we observed increased numbers of different macrophage subsets at birth and during the first week of life in NOD mice as compared to various control strains [4]. The co-localization of pancreatic macrophages with fibronectin is demonstrated in this report. Furthermore, adhesion and spreading of non-stimulated macrophages was favored by fibronectin-coated surfaces, whereas laminin-coated surfaces inhibited macrophage spreading, in accordance to what has been reported earlier [26]. Concomitantly, an increase in the protein level of fibronectin was found in the pancreas of NOD mice. Hence, it is conceivable that more macrophages accumulate in the pre-weaning NOD pancreas due to the higher content of pancreatic fibronectin. Since macrophages themselves are also able to produce and secrete ECM-proteins, it remains unclear whether the increased macrophage number in the pre-weaning NOD pancreas is the cause or actually the consequence of the increased fibronectin levels.

Adhesion of macrophages to matrix proteins activates different signal-transduction pathways, leading to the production of various cytokines, growth factors and matrix metallo-proteases that play a role in pancreas and islet development [7, 27]. Moreover, ECM proteins themselves play a significant role in pancreas and islet development. Fibronectin has been suggested to act as a pathfinder matrix for migrating endocrine progenitor cells in the human fetal pancreas [10] and the localization of fibronectin as described here, suggests that fibronectin may be used as a migratory matrix for maturing endocrine cells. Furthermore, interaction of endocrine precursor cells and mature endocrine cells with the surrounding ECM is of critical importance for a proper development of ducts and islets [28]. Here, we observed increased levels of the

ECM component fibronectin concomitantly with alterations in islet morphology in the pre-weaning pancreas of the NOD mouse.

In the rodent pancreas, islets develop relatively late in gestation and their remodeling takes place from birth to weaning [5]. The normal pre-weaning mouse pancreas contains low numbers of small, circular-shaped islets. By using morphometric analysis, we show the presence of higher percentages of endocrine areas in NOD pancreases due to larger islet sizes, throughout the pre-weaning period when compared to pancreases of control strains. In addition to size, we also analyzed islet shape. Pancreases of control strains were characterized by circular-shaped islets, whereas NOD pancreases showed higher percentages of irregular-shaped islets until weaning. Irregularity of pancreatic islet contours suggests a disturbance of the surrounding peripheral ECM, and the enhanced protein levels of fibronectin in the early pre-weaning NOD pancreas may contribute to these irregularities.

At 3 days of age, the irregular-shaped islets consisted mainly of large islets in NOD mice. At a later stage of postnatal pancreas development, i.e. at two weeks of age, significantly higher numbers of small irregular-shaped islets were observed in NOD mice. Since peaks of new islet development normally take place in rodents during this period [29], this might reflect an abnormal development of a new wave of differentiating islets in the NOD mouse.

In conclusion, our study shows the presence of increased levels of the ECM protein fibronectin in the early postnatal NOD pancreas. This increased fibronectin contents might favor the infiltration and/or retention of pancreatic macrophages, and appears in association with altered islet morphology, reflected by higher percentages of endocrine areas and larger islets that are also irregular in shape.

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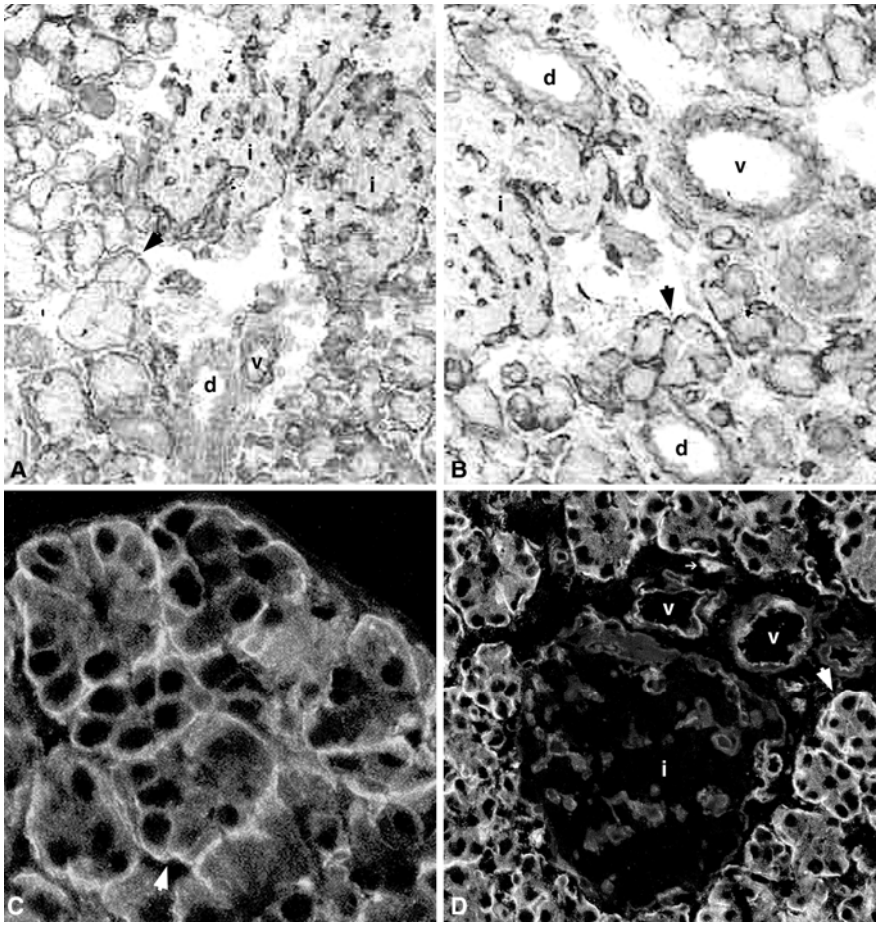

Figure 4.1

Upper panels show laminin labeling in the pancreas of a 3-day-old C57BL/6 mouse (A) and a 3-day-old NOD mouse (B). Laminin staining (red) is observed in the basement membranes of vascular (v) and ductal (d) structures and of the exocrine acini (arrowheads), (i) indicates islets. The sections were counterstained with hematoxylin-eosin. Original magnifications are 200x.

Lower panels depict fluorescent labeling of the α6 integrin chain of the α6β1 laminin receptor, examined by confocal microscopy C. The labeling of α6 (green) was evident in exocrine acinar cells (arrowhead), shown here for a NOD pancreas at 3 days of age, original magnification is 1000x. D. Alpha6-labeling (green) was observed at the basement membranes of capillaries (arrow) and exocrine cells (arrowhead), co-localizing (yellow) with laminin staining (red). Alpha6-labeling was absent on β-cells of the islet (i), shown here for a NOD pancreas at 2 wks of age, original magnification: 400x

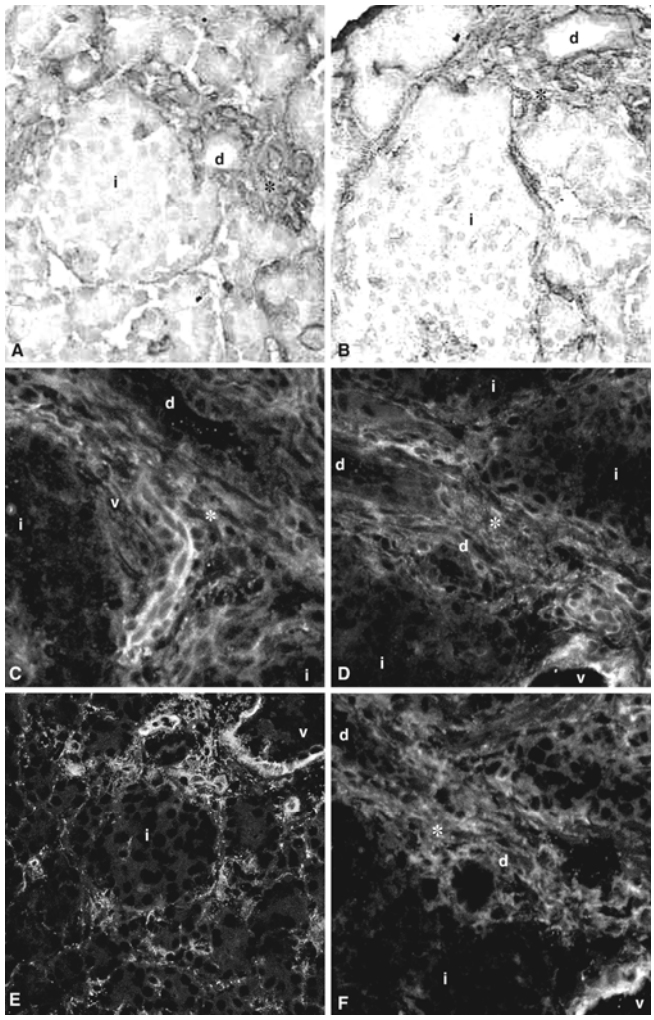

Figure 4.2

Upper panels show fibronectin immunolabeling in pancreases of C57BL/6 (A) and NOD (B) mice at 1 week of age. The fibronectin-labeling is seen around the islets (i), and staining for the protein is particularly strong around ducts (d) and at the islet ductal pole (∗). The sections were counterstained with hematoxylin-eosin. Original magnifications are 400x.

Middle panels show fluorescent labeling of the α4 integrin chain in areas of the islet-ductal pole, examined by confocal microscopy. Alpha4 labeling (green) was faintly detected in epithelial ducts (d) and on endocrine cells of the islet (i). More strongly stained α4-positive cells were identified in the fibronectin (red) positive matrix of the islet-ductal pole (∗) and in vascular structures (v), shown here for a C57BL/6 mouse pancreas at 2 weeks of age (C) and NOD pancreas at 2 wks of age (D), original magnification is 400x.

Bottom panels show fluorescent labeling of the α5 integrin chain, examined by confocal microscopy. Alpha5 labeling shows a pattern of expression comparable to that of α4, shown here at 2 weeks of age in C57BL/6 mice (E) and at two weeks of age for NOD mice (F). Symbols depicted indicate: i: islet, d: duct, v: vascular structures and ∗: islet-ductal pole, original magnifications are 400x.

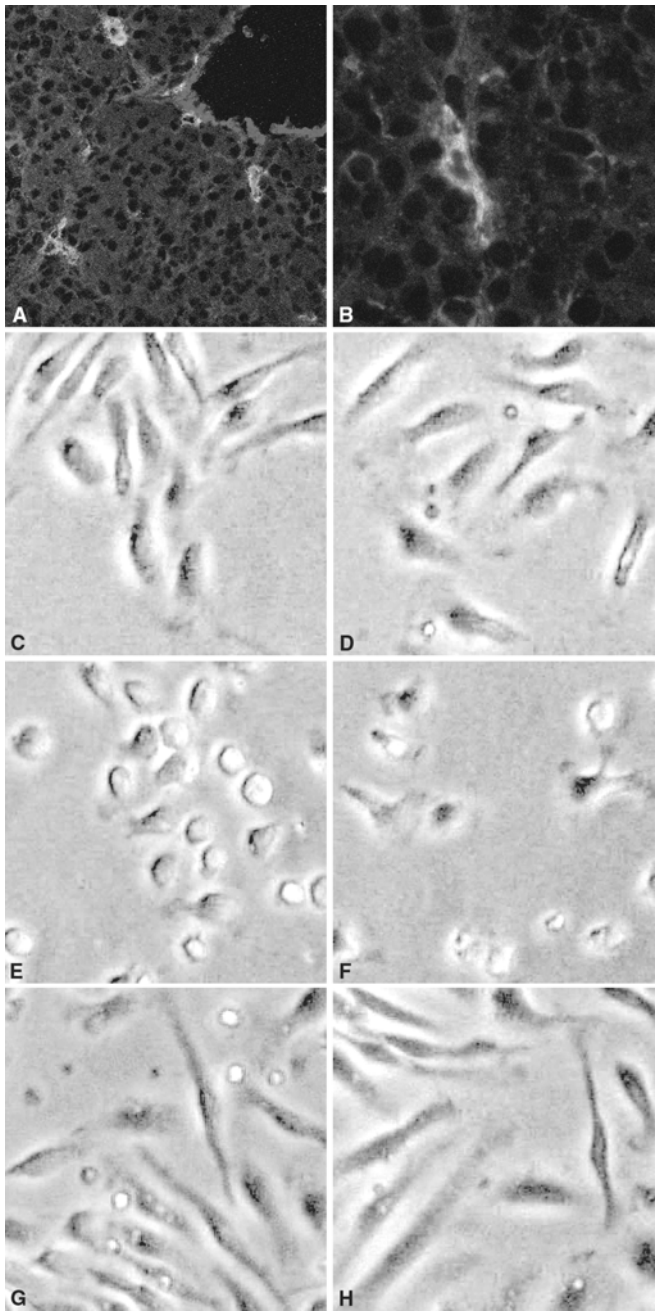

Figure 4.3

Upper panels correspond to immunofluorescent double-labeling of fibronectin and the macrophage-specific marker ER-MP23 in frozen sections of mouse pancreas, examined by confocal microscopy. A. Fibronectin labeling (red) is observed in the intra- and interlobular septa that contain several ER-MP23-positive histiocyte-type macrophages (green). Original magnification is 200x. B. A single ER-MP23-positive macrophage (green) is shown in close contact with fibronectin (red) in the intralobular septa. Original magnification is 1000x.

Lower panels show BMDMs of C57BL/6 mice (C, E and G) or NOD mice (D, F and H), subjected to albumin- (C-D), laminin- (E-F) or fibronectin- (G-H) coated surfaces. BMDMs exhibit a different morphology depending on the substrate and adherence is supported by albumin and fibronectin, but not by laminin.

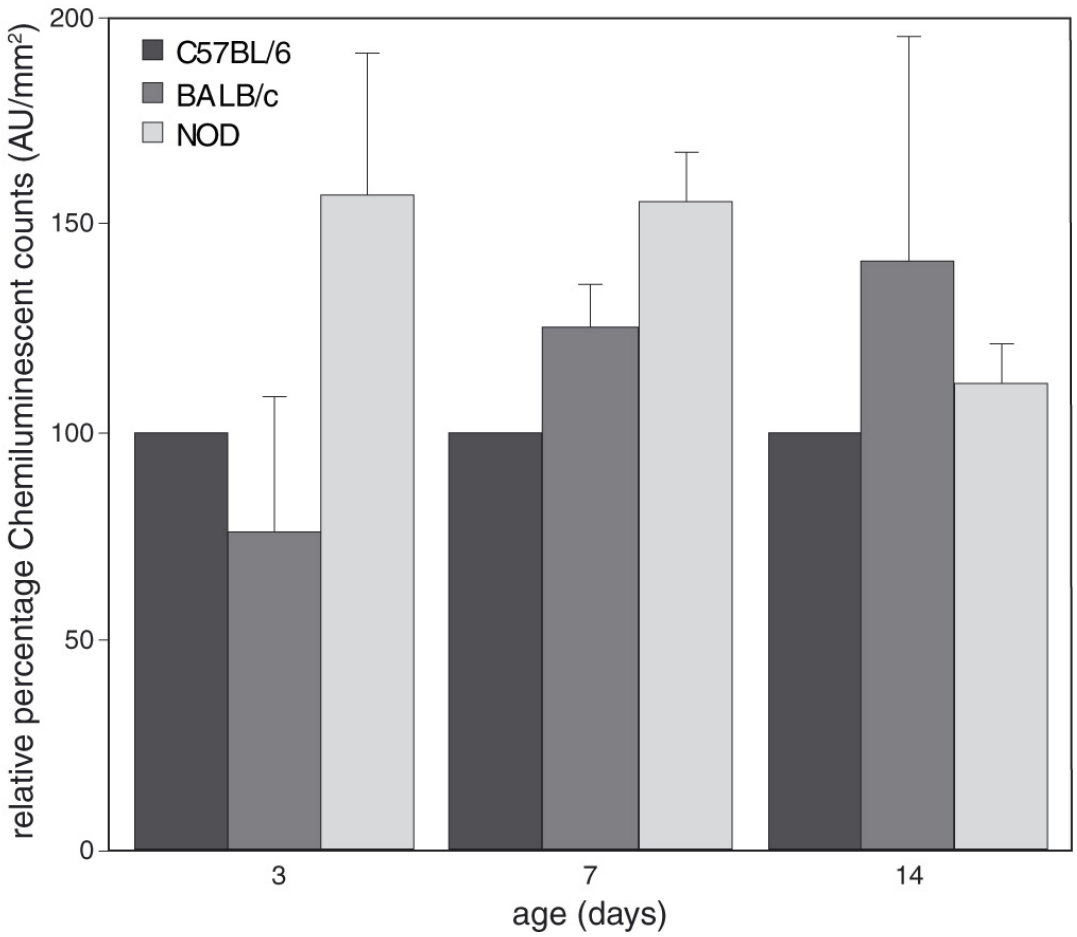

Figure 4.4

The graph shows the mean percentage of direct chemiluminescent counts (chemiluminescent counts AU/mm^2) ± SD of fibronectin protein levels, relative to that of C57BL/6 mice from 3 or 4 individual experiments, as was determined by immunoblotting when equal amounts of protein were resolved of total protein lysates of pooled pancreases (for each experiment n = 3 per strain per age) of sex- and age-matched C57BL/6, BALB/c and NOD mice at 3, 7 and 14 days of age.

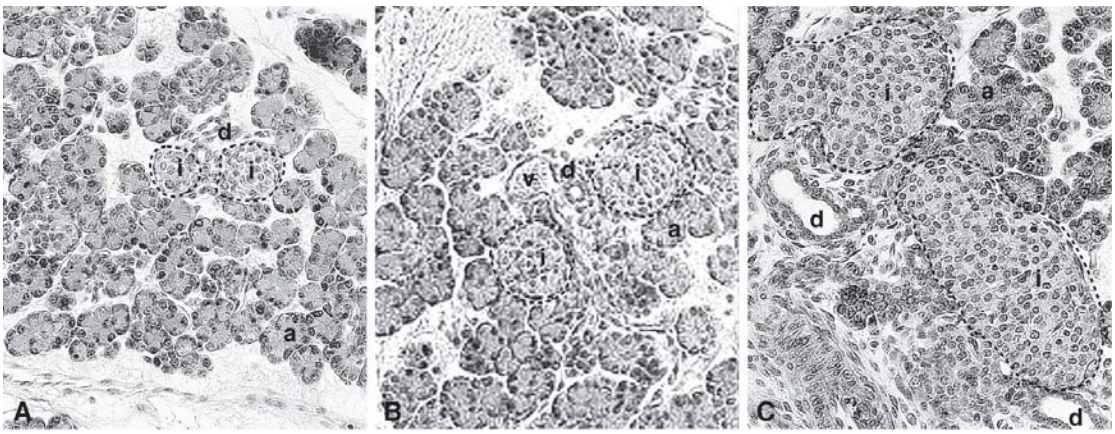

Figure 4.5

A. HE-stained paraffin section of the pancreas of a C57BL/6 mouse at 3 days of age showing loose connective tissue containing acini (a), and two small circular islets (i) aligning an epithelial duct (d) **B**. HE-stained paraffin section of the pancreas of a BALB/c mouse at 3 days of age, (v) indicates a vascular structure. C. HE-stained paraffin section of a 3-day-old NOD mouse pancreas, showing an irregular endocrine islet profile. Dashed lines show islet profiles. Original magnification: 200x.

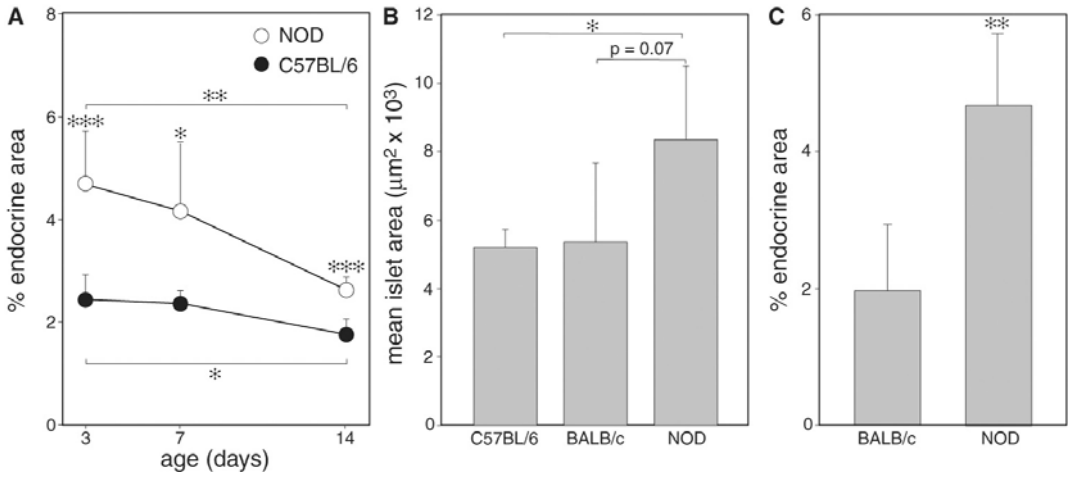

Figure 4.6

A. The percentage of endocrine area (related to the total pancreas surface) as a function of age for both C57BL/6 (●) and age-matched NOD (○) female mice. **B**. The mean islet area (in μm^2) at 3 days of age is represented for C57BL/6, BALB/c and NOD mice. **C**. The percentage of endocrine area (related to the total pancreas surface) at 3 days of age is represented for BALB/c mice and NOD mice. The data are expressed as the mean ± SD (n = 5 per strain per age), with * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$.

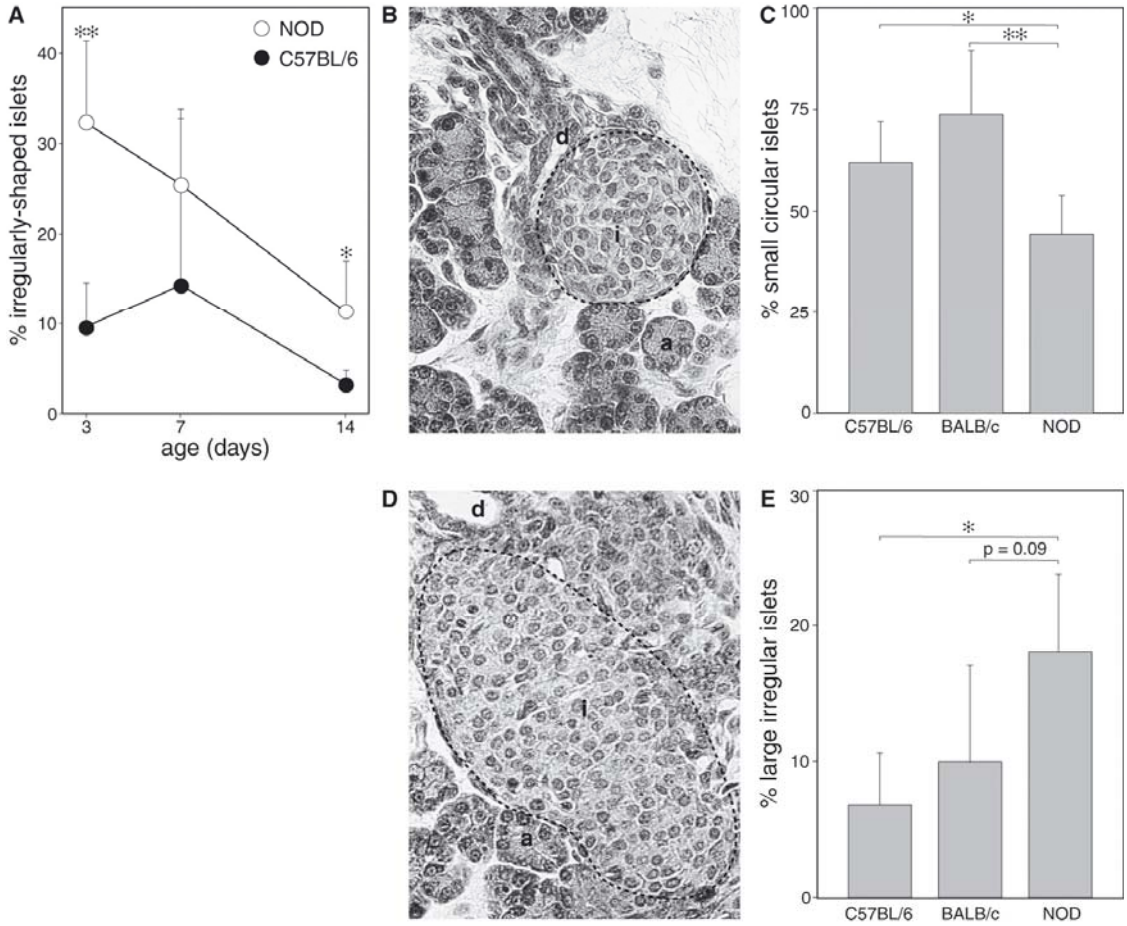

Figure 4.7

A. The percentage of irregular-shaped islets as a function of age for C57BL/6 (●) and age-matched NOD (○) female mice. **B**. HE-stained paraffin section of the pancreas of a C57BL/6 mouse at 3 days of age, demonstrating the circular shape of the islet. **C**. The relative percentage of small islets with a circular shape is represented for C57BL/6, BALB/c and NOD mice at 3 days of age. **D**. HE-stained paraffin section of the pancreas of a NOD mouse at 3 days of age showing the enlarged and irregular-shaped islet. **E**. The relative percentage of large islets with an irregular shape is represented for C57BL/6, BALB/c and NOD mice at 3 days of age. Original magnifications are 400x. Symbols depicted indicate: a: exocrine acini, i: islet and d: duct. In histologic sections, dashed lines show islet profiles. The data are expressed as the mean ± SD (n = 5 per strain per age), with * $p < 0.05$; ** $p < 0.01$.

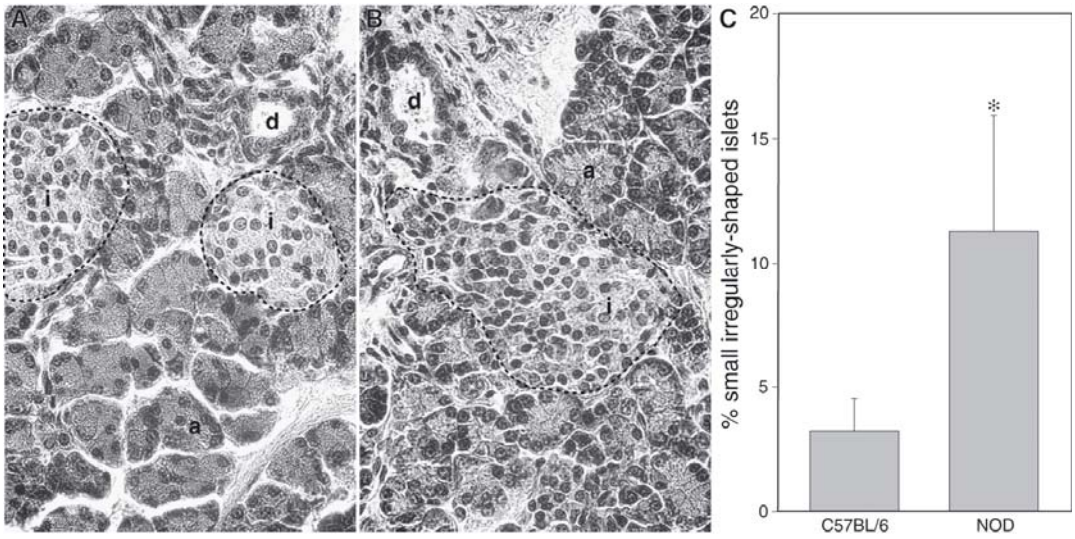

Figure 4.8

A. HE-stained paraffin section of the pancreas of a C57BL/6 mouse at two weeks of age showing the near circular shape of its islets, compared to that of the irregularly-shaped islet of the NOD mouse (**B**). Original magnifications are 400x. **C**. The percentage of small islets with an irregular shape is represented for C57BL/6 and NOD mice at two weeks of age. Symbols depicted indicate: a: exocrine acini, i: islet and d: duct. In histologic sections, islet profiles are shown by dashed lines. The data are expressed as the mean ± SD (n = 5 per strain per age), with * $p < 0.05$.

Chapter 5

Defective upregulation of CD49d in final maturation of NOD mouse macrophages

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Abstract

Macrophages are potent regulators of both innate and adaptive immunity. They play a central role in the development of autoimmune diabetes, and are among the first cells to appear in peri-islet infiltrates of NOD mice that develop diabetes spontaneously. Since efficient adhesion and migration are crucial for proper macrophage trafficking, we examined the migration and fibronectin adhesion capacity of NOD macrophages, as well as the regulation and expression of the fibronectin receptors α4β1 and α5β1.

When compared to macrophages from control strains, resident NOD macrophages showed a reduced ability to adhere to and migrate on fibronectin, a delayed clearance following peritoneal inflammation, and substantially lower expression levels of the α4β1 integrin α-chain, CD49d. NOD bone marrow-derived macrophages were specifically defective in the lipopolysaccharide-induced increase in CD49d expression. Moreover, the mitogen-activated protein kinase extracellular signal-regulated kinase-1/2 negatively regulated macrophage CD49d expression and strongly suppressed its expression in NOD macrophages.

The data presented herein indicate that the lipopolysaccharide-activated signaling cascade plays a critical role in CD49d expression of macrophages. Mature NOD macrophages are characterized by decreased CD49d expression and show defective CD49d-mediated adhesion to fibronectin.

Abbreviations used:

BMDMs, bone marrow-derived macrophages; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FN, fibronectin; MAPK, mitogen-activated protein kinase; MCP-1, macrophage chemoattractant protein-1; TG, thioglycollate; TLR, Toll-like receptor.

5.1 Introduction

Macrophages form a heterogeneous group of cells, distributed in various tissues. There they form a first line of defense against invading microbial pathogens and regulate both innate and adaptive immune responses. In the NOD mouse, a spontaneous animal model of autoimmune diabetes, macrophages are among the first cells to appear in peri-islet infiltrates, preceding diabetes development [1]. They play an important role in the development of disease, since the selective depletion of macrophages and DC as well as prevention of their intra-islet infiltration, abrogates diabetes [2, 3].

Several anomalies have been described for NOD macrophages that might contribute to the development of diabetes. NOD BM-derived macrophages (BMDMs) generated *in vitro*, fail to mature functionally and *in vivo* such a defect may result in a reduced ability to activate regulatory T-cells efficiently [4]. Furthermore, NOD BMDMs exhibit hyper-activation of the transcription factor NF-κB, leading to elevated expression of IL-12(p70), promoting a Th-1 driven immune response [5]. BMDMs represent a homogeneous pool of primary macrophages derived from BM precursors. Stimulation of these non-adherent precursors with M-CSF induces an adherent macrophage phenotype, associated with increased expression of the integrin-type fibronectin receptors α4β1 and α5β1 [6].

Integrins form a large family of heterodimeric transmembrane receptors comprised of α and β subunits that specifically recognize extracellular matrix (ECM)-components or cell surface receptors as counter ligands. The heterodimers α4β1 (CD49d/CD29) and α5β1 (CD49e/CD29) respectively recognize the CS-1 and RGD-sequence of fibronectin (FN), whereas α4β1 additionally mediates cell-cell adhesion via interaction with the addressin VCAM-1 [7, 8]. The regulation of integrin expression and activation is essential for efficient tissue trafficking of macrophages in inflammation. In this context, inflammation of the peritoneal cavity results in the disappearance of resident macrophages [9] and both α4β1 and α5β1 are involved in the regulation of macrophage-mesothelial adhesion and transmigration [10]. Regulation of α4β1 and α5β1 expression is also essential in the development of diabetes, since inhibition of the interaction of these integrins with their specific ligands prevents diabetes in both induced as well as spontaneous disease in NOD mice [11, 12].

Different functional aspects of NOD macrophages have been studied with an emphasis on the examination of their T-cell stimulatory capacity. Efficient adhesion and migration are crucial for proper macrophage trafficking. Since the maturation of NOD macrophages is defective, we reasoned that the expression of the α4β1 and α5β1 integrins and related adhesive features might be affected in NOD BMDMs. Therefore, we examined CD49d, CD49e and CD49f integrin α-chain expression profiles of mature resident macrophages and BM-precursors during their differentiation into BMDMs in NOD and control mice, in relation to their adhesive capacity. In addition, we studied the mechanisms involved in the regulation of CD49d and CD49e expression related to final macrophage maturation.

5.2 Materials and methods

5.2.1 Animals

Male and female C57BL/6, BALB/c, NOD, NOD.H-2^b, NOD.CD45.1 and F1[NOD x C57BL/6] raised in C57BL/6 as well as in NOD female mice, were fed *ad libitum* and bred under specific-pathogen-free conditions at Necker Hospital, France or ErasmusMC, the Netherlands following the ethical rules provided by the European Union. NOR mice were purchased from Harlan (Horst, the Netherlands). Mice were sacrificed by cervical dislocation.

5.2.2 Antibodies and reagents

For cytofluorometry, FITC-conjugated Ly6C (own culture) or PE-conjugated mAbs against mouse CD49d (clone R1-2 or 9C10), mouse CD49e (clone 5H10-27) or FITC-conjugated human CD49f (clone GoH3), were purchased from Pharmingen/Becton-Dickinson (Grenoble, France), and were incubated simultaneously with the biotinylated anti-macrophage mAb BM8 (BMA Biomedicals, Augst, Switzerland) or CD11b, detected with streptavidin-PeRCP (Pharmingen). Purified anti-CD49d, anti-CD49e or isotype control (Pharmingen) were used as blocking Abs in adhesion assays, or as first Abs for confocal microscopy followed by incubation with FITC-conjugated goat-anti-rat mAb (Jackson Laboratories, West-Grove, USA). For immunoblotting, first antibodies specific for ERK-1/2, pERK-1/2 (Sigma Co., St.Quentin Fallavier, France) and actin (Santa-Cruz Biotechnology, Le Perray en-Yvelines, France) were followed by incubation with horseradish peroxidase-conjugated swine anti-rabbit or goat-anti-mouse Abs (DAKO, Trappes, France).

Peritoneal macrophages or day 6 BMDMs were stimulated for 45 min (RT-PCR) or overnight (FACS-analysis) with 10 or 100 ng/ml LPS (Escherichia Coli Strain 055:B5 Sigma Aldrich) or 10 ng/ml murine recombinant IFN-γ (R&D systems Europe, Lille, France). The phosphorylation of ERK-1/2 was inhibited by the addition of 10 µM MEK-1/2 inhibitor UO126 (Promega France SARL, Charbonnieres, France) 1 h prior to LPS stimulation. The solvent DMSO was used as a control.

5.2.3 Cytofluorometry and confocal microscopy

Peritoneal macrophages were harvested by washing the peritoneal cavity with ice-cold PBS (Invitrogen, Cergy Pontoise, France). Blood monocytes were obtained by heart puncture. Single cell-suspensions of the thymus were prepared by passing the excised thymus through a 100 µm mesh cell strainer. Cells were counted for individual mice and adjusted cell-numbers of 3 mice/strain/age were pooled. Cells were directly stained for cytofluorometric analysis and incubations were performed in 0.1% BSA/PBS. Blood monocytes were identified by their low sidescatter and high expression of CD11b as was described recently [23]. Non-specific Ab adhesion was blocked by adding anti-FcRγII/III mAb (clone 2.4G2) or 1% normal mouse serum. Samples were analyzed in a FACScalibur flow cytometer (Becton-Dickinson) and results were processed with WinMDI software.

Adherent BMDM grown on glass coverslips (VWR International, Fontenay sous Bois, France) were fixed in 3% formaldehyde for 15 min and washed with PBS. Fixed BMDMs were incubated overnight with primary Abs. Subsequently, slides were washed with PBS-Tween-20 and incubated with appropriate fluorescent secondary Abs in the presence of 1.5% normal mouse serum. Nuclei were visualized by adding propidium iodide in the last washing step. Coverslips were then embedded in vectashield (Vector laboratories, Burlingame, USA), and examined by confocal microscopy, using the MS 510 Zeiss model device (Germany).

5.2.4 Immunoblotting

Protein lysates were made after washing adherent BMDMs with PBS, removing all PBS by aspiration and adding protein lysate buffer containing 10 mM Tris, 50 mM NaCl, 1% Triton X-100, 30 mM NP_2O_7, 50 mM NaF, 5 µM $ZnCl_2$, 100 µM Na_3VO_4, 1 mM DTT, 0.5 µM phenylmethylsulfonylfluoride, 5 nM okadaic acid, 0.5 mM benzamidine and EDTA-free protease-inhibitor cocktail (Complete mini, Roche, Mannheim, Germany). The protein contents of lysates were determined in duplicate with the bicinchoninic acid protein assay (Pierce, Rockford, USA). Ten µg of lysates were resolved in 4-12% gradient Precast Nupage Bis-Tris gels (Invitrogen, Paisley, USA) in standard Laemli's loading buffer (100 mM Tris, 4% SDS, 30% glycerol and 2-mercaptoethanol) containing 0.2 M dithiothreitol and transferred using semi-dry blotting on Hybond C-extra nitrocellulose protein-blots (Amersham, Buckinghamshire, UK). Blots were washed with Tris-buffered saline and stained with Ponceau Red to control the blotting efficiency. Non-specific binding sites were blocked with Tris-buffered saline containing 5% powder milk, followed by incubation with first Abs. Blots were washed and incubated with the appropriate peroxidase-conjugated secondary Abs. Peroxidase-activity was visualized using the supersignal kit with ECL-hyperfilms (Amersham). Colored kaleidoscope broad-range markers (Bio-Rad, Ivry, France) were used to indicate protein size. The chemiluminescence signal was exposed to Kodak films or counted directly by an electronically cooled LAS-1000 plus charge-coupled device camera system and analyzed with Image Gauche 4.0 software (Fuji Photo Film Co., Tokyo, Japan).

5.2.5 Real-time PCR

Total RNA was extracted from adherent BMDMs using RNAble reagent (Eurobio, Les Ulis, France) according to the manufacturer's instructions. RNA concentrations were measured by optical absorbance at 260 nm and the samples were subsequently stored at -80°C. Total RNA (2 µg) was reverse transcribed using a reverse transcription system (Promega) according to the manufacturer's instructions, in the presence of Oligo dT15 and random hexamers. Partial cDNAs for CD49d or CD49e integrin α-chains were amplified by using specific primer pairs (CD49d forward: 5' GAATCCAAACCAGACCTGCGA 3'; reverse 5' TGACGTAGCAAATGCCAGTGG 3'. CD49e forward: 5' CTGTGATTTTCCCAGTCAGCC 3'; reverse 5' TGCAGTTGCTGAGTCCTGTCA 3') by real-time PCR using Sybergreen reagent (Eurogentec, Herstal, Belgium) and β2-microglobulin or β-actin as internal reference. Amplification was measured with the ABI Prism 7700 Sequence Detection System and

analyzed with ABI Prism Sequence Detector v1.6.3 software (Applied Biosystems, Courtaboeuf, France).

5.2.6 *In vivo* macrophage disappearance response

Three-week-old mice, weighing 10-15 g, were injected i.p. with 50 μl/g 3% TG broth (Biomerieux, Marcy-Etoile, France). Peritoneal macrophages were harvested 6 or 18h after injection and stained as described above. Cells were kept on ice during the entire procedure. The experiment was repeated three times comprising a total of 6-12 mice per strain/group.

5.2.7 Macrophage adhesion and transmigration assays

Ninety-six-well flat-bottom culture plates or transwell filters (8 μm pore-size, Corning Costar Corporation, Cambridge MA) were coated overnight at 4°C with 10 μg/ml albumin (Sigma-Aldrich), fibronectin (Chemicon International, Temecula, USA) or CD49d or CD49e-specific FN-ligands (CS-1-peptide respectively RGD-peptide or an inactive analog (GRGESP) BACHEM Biochimie SARL, Voisins-le-Bretonneux, France). Wells or filters were washed 3 times with sterile PBS and non-specific sites were blocked with 1% BSA for 1h at 37°C. Wells were washed twice with PBS and once with RPM1-1640-glutamax completed with 0.5% FCS (Invitrogen, Cergy Pontoise, France). Freshly harvested peritoneal macrophages or day 7 BMDMs were added in a concentration of 5.10^4/ml in 100 μl RPMI-0.5% FCS and incubated for 30 min at 37°C. To standardize macrophage numbers, the percentage of $BM8^{hi}$ macrophages was determined in parallel, using cytofluorometry. After 30 min of adhesion, non-adherent cells were removed by gentle aspiration and washing with RPMI. Adherent cells were fixed and stained with the RAL-555 fast-staining kit (RAL REACTIFS, Bordeaux, France). For migration assays, 2.10^4/ml peritoneal cells were added in 100 μl RPMI-0.5% FCS in the upper compartment and incubated for 4 hrs at 37°C to allow migration to the lower compartment filled with 600 μl RPMI-0.5% FCS with or without 100 ng/ml MCP-1 (Peprotech, Rocky Hill NJ). Migrated cells were stained with BM8 to determine the absolute number of migrated macrophages. When blocking Abs were applied, adjusted macrophage numbers were incubated for 30 min on ice with 1 mg/ml of antibody prior to adhesion. Each condition was tested in triplicate in 2 or 3 independent experiments.

5.2.8 Culture of BMDMs with M-CSF

Two or 4-week old mice were sacrificed by cervical dislocation. Their femurs and tibiae were collected and muscle tissue was removed. Bone marrow single-cell suspensions were prepared as described previously [24] and cells were seeded in sterile culture dishes (Falcon) at $4x10^6$ cells/10 ml in RPMI-1640-glutamax, enriched with 10% FCS and 50 ng/ml recombinant murine M-CSF (R&D Systems, Abingdon, UK). Cultures were maintained for 7 days without refreshing culture media.

5.2.9 Statistical analysis

Data of disappearance and adhesion assays, as well as data of real-time PCR, were

expressed as mean ± SD or SEM; *P* values were calculated with the two-tailed Student's *t* test for unpaired values.

5.3 Results

5.3.1 Resident NOD macrophages express lower levels of membrane CD49d

We first examined the membrane expression levels of CD49d and CD49e as well as that of the laminin receptor α6-chain, CD49f. Peritoneal macrophages were identified by high expression of the macrophage-specific marker BM8 and, like most other peritoneal cells, expressed both CD49d and CD49e (Fig. 5.1A). By contrast, high levels of CD49f were only expressed by mature peritoneal macrophages (Fig. 5.1A). Interestingly, unlike blood monocytes (Fig. 5.1B), mature peritoneal NOD macrophages were characterized by lower CD49d membrane expression levels as compared to macrophages of C57BL/6 and BALB/c strains, independently of the sex (not shown) or age examined (Fig. 5.1B). No differences of the levels of the β1 integrin α-chains CD49e and CD49f were observed (Fig. 5.1C). Additionally, $BM8^+$ thymic macrophages of NOD mice showed lower CD49d membrane expression levels, as compared to thymic macrophages of control mice (Fig. 5.1D).

Peritoneal macrophages from male (not shown) or female F1[NODxC57BL/6] mice (Fig. 5.1E) expressed levels of CD49d intermediate to that of macrophages derived from both parental strains. This suggests that the macrophage CD49d membrane expression levels are inherent to the NOD genetic background. Interestingly, peritoneal macrophages of other diabetes-resistant strains like congenic NOD.H-2^b or NOR mice also exhibited higher levels of CD49d as compared to the parental NOD strains (table 5.1). By contrast, macrophages of 4-week old congenic NOD.CD45.1 mice that develop diabetes, expressed levels of CD49d similar to parental NOD strains (table 5.1).

Table 5.1 Mean fluorescence intensity (MFI) of CD49d in congenic NOD strains.

	Strain	MFI CD49d±SD	MFI isotype±SD	n (mice)
A	C57BL/6	360.24±25.42	2.55±0.01	6
	NOD	226.94± 9.54	7.48±3.10	6
	NOD.H-2^b	284.23± 3.86	2.48±0.14	6
B[a]	BALB/c	1342.09±114.59	31.40± 9.93	3
	NOD	684.81± 29.03	33.00±11.85	2
	NOR	1194.59±190.52	52.53±18.59	4
C	C57BL/6	311.89±27.87	4.19±0.03	6
	NOD	172.83± 1.63	3.97±0.01	6
	NOD.CD45.1	173.45± 9.00	2.70±0.16	6

[a]Analysis was performed on a different flow cytometer.

5.3.2 Resident NOD macrophages show delayed disappearance upon induction of inflammation

Inflammation of the peritoneal cavity results in the disappearance of resident macrophages [9]. This regulated process of macrophage adhesion and mesothelial transmigration depends, at least partly, on the interaction of α4β1 and α5β1 [10]. We examined the *in vivo* migratory and adhesive behavior of resident peritoneal macrophages in 3-wk-old NOD mice and C57BL/6 mice, in response to inflammation of the peritoneal cavity induced by thioglycollate (TG). For both mouse strains the frequency of resident $BM8^+$ macrophages decreased in response to TG, 6 and 18h after TG-injection (Fig. 5.2A). However, total numbers of peritoneal cells increased due to the influx of inflammatory monocytes and granulocytes (not shown). To correct for infiltrating cells, absolute numbers of $BM^{hi}CD49f^+$ resident macrophages were determined and the percentages of macrophages as compared to the steady state (non-inflamed) situation were calculated. The number of resident macrophages decreased by 70% in C57BL/6 mice, 6h after TG injection (Fig. 5.2B) with a further decrease to 90% after 18h. In NOD mice, a decrease of only 40% was observed after 6h as compared to the non-inflamed situation (Fig. 5.2B) and numbers of resident NOD macrophages hardly decreased thereafter.

5.3.3 NOD-macrophages show a defective interaction with fibronectin

Lower expression of CD49d on $CD34^+$ peripheral blood progenitor cells resulted in decreased adhesion to FN [13]. Therefore, we examined the capacity of NOD peritoneal macrophages to adhere and migrate to FN.

Resident peritoneal macrophages from both C57BL/6 and NOD mice adhered to FN (Fig. 5.3A). However, NOD macrophages did so in much lower numbers than C57BL/6-derived peritoneal macrophages, whereas only a minor difference was observed in the α4β1/α5β1-independent adherence to albumin, used here as an unrelated protein (Fig. 5.3A). Similar results were obtained when macrophages were allowed to adhere to the CD49d-specific ligand CS-1 (Fig. 5.3B), whereas no differences were observed in the adhesion to the CD49e-specific RGD containing peptide, an unrelated control peptide or albumin (Fig. 5.3B). When the interaction of α4β1 with FN was inhibited by the addition of different CD49d-specific blocking antibodies, adhesion to FN was significantly impaired for macrophages of C57BL/6 mice (Fig. 5.3C). Addition of the CD49d-specific antibodies did not affect the adherence of NOD macrophages to FN (Fig. 5.3C). These data show that, unlike C57BL/6 macrophages, CD49d hardly contributed to the adhesion of NOD macrophages to FN.

Furthermore, C57BL/6 macrophages readily migrated through FN-coated filters as compared to albumin-coated filters, whereas NOD macrophages barely migrated through the FN-coated filters (Fig. 5.3D). Addition of the chemokine macrophage chemoattractant-protein-1 (MCP-1) slightly stimulated the migration of C57BL/6-derived macrophages, but did not rescue the migration of NOD macrophages. Importantly, CD49d-specific antibodies almost completely prevented the MCP-1-stimulated migration of C57BL/6-macrophages, indicating that, under these conditions migration of macrophages on FN is mainly mediated by CD49d.

5.3.4 NOD-derived BMDMs fail to increase CD49d following LPS-induced maturation

Membrane density of CD49d and CD49e closely correlates with the stage of macrophage maturation [6]. We examined the integrin expression during the M-CSF-stimulated development of BMDMs from BM-precursors. No difference was observed in the expression of CD49d, CD49e or CD49f during the development of BMDMs, when NOD-derived BMDMs were compared to BMDMs from C57BL/6 or BALB/c mice (not shown).

BMDMs are not directly comparable to peritoneal macrophages, since the latter represent a fully differentiated population of cells. In keeping with their developmental stage, peritoneal macrophages were unable to change their integrin profile upon overnight stimulation with LPS or IFN-γ *in vitro* (not shown). We therefore used the homogeneous population of BMDMs as a model to examine the expression of CD49d and CD49e upon the induction of terminal macrophage development by stimulation with LPS or IFN-γ. Stimulation of BMDMs from both C57BL/6 and NOD mice with IFN-γ led to an increase in CD49d membrane expression (Fig. 5.4A). By contrast, NOD BMDMs failed to increase CD49d upon LPS stimulation, unlike BALB/c (not shown) and C57BL/6-derived BMDMs (Fig. 5.4A). Neither prolongation of the M-CSF cultures for up to 10 days, stimulation with higher concentrations of LPS, nor prolonged duration of LPS stimulation, affected the CD49d expression in NOD-derived BMDMs (not shown). They did, however, respond to LPS with an increase in the surface expression of CD49e (Fig. 5.4A) and a decrease in the expression of CD49f (not shown), similar to what was observed for BMDMs of the control strains.

To examine whether the increase in integrin surface expression is regulated at the level of gene transcription, we analyzed CD49d and CD49e mRNA expression of non-stimulated or LPS-stimulated BMDMs by real-time PCR. CD49e mRNA levels increased upon LPS-stimulation in both C57BL/6 as well as in NOD BMDMs (not shown). The levels of CD49d mRNA also increased upon LPS stimulation in both strains. However, this increase was two-fold higher for C57BL/6 BMDMs as compared to the increase in NOD BMDMs (Fig. 5.4B).

5.3.5 Differential CD49d distribution in BMDMs following LPS stimulation

To determine the implications of LPS stimulation, we examined integrin membrane distribution and FN-adhesive properties of LPS-stimulated NOD and C57BL/6 BMDMs.

LPS stimulation induced changes in BMDM-morphology (Fig. 5.5A), characterized by swelling of the cell body and the formation of more and thinner protrusions. CD49d (Fig. 5.5A) and CD49e (not shown) molecules re-localized upon LPS stimulation in C57BL/6 BMDMs, from a diffuse distribution to a linear staining on the adherent cell-edges. This CD49d redistribution was not observed in LPS-stimulated NOD BMDMs, although their change in cell morphology was evident (Fig. 5.5A).

The capacity to adhere to FN increased almost 3-fold after stimulation with LPS for C57BL/6 BMDMs and to a lower extent (~2.2-fold) for NOD BMDMs (Fig. 5.5B). Interestingly, higher numbers of LPS-stimulated C57BL/6 BMDMs adhered to CS-1

peptides and FN as compared to LPS-stimulated NOD BMDMs, whereas no differences were observed in the numbers of BMDMs adherent to control and RGD-peptides or albumin (Fig. 5.5C). A significant decrease in the numbers of adherent LPS-stimulated C57BL/6 BMDMs was observed when the interaction of α4β1 with FN was inhibited by the addition of CD49d-specific antibodies. By contrast, CD49d-specific antibodies hardly affected the adherence of LPS-stimulated NOD BMDMs to FN (Fig. 5.5D), similar to what was observed for mature peritoneal macrophages. Numbers of FN-adherent LPS-stimulated BMDMs from both strains decreased when the interaction of α5β1 was blocked or when a combination of CD49d and CD49e-specific antibodies was used (Fig. 5.5D). Thus, the adhesion to FN was mediated by both α4β1 and α5β1 for C57BL/6 BMDMs and foremost by α5β1 for NOD BMDMs, confirming earlier observations of mature peritoneal NOD macrophages.

5.3.6 Inhibition of ERK-1/2 phosphorylation enhances CD49d expression in both NOD and C57BL/6 derived BMDMs

LPS acts via CD14/Toll-like receptor-4 (TLR-4)-MD2 complexes on the cell surface [14]. The mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK)-1/2, downstream of MEK-1/2, belongs to one of the major signaling-pathways that become activated upon TLR-4 stimulation by LPS. We therefore studied the expression of phosphorylated-ERK-1/2 (pERK-1/2) in BMDMs in response to LPS stimulation.

Day 7 BMDMs were analyzed for CD49d and CD49e membrane levels after LPS stimulation, with or without simultaneous incubation with the MEK-1/2 inhibitor UO126. The inhibition of ERK-1/2 phosphorylation, simultaneously with overnight LPS stimulation, enhanced the membrane levels of CD49d in both C57BL/6 and NOD BMDMs (Fig. 5.6A). Levels of CD49d expression did not change when BMDMs were incubated with DMSO or UO126 alone (Fig. 5.6A). Although CD49d membrane levels of NOD BMDMs remained lower as compared to that of C57BL/6 BMDMs, the magnitude of relative increase in CD49d expression (compared to LPS alone) was similar in both strains.

Immunoblot analysis showed that pERK-1/2 was present in BMDMs of both strains as early as 15 min after addition of LPS (Fig. 6B). No pERK-1/2 was detected in non-stimulated cells (Fig. 5.6B), cells incubated with UO126 alone or BMDMs incubated with IFN-γ (not shown). Interestingly, a strong pERK-1/2 signal appeared in LPS-activated NOD BMDMs with earlier kinetics as compared to C57BL/6 BMDMs, when equal amounts of protein were loaded (Fig. 5.6B). Additionally, when related to total ERK protein, the pERK-1/2 signal in LPS-stimulated NOD BMDMs also appeared stronger than that of C57BL/6 BMDMs, (Fig. 5.6C).

Furthermore, CD49d mRNA significantly increased in C57BL/6 and NOD BMDMs when stimulated with LPS and UO126, as compared to LPS stimulation alone (Fig. 5.6D). Together, these data show that LPS-induced ERK-1/2 activation negatively regulated macrophage CD49d expression and strongly suppressed the enhancement of CD49d expression of NOD BMDMs.

5.4 Discussion

NOD macrophages display various abnormalities [4, 5]. Here, we show a substantially lower expression level of the α4β1 integrin α-chain CD49d in mature resident NOD macrophages as compared to control strains. Additionally, α4β1 appeared functionally defective in NOD macrophages, ascertained by their reduced adhesion to and migration on FN and supported by the higher numbers of resident macrophages that remained in the NOD peritoneum upon induction of inflammation, a process previously shown to be partially α4β1-dependent [10].

The differentiation of BM-precursors into BMDMs is associated with increased expression levels of α4β1 and α5β1 [6]. Here, we show a further increase of CD49d expression levels in BMDMs upon induction of final maturation by stimulation with IFN-γ or LPS. Non-stimulated NOD BMDMs and peripheral blood monocytes expressed CD49d levels similar to their C57BL/6 counterparts, whereas LPS-matured NOD BMDMs were defective in the increase of CD49d expression, like fully developed peritoneal NOD macrophages. This impaired increase in CD49d of NOD-BMDMs was LPS-specific, since stimulation with IFN-γ did increase the levels of CD49d. The defective CD49d increase was not related to a general lack of LPS-responsiveness, since NOD-BMDMs did respond to LPS stimulation with an increase in CD49e mRNA and membrane levels, similar to what was observed in control BMDMs. In this respect, it is noteworthy that CD49e-functionality is regulated differently from that of CD49d, as has been reported earlier [15]. Our data show that both CD49d function, as well as the upregulation of its expression by LPS is defective in NOD macrophages, while that of CD49e appears unaffected.

Maturation and increase of CD49d expression in human DC depends on the phosphorylation of p38 MAPK [16], and enhanced maturation of these cells was observed upon inhibition of ERK-1/2 phosphorylation [17]. Although ERK-1/2 and p38 MAPK are both phosphorylated upon LPS-stimulation, these signaling molecules usually do not operate in synergy. Moreover, direct “cross-talk” between ERK-1/2 and p38 MAPK resulted in the selective mutual suppression of their activity [18]. High levels of pERK-1/2 were observed early after LPS-stimulation in NOD BMDMs. Inhibition of ERK-1/2 phosphorylation allowed the increase of CD49d membrane levels and corresponding mRNA expression, in both C57BL/6 and NOD BMDMs. Interestingly, IFN-γ did upregulate CD49d in NOD macrophages and no pERK-1/2 was detected early after stimulation with IFN-γ in BMDMs derived from neither C57BL/6 nor NOD mice (not shown). Hence, LPS-induced ERK-1/2 phosphorylation negatively regulates macrophage CD49d expression and strongly suppresses the increase of CD49d upon final maturation in NOD macrophages. The coordinated regulation of the balance between activated MAPKs is believed to fine-tune the cellular response to a given stimulus. This balance may be disturbed in NOD macrophages upon stimulation with LPS, leading to high levels of pERK-1/2 and consequent suppression of CD49d expression. Stimulation of NOD macrophages was previously reported to result in hyper-activation of NF-κB [5]. NF-κB might be involved in the regulation of CD49d expression. However, we show that inhibition of ERK-1/2 phosphorylation alone was sufficient to increase CD49d expression

in NOD BMDMs. LPS-induced increase in NF-κB binding activity and increase in the levels of IL-12p40 can be regulated independently of ERK-1/2 activation [19]. Furthermore, NF-κB hyper-activation in NOD macrophages was observed upon activation with various stimuli, and not exclusively restricted to activation with LPS [5]. Thus, NF-κB hyper-activation in NOD macrophages is probably not involved in the defective CD49d expression following stimulation with LPS.

LPS is a well-studied exogenous activator of TLR-4. Aberrant TLR-4 responsiveness of NOD macrophages may lead to the unbalanced production of cytokines and, as is shown here, plays a critical role in the expression of relevant adhesion molecules like CD49d. Characteristic levels of CD49d and CD49e membrane expression generally reflect phenotypic features of fully differentiated cells and although the functional properties of α4β1 and α5β1 may change upon stimulation, the integrin expression profile usually remains unaffected [20]. Neither LPS nor IFN-γ affected CD49d expression profiles of peritoneal NOD or control macrophages, indicating their fully differentiated phenotype. The encounter of TLR-4 ligands during final macrophage maturation *in vivo* will enhance levels of CD49d in macrophages of control mice. However, activation of the TLR-4 pathway during maturation of NOD macrophages does not lead to enhanced CD49d expression. Fully developed peritoneal and thymic NOD macrophages indeed expressed lower levels of CD49d *in vivo* as compared to various controls strains, in accordance with the idea that TLR-4-signaling plays a role in steady-state macrophage maturation.

The distinct regulation of α4β1 and α5β1-mediated interaction determines their ability to independently mediate migration and adhesion, influenced by the local microenvironment. Low FN-concentrations preferentially stimulate the usage of α5β1 for migration of melanoma cells, whereas α4β1-usage is favored when FN-concentrations increase [15]. Our results show that NOD macrophages are hampered in their CD49d-mediated migratory capacities. Increased numbers of resident macrophages accumulate in the pancreas of neonatal and young NOD mice, even before the onset of insulitis [21]. It would be interesting to examine whether the impaired CD49d-mediated adhesion and migration is involved in the early retention of the macrophages in the pancreas. Such a prolonged local retention may affect the pancreatic microenvironment and favor diabetes development.

Overall, data presented herein show that mature NOD macrophages have decreased CD49d expression and defective CD49d-mediated adhesion to fibronectin. Considering our recent findings showing decreased α5β1 expression and functions in NOD thymocytes [22], it is likely that NOD mice bear multiple fibronectin receptor defects in their distinct hemopoietic lineages.

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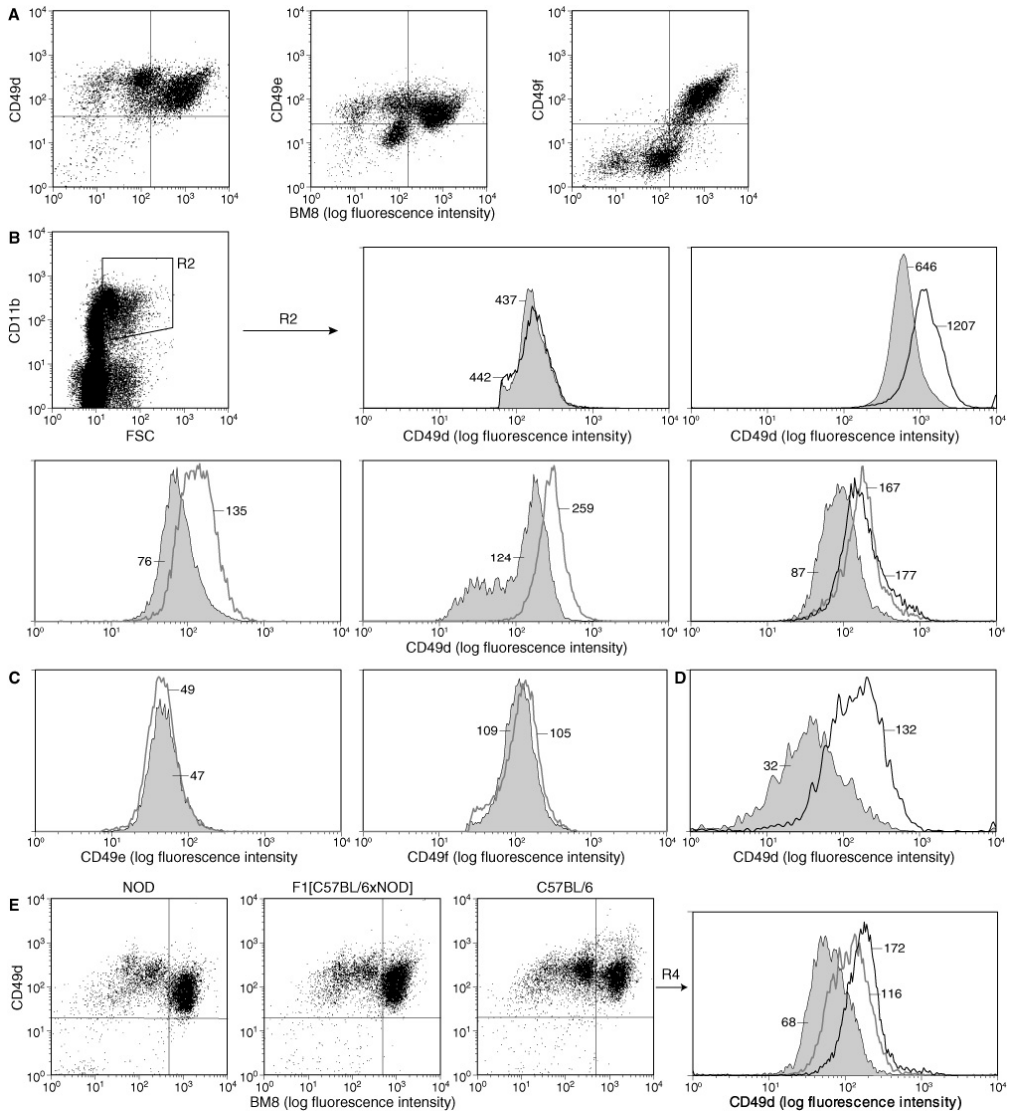

Figure 5.1 NOD macrophages exhibit low levels of surface CD49d.

A. Dot-plot analysis after cytofluorometry of peritoneal macrophages of 2-wk old C57BL/6 mice stained with BM8 (upper right quadrant) express CD49d (left plot), CD49e (middle) and CD49f (right plot).

B. Upper panels: $FSC^{hi}CD11b^{hi}$ blood monocytes (**dot plot, left**) were gated and CD49d levels (**middle histogram**) of 10-wk-old NOD mice (closed graphs) were compared to that of age-matched BALB/c mice (open graphs). Peritoneal $BM8^{hi}$ macrophages of the same mice were also analyzed for CD49d expression (**right histogram**) using identical settings (NOD, closed graphs; BALB/c, open graphs).

Lower panels: histograms show CD49d membrane levels of macrophages of NOD mice (closed graphs) compared to those of C57BL/6 mice (open graphs) at 2 wks (**left**) or 14 wks (**middle**) of age and compared to C57BL/6 (grey) and BALB/c (black) at 4 wks of age (**right**).

C. Histograms show CD49e and CD49f levels of NOD (closed graphs) and C57BL/6 (open graphs) peritoneal macrophages at 2 wks of age.

D. Histogram shows CD49d expression of $BM8^{+}$ thymic macrophages of NOD (closed graphs) and C57BL/6 (open graphs) mice at 3 wks of age.

E. Dot plots and histograms show CD49d membrane levels of $BM8^{+}$ peritoneal macrophages of NOD (closed graph), F1[NOD x C57BL/6] (grey, open graph) and C57BL/6 (open graph) mice at 2 wks of age.

Experiments were performed with pools of macrophages for young mice and comprised at least 5 mice/strain/age (n=12 animals per strain). For older mice, cells from individual mice were analyzed. Results are given for one representative experiment, selected out of two or more independent experiments. The MFI is depicted for each analysis.

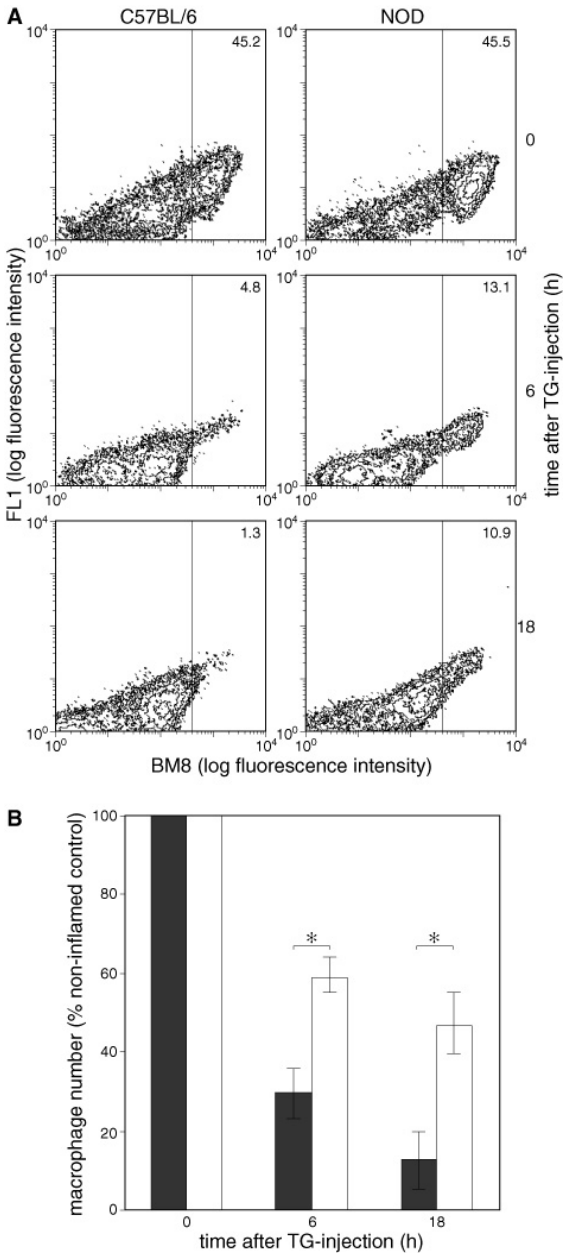

Figure 5.2 Resident NOD peritoneal macrophages show delayed disappearance following peritoneal inflammation.

A.Density plots show peritoneal cells stained with BM8 from 3-wk-old C57BL/6 or NOD mice before and after TG-injection. Frequences of $BM8^{hi}$ macrophages are depicted. Results are given for one representative experiment, selected out of a series of three independent experiments.

B. Percentages of numbers of $BM8^{hi}$ resident macrophages in C57BL/6 (closed bars) and NOD mice (open bars) that remained in the peritoneum after TG injection as compared to the non-inflamed steady state situation, defined as 100% at t=0h. Data are expressed as the mean of resident macrophages (% non-inflamed control) ± SEM from mice of two experiments with * $p < 0.05$ (n=6 or more/strain for each given timepoint).

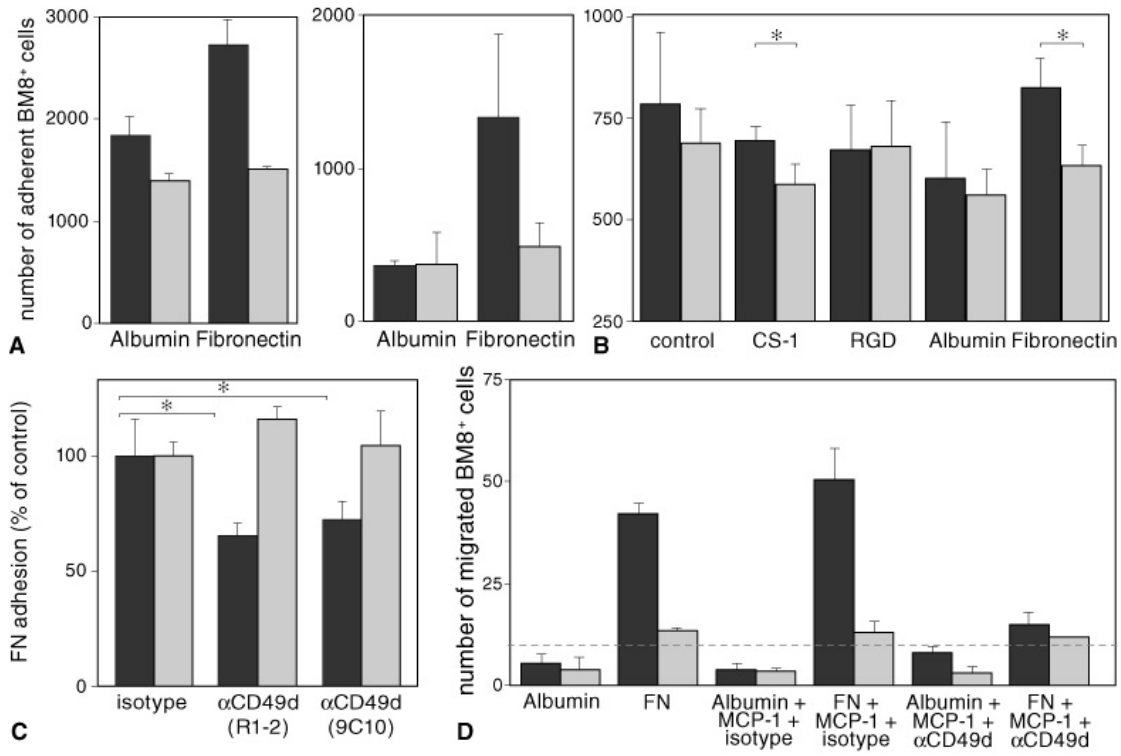

Figure 5.3 NOD-derived peritoneal macrophages show defective CD49d-mediated interaction with fibronectin.

A. Graphs show standardized numbers of albumin or fibronectin-adherent $BM8^+$ C57BL/6 macrophages (black bars) or NOD macrophages (grey bars) at 2 wks (**left**) or 14 wks of age (**right**).

B. The graph shows standardized numbers of $BM8^{hi}$ C57BL/6 macrophages (black bars) or NOD macrophages (grey bars) adherent to control-, CS-1- or RGD-peptide, albumin or fibronectin.

C. The graph shows standardized numbers of adherent $BM8^+$ cells from 4-wk-old mice after incubation with CD49d-specific or an unrelated isotype-matched antibody. Adherent cells were counted and related to the number of adherent cells when no antibody was added.

D. The graph shows standardized numbers of $BM8^+$ peritoneal cells of C57BL/6 (black bars) or NOD mice (grey bars) that migrated through FN- or albumin-coated transwell filters with or without the addition of MCP-1and CD49d-specific or isotype-matched antibody.

Data are expressed as the mean±SD with * $p < 0.05$ of triplicates of one representative experiment (n=5/strain), out of two or more independent experiments.

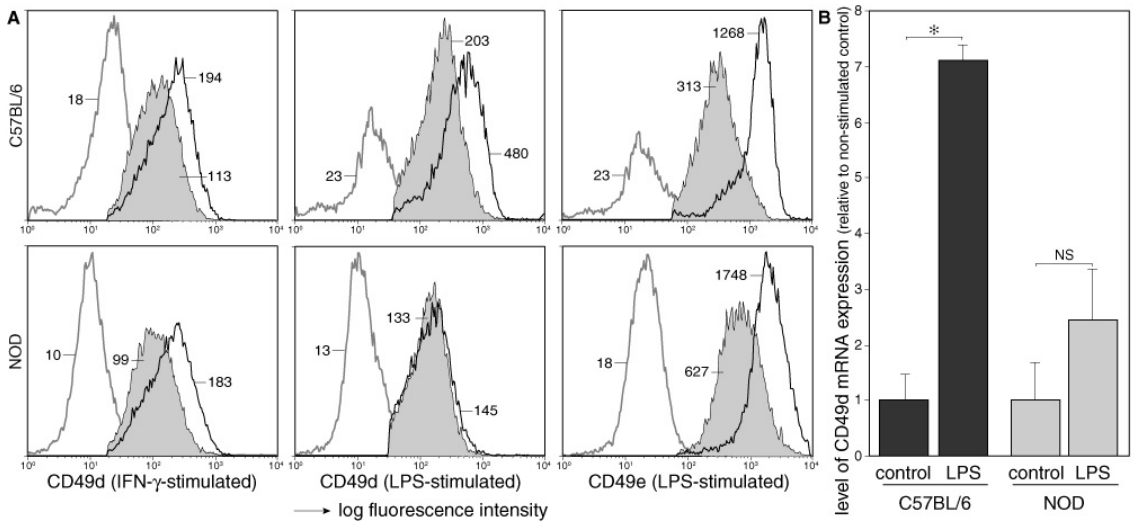

Figure 5.4 NOD-derived BMDMs fail to enhance CD49d upon LPS stimulation.

A. Cytofluorometry profiles of CD49d or CD49e. Grey lines represent isotype-matched controls. The MFI is depicted for each analysis.

Left graphs: CD49d-levels of C57BL/6-derived BMDMs (**upper panels**) and NOD-derived BMDMs (**lower panels**), not stimulated (closed graphs) and IFN-γ-stimulated (open graphs).

Middle graphs: CD49d-levels of non-stimulated BMDMs (closed graphs) compared to LPS-stimulated BMDMs (open graphs).

Right graphs: CD49e-levels of non-stimulated BMDMs (closed graphs) compared to LPS-stimulated BMDMs (open graphs).

B Relative levels of CD49d mRNA expression determined by real-time PCR of LPS-stimulated C57BL/6-(black bars) and NOD-(grey bars) derived BMDMs as compared to non-stimulated BMDMs ± SD of triplicates from one representative experiment, out of two or more independent experiments. * $p < 0.05$; NS = not significant.

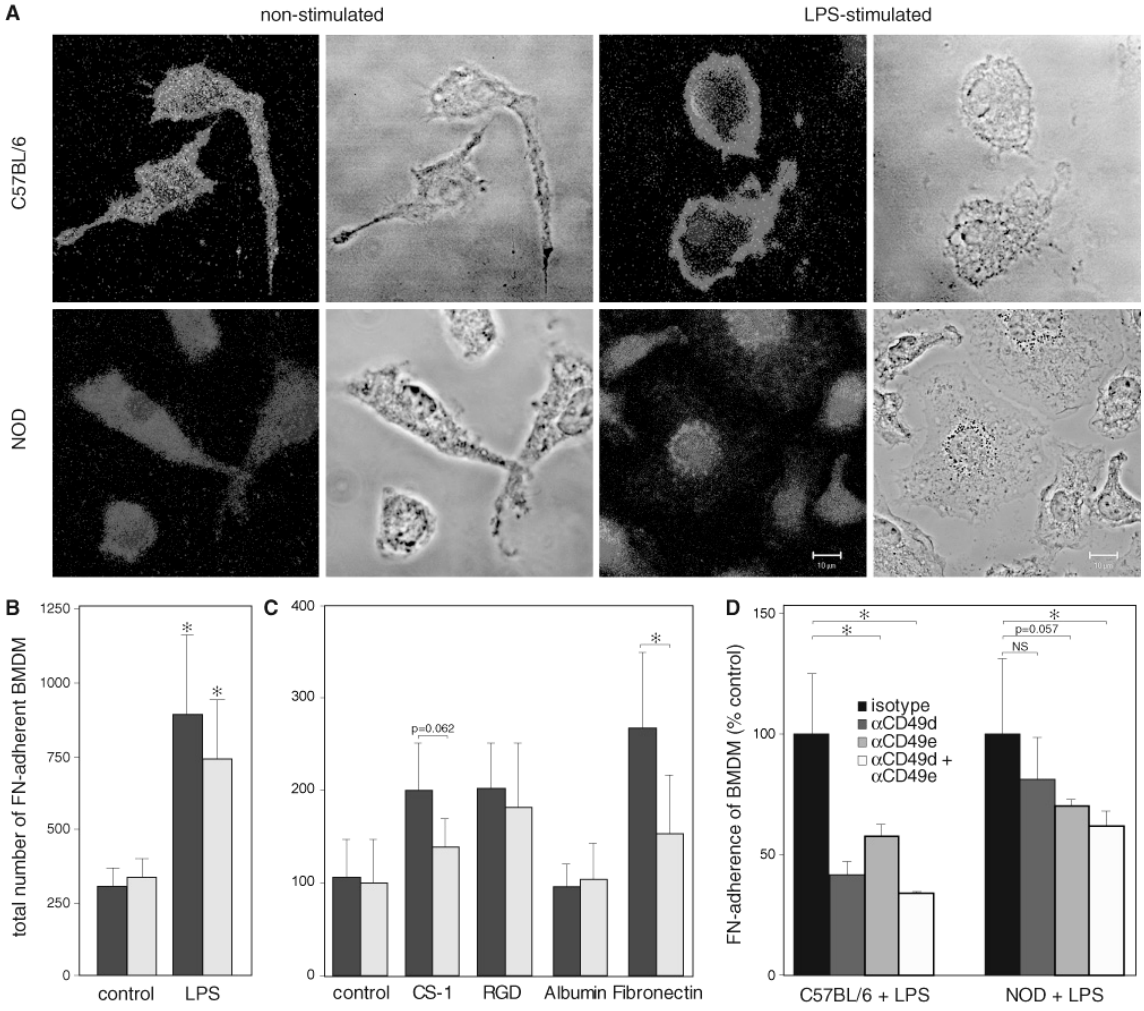

Figure 5.5 Differential CD49d distribution in BMDMs following LPS stimulation.

A. Confocal microscopy analysis of CD49d expression (green) of adherent day 7 C57BL/6-derived BMDMs (**upper panel**) and NOD-derived BMDMs (**lower panel**) before (**left**) and after (**right**) overnight stimulation with LPS. Nuclei are stained with propidium iodide (red). Original magnification: x1000.

B. The graph shows standardized numbers of non-stimulated or LPS-stimulated day 7 BMDMs derived from C57BL/6 (black bars) and NOD (grey bars) mice, adherent to FN.

C. The graph shows standardized numbers of LPS-stimulated day 7 BMDMs derived from C57BL/6 (black bars) and NOD (grey bars) mice adherent to control-peptide, CS-1-peptide, RGD-peptide, albumin or FN.

D. The graph shows standardized numbers of FN-adherent LPS-stimulated day 7 BMDMs from C57BL/6 or NOD mice incubated with specific blocking antibodies prior to FN adhesion. Adherent cells were counted and related to the number of adherent cells when no antibody was added.

Data are expressed as the mean ± SD of triplicates from one representative experiment, out of two or more independent experiments. * $p < 0.05$; NS = not significant.

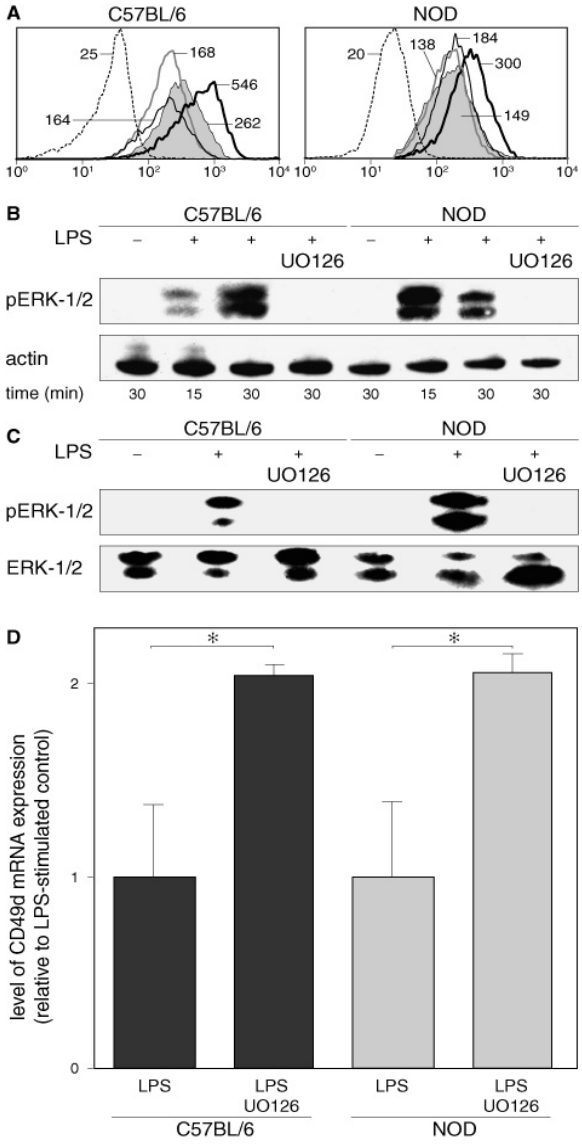

Figure 5.6 Inhibition of ERK-1/2 phosphorylation enhances membrane CD49d expression in LPS-stimulated BMDMs.

A. CD49d levels of BMDMs were determined by cytofluorometry after overnight LPS-stimulation (closed graphs) or LPS stimulation and simultaneous inhibition of ERK-1/2 phosphorylation by UO126 (open graphs). Cells incubated with DMSO (grey lines), UO126 (thin black line) or isotype-matched irrelevant mAb (dashed line) served as controls.

B. Immunoblots of BMDM-lysates, non-stimulated or stimulated with LPS for different duration with or without UO126, stained for pERK-1/2 or actin.

C. Immunoblots of BMDM-lysates, non-stimulated or stimulated for 15 min with LPS with or without UO126, stained for pERK-1/2 or total ERK-1/2.

D. Relative levels of CD49d mRNA expression determined by real-time PCR. Compared to LPS-stimulated BMDMs, a 2-fold increase in the level of CD49d mRNA was observed in both C57BL/6 BMDMs and NOD BMDMs, when the MEK-1 inhibitor UO126 was added. Data are expressed as the CD49d mRNA levels from LPS plus UO126-stimulated BMDMs over CD49d mRNA levels from LPS-stimulated BMDMs ± SD * $p < 0.05$.

Chapter 6

Fibronectin receptor defects in NOD mouse Leukocytes: possible consequences for Type 1 Diabetes

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Abstract

Integrins of the very late antigen (VLA) family mediate leukocyte traffic to lymphoid organs under physiological conditions and in chronic inflammatory situations such as autoimmunity. Accordingly, the current thinking is of a positive correlation between VLA expression and capability of the generation of autoimmunity. Herein we discuss recent findings on the defective expression of integrin-type fibronectin receptors α4β1 (VLA-4) and α5β1 (VLA-5) in the non-obese diabetic (NOD) mouse, a murine model of autoimmune insulin-dependent diabetes mellitus. As compared with normal animals, NOD thymocytes (including the $CD4^+CD25^+$ T regulatory cells) exhibit a decrease in the membrane expression of α5β1, resulting in a functional impairment of fibronectin-mediated interactions, including cell migration. Interestingly, thymocytes that are trapped within the giant perivascular spaces seen in NOD thymus are consistently α5β1negative, suggesting that the progressive arrest of mature cells can be related to the α5β1defect. Peripheral T cells also exhibit decreased α5β1membrane expression and impaired fibronectin-driven migration.

Additionally, we observed a defect in α4β1fibronectin receptor expression in NOD macrophages. Peritoneal, bone marrow-derived precursor as well as thymic macrophages of NOD mice showed an impaired upregulation of α4-integrin chain expression, dependent on the level of macrophage maturation. Overall these data lead to the notion that NOD leukocytes bear distinct fibronectin receptor-mediated cell migration defects, which may be involved in the pathogenesis and/or pathophysiology of the autoimmune events seen in NOD mice.

Further studies will be helpful to define whether or not this concept can be applied for other autoimmune diseases.

6.1 Introduction

Traffic of leukocytes is regulated by the specific interactions of integrins and other adhesion molecules. Integrins mediate not only the homing of leukocytes to lymphoid organs under steady state conditions, but also the specific recruitment of leukocytes to peripheral organs in chronic inflammatory situations such as autoimmunity. Integrins are heterodimeric cell-surface molecules formed by one alpha and one beta chain that can effectively establish contacts of leukocytes with other cells or the extracellular microenvironment. Such interactions result in activation of signaling pathways within the leukocytes, yielding distinct biological responses, including cell migration and cell activation. The entire family of integrins has 24 different members that comprise 18 alpha and 8 beta chains identified so far [1]. The largest subgroup of integrins, the so-called very late antigens (VLA), share the same β1 chain (CD29) that can be coexpressed in combination with 12 different α chains (CD49), such as α1β1 (VLA-1 or CD49a/CD29), α2β1 (VLA-2 or CD49b/CD29), etc. Unlike the β2 integrins that are only expressed on leukocytes, β1 integrins are expressed on a variety of cells, and a common feature of the VLA family is the specific binding to extracellular matrix (ECM) ligands. Some β1 integrins specifically recognize different ligands, while different β1 integrins have been identified that bind to the same ECM component. Alpha4β1 (CD49d/CD29) and α5β1 (CD49e/CD29) are both fibronectin receptors that display specificity for distinct regions of this ECM-protein, whereas α4β1 additionally recognizes the vascular endothelial cell adhesion molecule VCAM-1.

In general, overexpression of integrins, rather than their decreased expression, is related to an enhanced susceptibility to chronic inflammation and autoimmunity [2]. Increased levels of α1, α1, α3, α4, α5 and α6 integrin chains are expressed on the surface of lymphocytes from synovial fluid of rheumatoid arthritis patients when compared to of healthy controls [3]. Engagement of the α1β1 integrin with its ligands laminin and collagen regulates the progression of the inflammatory process of hapten- and dextran sodium sulfate induced colitis [4, 5], while both α4β1and α5β1 fibronectin receptors have been implicated in the pathogenesis of several autoimmune diseases such as experimental encephalomyelitis, as well as autoimmune diabetes [6, 7]. In non-obese diabetic (NOD) mice, an important murine model of autoimmune insulin-dependent diabetes mellitus (also referred as type 1 diabetes), autoreactive as well as regulatory T-cells use α4β1 and α5β1-mediated pathways for their recruitment to the inflamed pancreas [8]. Thus, the current thinking is of a positive correlation between VLA expression and capability of autoimmunity generation. In this review we will discuss recent findings on the defective expression of integrin-type fibronectin receptors α4β1 and α5β1 in NOD mice, and the possible relationship with the pathogenesis of the autoimmune diabetes. Nevertheless, in order to better understand the data summarized below, it seems worthwhile to briefly provide a general background on the autoimmune insulitis.

The early phases of pancreatic immune infiltration progress asymptomatically and intraislet infiltration of mononuclear cells (insulitis, a characteristic feature of type 1

diabetes) comprises mostly $CD4^+$ and $CD8^+$ T lymphocytes, dendritic cells (DC) and macrophages [9-11]. The multistep process that finally results in clinical diabetes involves both lymphoid as well as myeloid leukocyte lineages (Fig. 6.1).

Most of our current understanding of mechanisms involved in type 1 diabetes development has derived from studies in small-animal models that develop diabetes spontaneously, such as the NOD mouse. In this animal, the first signs of hyperglycemia can be observed as early as 12 weeks of age. Incidence of overt diabetes is as high as 60-90% around 20 weeks of age in female NOD mice and, in accordance with the human situation, tends to be lower in males [12, 13].

Despite the fact that the pancreas is the main target organ severely affected by the immune system, extra-pancreatic signs of autoimmunity such as autoimmune thyroiditis and adenosialitis are observed in NOD mice, being also frequently diagnosed in type 1 patients [14-16]. This indicates a lower threshold for the development of autoimmunity and strongly suggests that faulty immune control is underlying autoimmunity in NOD mice.

Alpha4β1 mediated interactions play a pivotal role in early and late phases of IDDM, whereas the cooperative interaction of α4β1 and α5β1 is implicated in the intraislet infiltration of autoreactive lymphocytes [17, 18]. Beta-1 and its associated alpha chains α4 and α5, are expressed early in embryogenesis and essential for normal development. Deficiency of β1, α4 or α5 integrin chains resulted in embryonic lethality due to the impairment of different developmental processes [19-21]. Deficiencies within the hematopoietic compartment resulted in distinct phenotypic characteristics. Alpha-4 expression is required for the proper differentiation of erythroid, myeloid and lymphoid progenitors [22]. Alpha-5 is essential for vasculogenesis [21], while only lack of the β1 chain severely affected the migration of hematopoietic progenitors to the fetal liver, without directly affecting their differentiation [23]. Alpha44β1 and α5$\beta$are also expressed on adult hematopoietic stem cells and later on observed on subsets of lymphocytes, granulocytes and monocytes, the blood-born precursors of macrophages and DC [24, 25]. Since α4β1- and α5β1-regulated pathways efficiently mediate migration of autoreactive T lymphocytes in NOD mice, this might imply that the imbalanced expression of certain integrins may favour autoimmunity by promoting leukocyte migration. Accordingly, overexpression of the α4-chain of α4β1 enhances endothelial adhesion and splenic homing of T cells, and induces antigen-independent antigen presenting cell (APC)-mediated proliferation, and T-cell autoreactivity [2].

In addition to lymphocytes, macrophages are also critically involved in the pathophysiology of type 1 diabetes in NOD mice: depletion of peripheral macrophages as well as the prevention of intra-islet infiltration of macrophages abrogated diabetes [26, 27]. Macrophages appear early in the pancreas and the first pancreatic accumulations of these cells in NOD mice were seen in the peri-vascular areas, by 3 weeks of age, before the establishment of lymphocyte infiltration [11]. These resident macrophage subsets are later on observed surrounding the islet-periphery, while lymphocytes were still absent. Defective maturation of macrophages and of dendritic cells has been described in NOD mice [28, 29], and in type 1 diabetes patients [30]. This is correlated with a reduced

ability of antigen presentation by NOD mouse APC [31] and may lead to the inefficient induction of negative selection in the thymus, and/or to the impaired activation of peripheral regulatory T cells.

6.2 NOD mouse thymocytes exhibit a fibronectin receptor defect

Previous studies showed that the thymic microenvironment of NOD mice is altered, with phenotypic and structural abnormalities of epithelial cells both in the cortex and medulla, and an enhancement of the ECM network, which is denser in both the cortical and medullary regions of the thymic lobules [32-34]. Additionally, we showed that these animals progressively develop giant perivascular spaces (PVS) around medullary vessels (Fig. 6.2). Such giant PVS are filled with lymphocytes, which are found intermingled with a novel ECM-containing intra-PVS [32, 33]. The appearance of giant PVS within NOD thymus was later confirmed by others as well [36]. We further demonstrated that most of the intra-PVS are mature thymocytes, although clusters of B cells can occasionally be found. Long-term pulse chase experiments with bromodeoxyuridine revealed that these mature thymocytes can be trapped along with time, within these giant PVS [37].

Interestingly, we showed that the formation of giant PVS in NOD mouse thymus was not due to the thymic epithelium, but was dependent of a hemopoietic-derived cell type [34]. Overall, these data pointed to a defect in thymocyte traffic, resulting in the slow, but progressive accumulation of mature cells within PVS in the medulla of thymic lobules. Considering previous data from our and other laboratories, showing that ECM-mediated interactions play a relevant role in thymocyte migration [38, 29], we raised the hypothesis of a defect in such interactions taking place in the thymus of NOD mice.

In this respect, we recently searched for the expression of integrin-type receptors in thymocytes, including the laminin receptor $\alpha6\beta1$ integrin (VLA-6 or CD49f/CD29), as well as the fibronectin receptors $\alpha4\beta1$ and $\alpha5\beta1$. When we compared NOD thymocytes with cells from other normal and autoimmune mouse strains, we found a consistent decrease in the membrane expression of $\alpha5\beta1$ in NOD mice (Fig. 6.3). Interestingly, such a defect, also detected at the $\alpha5$ integrin chain mRNA, appeared in the $CD25^-CD44^-$ late stage of $CD4^-CD8^-$ thymocyte development, and persisted along with maturation being even more conspicuous in mature CD4 or CD8 single positive cells. Moreover, it persisted throughout the development of the animals until ageing [40]. Interestingly, $\alpha5\beta1$expression in NOD-derived thymic epithelial cells was normal, as compared to TEC derived from C57BL/6 or BALB/c mice, indicating a degree of specificity in the intrathymic defect of $\alpha5\beta1$expression that concerns the lymphoid compartment.

We also evaluated $\alpha5\beta1$within specific regulatory T cell populations, such as mature $CD4^+CD62L^+$ and $CD4^+CD25^+$ cells, known to prevent diabetes onset in NOD mice [41, 43]. In fact, both $CD4^+CD62L^+$ and $CD4^+CD25^+$ cells in the NOD thymus exhibited a decrease in $\alpha5\beta1$membrane expression, as compared to normal animals. For example, among $CD4^+$ SP thymocytes, the proportion of $CD25^+\alpha5\beta1^+$ cells was three times lower in the NOD thymus, whereas $CD25^-\alpha5\beta1^+$ cells were twice less frequent in

NOD than in BALB/c thymuses. This is in keeping with the data showing that thymocytes bearing the regulatory phenotype $CD4^+CD25^+$ are progressively accumulated within the NOD thymus [41].

We further showed that the phenotypic defect of $\alpha5\beta1$expression seen in NOD thymocytes, did result in a functional impairment of fibronectin-mediated interactions. NOD thymocyte migration was diminished in thymic nurse cells, lymphoepithelial structures that can be used to evaluate thymocyte migration (particularly cortical thymocytes) in the context of thymic epithelial cells [39, 44]. It should be noted that we had previously shown that transit of thymocytes in TNCs is partially mediated by fibronectin and $\alpha5\beta1$: both lymphocyte release from thymic nurse cells and reconstitution of lymphoepithelial complexes with immature thymocytes plus TNC-derived epithelial cultures were enhanced by the ligand, and blocked by the addition of anti-fibronectin or anti-$\alpha5\beta1$antibodies [45]. Moreover, as compared with cells derived from normal mouse strains, the ability of NOD thymocytes to adhere onto fibronectin was significantly decreased, an effect that was actually secondary to the $\alpha5\beta1$ (and not $\alpha4\beta1$) defect, as the remaining adhesion could be inhibited by the $\alpha4\beta1$-blocking peptides but not by the antagonistic peptide of $\alpha5\beta1$ (an RGDS-containing peptide). Furthermore, such $\alpha5\beta1$-related fibronectin adhesion defect comprised both immature and mature thymocyte subsets. Lastly, fibronectin-driven migration of NOD thymocytes was also impaired, particularly in terms of mature CD4 or CD8 single positive subsets [40].

This finding points to a putative arrest of some thymocytes within the organ. Interestingly enough, when we examined $\alpha5\beta1$expression *in situ*, we found that thymocytes being trapped within the giant PVS were consistently $\alpha5\beta1$negative, suggesting that the progressive arrest of mature cells within PVS can be related to the $\alpha5\beta1$defect.

6.3 Is the defect in $\alpha5\beta1$ maintained in NOD peripheral T cells?

An obvious issue arising from the data summarized above concerns the levels of VLA expression in the periphery of the NOD immune system. In fact, we recently noticed decreased $\alpha5\beta1$membrane expression in splenocytes and lymphocytes from mesenteric and pancreatic lymph nodes (Fig. 6.4A). Interestingly enough, we observed impaired fibronectin-driven migration of lymphocytes from NOD pancreatic lymph nodes as compared to controls (Fig. 6.4B). It should be noticed that, in contrast to what happens in the thymus where we found enhanced numbers of thymocytes bearing the $CD4^+CD25^+$ regulatory phenotype, in NOD mice when compared with C57BL/6, the numbers of regulatory T cells in NOD mesenteric and pancreatic lymph nodes are diminished (Fig. 6.4C), whereas no changes are seen in the spleen. Additionally, these $CD4^+CD25^+$ cells exhibit much less $\alpha5\beta1$ than their counterparts in normal animals (Fig. 6.4D).

6.4 NOD macrophages also show aberrant fibronectin receptor expression

As was mentioned before, macrophages are relevant players in the pathogenesis of type 1 diabetes in the NOD mouse as well, but the exact level of their

involvement is not entirely resolved so far. NOD macrophages appear to be functionally disturbed in terms of maturation, phagocytosis and antigen presentation [31, 46, 47]. Interestingly, increased numbers of macrophages and DC were observed in the early preweaning pancreas of NOD mice as compared to pancreases of other mouse strains [48]. These resident APC subsets are located in close apposition to the ECM-containing connective lining, preceding their trafficking to, and accumulation at, the islet periphery during insulitis (Fig. 6.5).

In this respect, we recently evaluated whether these cells exhibit a VLA defect, similar to lymphocytes. We actually observed a defect in fibronectin receptor in NOD macrophages that affected the expression of $\alpha4\beta1$, but not that of $\alpha5\beta1$, and this feature did not depend on a particular tissue environment. Peritoneal macrophages, bone marrow-derived precursor, as well as thymic macrophages of NOD mice showed an impaired upregulation of CD49d, which was dependent on the level of macrophage maturation. Accordingly, this was accompanied by a decrease in CD49d-, but not CD49e-mediated adhesion of NOD mouse macrophages to fibronectin (Geutskens *et al.*, chapter 5).

Non-adherent myeloid precursors acquire an adherent macrophage phenotype upon maturation, associated with increase in $\alpha4\beta1$and $\alpha5\beta1$ fibronectin receptors [49]. The affected $\alpha4\beta1$expression might thus relate to the earlier reported impaired maturation of NOD macrophages. However, $\alpha4$-null/RAG-$2^{-/-}$ chimeric mice that were deficient in $\alpha4$ expression showed a delayed development of myeloid and lymphoid lineages, resulting in significantly decreased numbers of monocytes and their precursors [50]. This suggests that $\alpha4\beta1$ may be essentially involved in macrophage maturation.

6.5 Is there a relationship between the fibronectin-receptor defects in NOD leukocytes and the appearance and/or maintenance of type 1 diabetes?

Impaired $\alpha5\beta1$ and $\alpha4\beta1$ fibronectin receptor expression and function, was observed in NOD thymocytes and macrophages, respectively. Defective integrin expression and the consequent impaired migration of leukocytes may lead to their inappropriate retention and stimulation of cells, possibly resulting in local damage of the tissue microenvironment. This could in particular be important considering the early macrophage accumulation preceding insulitis in NOD mice.

Neonatal apoptosis of pancreatic β-cells is regarded as an important trigger for the initiation of IDDM [51] and apoptotic cells should be cleared and processed efficiently to avoid local inflammation. The involvement of $\alpha4$-integrin in the uptake of apoptotic leukocytes was recently described for endothelial cells [52]. As macrophages are closely related to endothelial cells and given that phagocytosis of apoptotic cells of NOD macrophages is defective [47], it is conceivable that lower $\alpha4$ expression of NOD macrophages is involved in the reduced phagocytic function of these cells.

Alpha4$\beta1$ and $\alpha5\beta1$ also exhibit additional functions that bear significance to the induction and progression of autoimmunity in general. In the thymus, activation-induced cell death of $CD4^+CD8^+$ thymocytes appeared to be mediated by $\alpha5\beta1$ (but not by $\alpha4\beta1$) and fibronectin [53]. Therefore, impaired interaction of $\alpha5\beta1$ of NOD thymocytes

potentially affects the negative selection process in the thymus and may favour the generation of an autoimmune susceptible T-cell repertoire. In addition, the likely $\alpha5\beta1$-related arrest of $CD4^+CD25^+$ in thymus, and consequent low numbers of these cells being settled in the periphery, would favor expansion of the autoreactive T cells.

In a second vein, it has been reported that the joint binding of $\alpha5\beta1$ and $\alpha4\beta1$ to fibronectin contributes to CD3-mediated proliferation of $CD4^+$ T-cells in a synergistic manner [54-56]. Additionally, increased proliferation mediated by fibronectin was observed for mononuclear cells isolated from the lamini priopria of mice suffering from hapten-induced colitis, but not from cells of control mice [4]. Whether this fibronectin-mediated interaction has different significance for different $CD4^+$ T-cell subsets is currently not known, but represents an interesting topic of research in view of the deficient regulatory T-cell populations observed in NOD mice.

The impaired fibronectin receptor expression in NOD leukocytes may be secondary to the affected intracellular signaling pathways, the so-called inside-out signaling, or to an inefficient contact with the specific microenvironmental niche (outside-in signaling). In this respect, it should be pointed out that cell trafficking is not solely decided by the cellular integrin expression pattern, but by the tissue microenvironment as well, including its distinct ECM molecules, which can concentrate cytokines, chemokines and growth factors (thus favouring their presentation to migrating leukocytes). For example, melanoma cells use $\alpha5\beta1$ for their migration on low fibronectin concentrations, switching to $\alpha4\beta1$-usage when fibronectin concentrations increase [57]. Bearing in mind the undisturbed homing of autoreactive T-lymphocytes and inflammatory macrophages to the pancreas in NOD mice, it is conceivable that defective fibronectin receptor expression affects some subsets to a greater extent than others, or defects are possibly being overruled by local inflammatory conditions.

In most cases, treatment of NOD mice with antibodies against $\alpha4$- and/or $\alpha5$-integrin alpha chains to prevent the interaction with their corresponding ligands was applied chronically or at later stages, when initiation of auto-reactivity against β-cells already took place [8, 17, 18]. As autoreactive effector T-cells use $\alpha4\beta1$ and $\alpha5\beta1$ for their specific homing to the pancreas [7, 8], it is difficult to draw conclusions on the role of these integrins in the early phases of IDDM.

Thus, at the moment we cannot exclude the possibility that the defective expression of fibronectin receptors in NOD leukocytes actually contributes to the susceptibility of these animals to develop autoimmunity in general, and not type 1 diabetes in particular.

6.6 Concluding remarks and remaining questions

The series of data discussed herein leads to the concept that NOD leukocytes bear cell migration-related defects. Considering that thymocyte migration likely results from a combined action of, at least, ECM-mediated and chemokine-mediated interactions [39, 44], it will be worthwhile to study the intrathymic expression of chemokines and chemokine receptors by NOD thymic cells, as well as the responsiveness of NOD lymphocytes and macrophages to distinct chemokines, in combination or not with ECM

molecules. We are presently approaching this issue, and preliminary data suggest an increased migratory activity of NOD thymocytes to the chemokine CXCL12 (Mendes-da-Cruz et al, unpublished data), possibly as a compensatory event of the reduced fibronectin-driven migration.

Additionally, the results presently available in the literature are scarce regarding the migratory capacities of NOD peripheral T cells. Yet, it is clear that chemokines are relevant for the intra-islet cell infiltration [58, 59]. Again, the combined role of ECM and chemokines should be investigated to better understand the various biological interactions driving these cells towards the pancreas parenchyma.

Resident pancreatic macrophages are the first APC subsets involved in the direct communication with the endocrine islets [11]. Their ontogeny is not known, but they may derive from precursors that reside locally in the pancreas. A detailed study combining the ontogeny of pancreatic macrophages and pancreatic ECM compartment may give additional insights in the local ongoing processes related to integrin-ECM-mediated interaction and diabetes pathogenesis. This issue is presently under investigation in our laboratory. Furthermore, clarification of the mechanisms underlying the defective fibronectin receptor, as the dissection of inside-out signaling pathways involved, can possibly relate these defects to aberrancies observed in NOD thymocytes and macrophages, and contribute to the comprehension of the role of these integrins in the pathogenesis of diabetes.

In conclusion, the data discussed above raise the possibility that, at least in NOD mice, impaired fibronectin- receptor-mediated interactions may be involved in the generation and/or progression of the autoimmune response. Further functional studies in NOD mice, integrating integrin defects with regulatory T cells and phagocytosis and/or antigen presentation will throw some more light on a putative dual role of fibronectin receptors in autoimmune diabetes. Additionally, with the analysis of other experimental models for autoimmune diseases, such as experimentally induced colitis and encephalitis, we will be able to evaluate whether the hypothesis herein raised for NOD mice, can be applied more widely, in the general outcome and control of autoimmunity.

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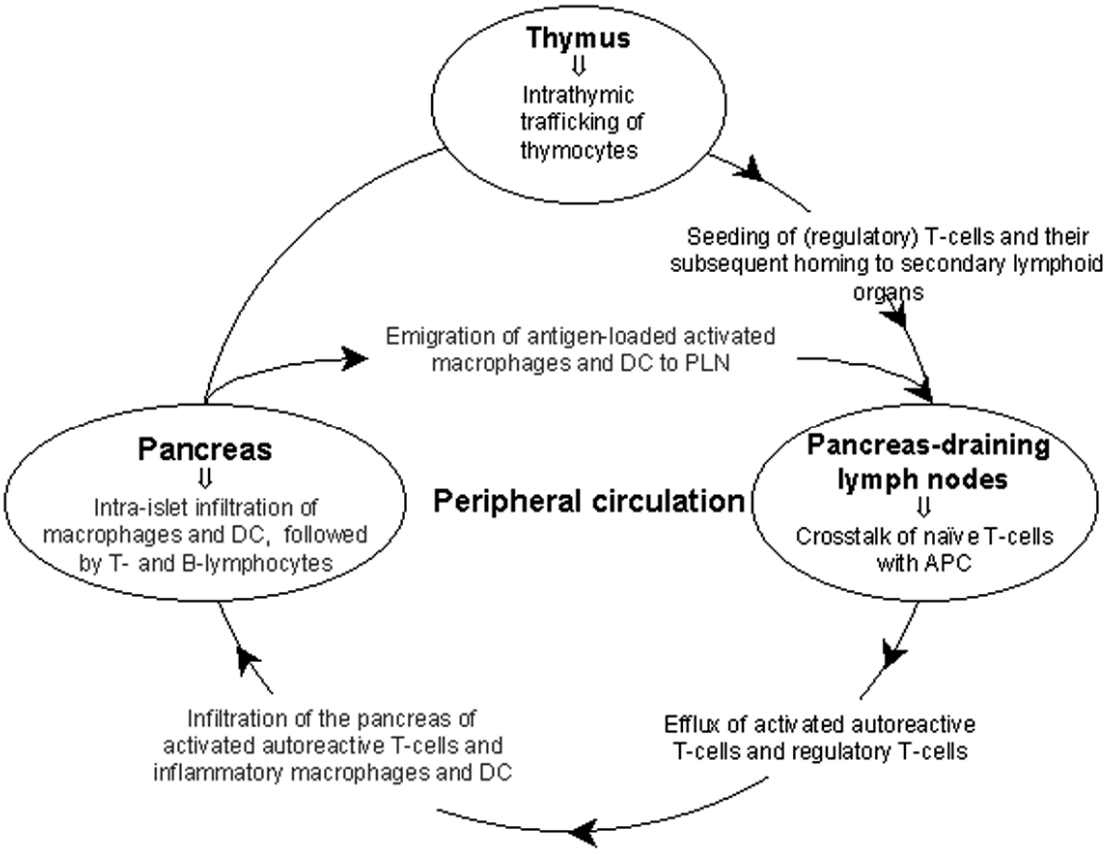

Figure 6.1 Thymic-pancreatic pathways of lymphocytes, macrophages and dendritic cells in normal and diabetogenic situations.

Thymus-derived autoreactive as well as regulatory cells exit the organ, seeding pancreatic lymph nodes. Antigen-loaded DC migrate from the pancreas to the draining lymph nodes where they activate T cells, leading to the efflux of the latter towards the endocrine islets, together with DC and macrophages, forming the infiltrate that will ultimately be responsible for the destruction of insulin-producing β cells. APC, antigen presenting cells; PLN, pancreatic lymph node.

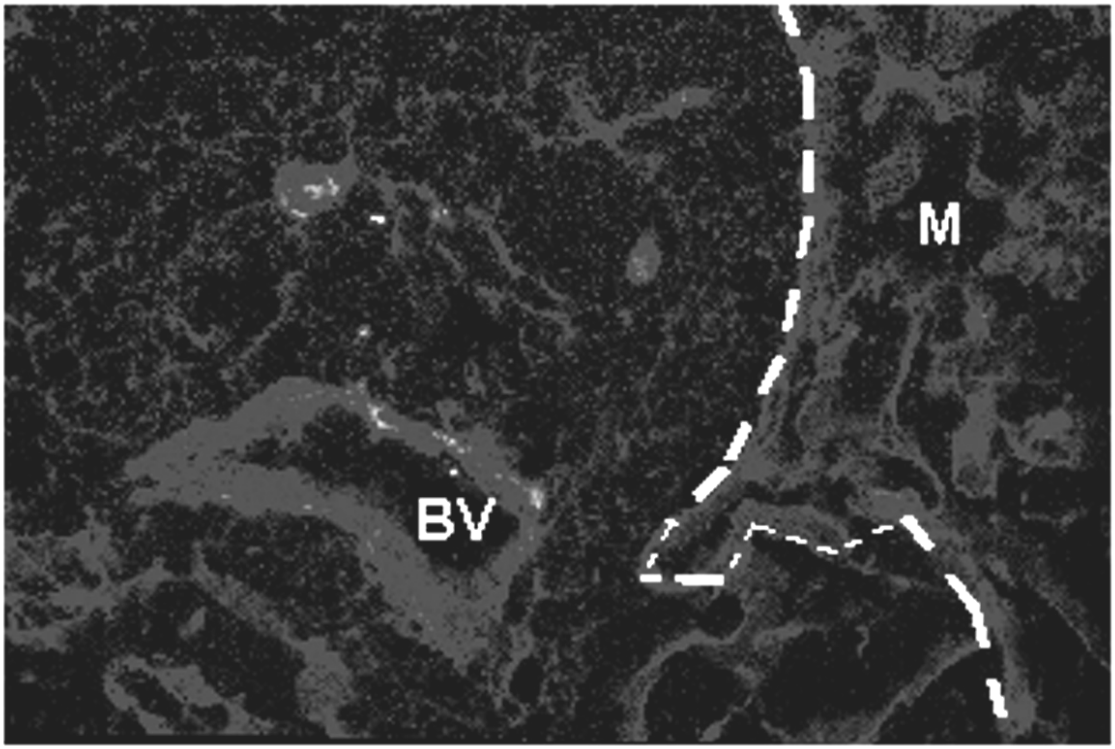

Figure 6.2 Giant perivascular space (PVS) in NOD thymus.
Immunofluorescence showing the fibronectin network in the medullary region of a thymic lobule. Dotted line represents the boundary between the PVS and the medullary parenchyma (x 500). BV, Blood vessel; M, medulla.

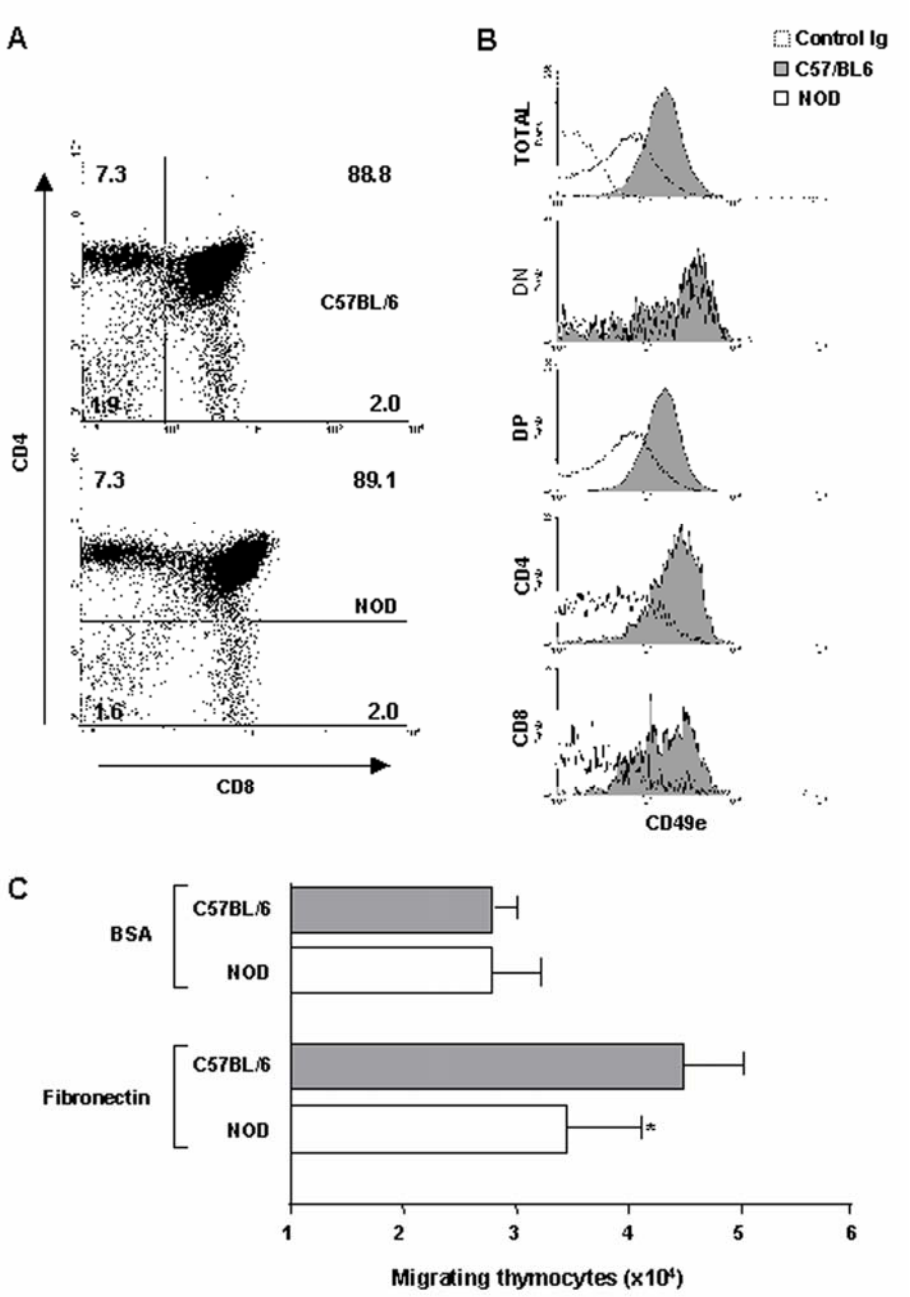

Figure 6.3 Decreased VLA-5 expression on NOD thymocytes.
Cytofluorometric dot plots for detection of CD4 and CD8 (**A**) reveal that in NOD mice stages of thymocyte differentiation defined by these markers are rather similar to a normal mouse (C57BL/6). Yet, as seen in panel **B**, there is a reduced level of membrane expression of VLA-5 (herein revealed with an anti-CD49e antibody) on $CD4^+CD8^+$ immature thymocytes, as well as in the $CD4^+$ and $CD8^+$ single positive mature subsets. As seen in panel **C**, such phenotypic abnormality results in a diminished capacity of NOD thymocytes to migrate under fibronectin stimulation, although no changes were observed in migration through bovine serum albumin (BSA), herein used as a non-specific stimulus. In this experiment (n=4) 2.5 million thymocytes were led to migrate through transwell chambers during 3h. * $P<0.05$, using Student's *t* test. Ig, immunoglobulin.

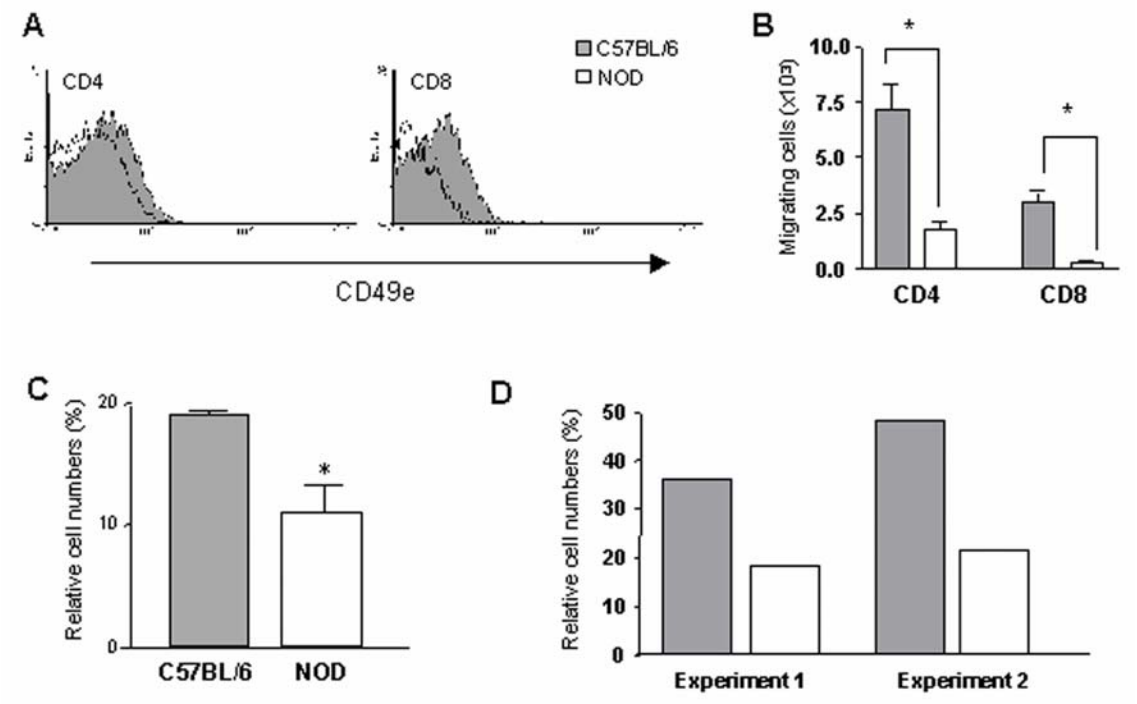

Figure 6.4 Impaired fibronectin-driven migration of NOD T cells: correlation with decreased VLA-5 expression in NOD peripheral T cells.

Panel **A** depicts cytofluorometric profiles revealing that VLA-5 expression is decreased in $CD4^+$ and $CD8^+$ T cells from pancreatic lymph nodes in 12 week-old female NOD mouse (open curves), as compared to control C57BL/6 (gray curves). As revealed in panel **B**, both CD4 and CD8 lymphocytes exhibit a reduced fibronectin-driven migratory ability.
In panel **C**, we can see that pancreatic lymph nodes from NOD mice have lower numbers of $CD4^+CD25^+$ T cells, when compared to C57BL/6 animals (* $P<0.05$), whereas panel **D** shows that the relative numbers of $CD4^+CD25^+$VLA-5^+ cells is also decreased, as seen in two separate experiments.

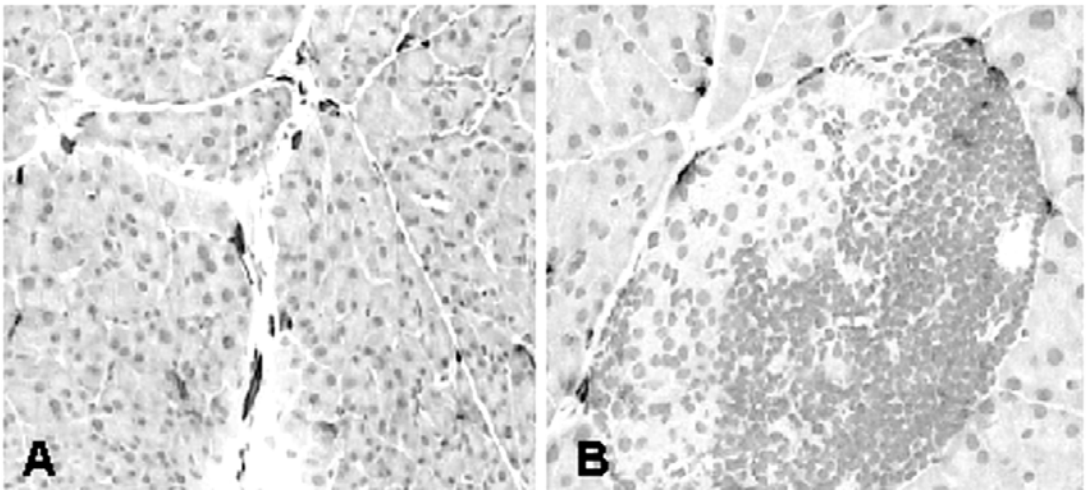

Figure 6.5 Resident pancreatic macrophages in early and advanced stages of diabetes.

Panels show ER-MP23-positive resident macrophages in the connective lining of the pancreas of a young non-diabetic female NOD mouse (A) and ER-MP23-positive macrophages surrounding the islet periphery in a prediabetic NOD mouse (B) (x250).

Chapter 7

Conclusions, general discussion and future directions

7.1 Conclusions

The studies that are described in this thesis were designed to gain insight into the origin and function of pancreatic macrophages and dendritic cells (DC) in relation to their involvement in autoimmune diabetes. Macrophages and DC are thought to be principally involved in the initiation of type 1 diabetes (T1D) via their activation of naïve T-cells to diabetogenic T-cells, and the continuation of the pathogenic process by directly mediating β-cell damage. Macrophages and DC are among the first immune cells observed in peri-islet infiltrates and they remain present in significant numbers in the pancreas during the entire diabetogenic process [1,2].

In addition to these functions that were described previously, we show in chapter 2 that pancreatic macrophages and DC are also important for the maintenance of the inflammatory islet infiltrations in non-obese diabetic (NOD) mice. Treatment with clodronate-loaded liposomes induced a temporary depletion of macrophages and DC from the pancreas. Interestingly, the depletion of macrophages and DC was accompanied by the complete disappearance of lymphocytes from the pancreas, despite the earlier presence of an established lymphocytic insulitis. Lymphocytes did not return to the pancreas until the newly recruited macrophages and DC were observed around 28 days after clodronate treatment. These results indicate an additional role of pancreatic macrophages and DC in the diabetogenic process: not only are these cells involved in the early initiation of the autoimmune response and the inflammatory damage to the β-cells at later stages, they are also involved in the recruitment to and retention of lymphocytes to the inflammatory pancreatic lesions.

In the second study (chapter 3) we phenotypically characterized the macrophage compartment to gain more insight into their origin. Macrophages in the non-inflamed pancreases of BALB/c and C57BL/6 mice essentially consisted of two phenotypically distinct subsets. The majority of the pancreatic macrophages were pancreas-resident $MMGL^+sialoadhesin^+CD45^+$ cells, whereas only a very small percentage of these cells additionally expressed F4/80, a marker generally expressed by mature macrophages [3]. Interestingly, macrophages in the fetal mouse pancreas were observed from E14.5 on and exclusively identified by the uniform expression of F4/80. Our experiments indicated that the fetal $F4/80^+$ macrophages were seeded in the pancreas as $F4/80^-$ precursors: these cells differentiated to $F4/80^+$ cells *in vitro* in cultured fetal pancreas explants even without the addition of the macrophage growth factor macrophage colony stimulating factor (M-CSF). Addition of exogenous M-CSF resulted in a 3-fold increase in macrophage number in the cultured explants. Interestingly, a simultaneous increase in he number of insulin-producing cells was observed, which indicates a positive contribution of the pancreatic macrophages to the development of endocrine tissue. Noteworthy, labeling of MMGL or sialoadhesin was not detected in the macrophages that had differentiated in the *in vitro* cultured pancreas explants. These markers were also not observed in the fetal pancreas before E17.5, approximately 2 days prior to birth. The $MMGL^+sialoadhesin^+CD45^+$ cells are possibly seeded during later stages of gestation and the presence of these cells is continued after birth.

Considering the early recruitment and accumulation of macrophages in the pre-weaning NOD pancreas [4], we examined the microenvironment of the pancreatic macrophages more closely. Extracellular matrix (ECM) plays an important role in the tissue trafficking, retention and differentiation of macrophages as well as in the development of the postnatal endocrine pancreas. In the study described in chapter 4 we examined the expression and distribution of laminin and fibronectin (FN), two major ECM proteins, in conjunction with the pancreas morphology. We show here that laminin labeling is mainly associated with exocrine tissue, whereas FN labeling is localized to the islet-ductal pole, islet periphery and intralobular septa. Interestingly, pancreatic macrophages were essentially found at sites of intense FN labeling. Remarkably, the protein expression level of FN was significantly increased in NOD pancreases at early stages of postnatal development. This increase coincided with altered islet morphology, reflected by enlarged and irregular shaped islets and increased percentages of total endocrine area as compared to that of control strains. Thus, the pancreas of the NOD mouse shows morphological abnormalities in the endocrine tissue during a critical time-window for the initiation of islet-autoimmunity.

Since efficient adhesion and migration are crucial for proper macrophage trafficking, we examined in the last study (chapter 5) the FN-dependent adhesion and FN-supported migration capacity of NOD macrophages. Unexpectedly, resident NOD macrophages showed a delayed disappearance of the macrophages following peritoneal inflammation and a reduced ability to adhere to and migrate on FN-coated surfaces. This was related to the substantially lower expression of the α4β1 integrin α-chain, CD49d of NOD macrophages as compared to macrophages from control strains. Furthermore, we show that NOD bone marrow-derived macrophages were specifically defective in the lipopolysaccharide (LPS)-induced increase in CD49d expression. Moreover, activation of the mitogen-activated kinase extracellular regulated kinase-1/2 (ERK-1/2) negatively regulated macrophage CD49d expression. Increased expression of this kinase strongly suppressed CD49d expression in NOD macrophages. These last results show that the LPS-activated signaling cascade plays a critical role in CD49d expression. This signaling pathway does not function properly in NOD macrophages, resulting in lower CD49d expression *in vitro* and possibly defective FN-mediated adhesion and migration that was observed *in vivo*.

The implications of these findings are discussed in the following paragraph.

7.2 General discussion

Matrix-macrophage interactions in NOD mice: miscommunication leads to misconduct

Type 1 diabetes (T1D) is caused by an immune-mediated destruction of the insulin-producing β-cells in the pancreas. The inflammatory lesions are characterized by intra-islet infiltration of autoreactive T and B-lymphocytes, a process generally referred to as insulitis. Insulitis is preceded by the accumulation of macrophages and dendritic cells (DC) at the islet periphery [1,2]. These innate immune cells are thought to be the leading actors in the initiation and progression of T1D pathogenesis.

In view of their pleiotropic functions, macrophages are particularly interesting since they may participate at different levels in the diabetogenic process. Macrophages are among the first immune cells that arrive at the islet periphery [1,2]. Upon stimulation macrophages may acquire the capacity to traffick to tissue-draining lymph nodes [5,6]. The macrophages that remain "on site" can reactivate infiltrating autoreactive T-cells within the pancreas. These activated T-cells possibly continue the inflammatory process together with the activated macrophages by producing a wide range of different damaging mediators. Finally, when the insulin-producing β-cells have been destroyed, macrophages are required to clear the debris of dead cells.

In spite of the important role in diabetogenesis that was suggested for pancreatic macrophages, they have not been fully characterized. Moreover, the ontogeny and precise function of pancreatic macrophages is not known. Therefore, our first aim was to characterize the pancreatic macrophage compartment and to gain more insight into the origin of these cells.

Macrophages belong to the mononuclear phagocyte system and comprise a diverse population of cells (see also chapter 1, table 2). Monocytes are blood-borne precursors of macrophages and are able to infiltrate peripheral tissues in response to acute or chronic inflammatory stimuli and chemoattractants like chemokines [7]. Upon their entry in the peripheral tissues, monocytes will differentiate into either DC or exudate macrophages [8]. It is not certain whether, under non-inflammatory conditions, the tissue macrophage population will be replenished by blood monocytes as well.

In the current view [9-12], inflammatory macrophages are recruited to the pancreas from the circulation in response to inflammatory mediators and chemoattractants released by the damaged pancreatic tissue. In the following sections I would like to discuss an alternative view. Based upon the results presented I would like to suggest that the first activated macrophages observed in the pancreas derive from responsive (immature) resident macrophages that are activated locally by the pancreas tissue itself, without the absolute requirement of a damaging insult.

7.2.1 High numbers of F4/80$^+$ macrophages are exclusively observed in the pancreas of NOD mice

Macrophages are observed in the murine pancreas from birth onwards as identified by the expression of different macrophage-specific cell surface receptors [4]. We have identified two phenotypically distinct macrophage subsets (chapter 3) that differ in the expression of the general macrophage marker F4/80. The majority of the pancreatic macrophage compartment of BALB/c and C57BL/6 mice is comprised of pancreas-resident MMGL$^+$sialoadhesin$^+$CD45$^+$ histiocytes that are located in the connective tissue of inter- and intralobular septae. Only a small percentage additionally expresses the general macrophage marker F4/80. Thus, F4/80-expressing cells are almost absent in the non-inflamed pancreas, but interestingly, abundantly present in the pancreas of non-obese diabetic (NOD) mice, before lymphocytes are present [1,2]. Macrophages expressing F4/80 are observed massively before the onset of destructive insulitis in the NOD pancreas, and their presence is particularly associated with islet destruction.

This suggests that F4/80 identifies a particular subset of inflammatory macrophages in the pancreas that are virtually absent under non-inflammatory conditions.

7.2.2 Pancreatic macrophages do not appear to be repopulated by blood monocytes directly

Some insight into the origin of the pancreas macrophages is given by the phagocyte depletion study described in chapter 2. Pancreatic macrophages were depleted by intraperitoneal injection of clodronate-loaded liposomes (lip-Cl_2MDP) in NOD and C57BL/6 mice. Interestingly, we observed that blood monocytes and splenic macrophages disappeared almost instantly, whereas the disappearance of pancreatic macrophages and DC was not observed until 7 days after treatment. This may indicate that the clodronate did not (directly) reach the pancreatic antigen presenting cells (APC), and their disappearance can thus be the result of normal cell turnover.

Consistent with the delayed disappearance, the return of the pancreatic macrophages also took much longer as compared to the blood and spleen, despite the presence of established insulitis before the injection of lip-Cl_2MDP. MMGL$^+$ as well as F4/80$^+$ macrophages did not reappear in the NOD pancreas until 3 weeks after the complete reconstitution of monocytes and macrophages in blood and spleen, respectively. This supports the view that pancreas macrophages are not replenished by blood monocytes directly, but derive from macrophages or precursors that reside elsewhere. This may be either precursors in the pancreas itself or in nearby organs like the omentum or the peritoneum. Under inflammatory conditions macrophages are capable to traffic directly from the peritoneum to the pancreas, as was observed for Cytomegalovirus-infected peritoneal macrophages in the rat [13]. If the pancreatic macrophages indeed derive from the peritoneum, the delay in the reconstitution of the pancreatic macrophage compartment may be explained by a slow replenishment from the peritoneum, the formation of a peritoneal depot of lip-Cl_2MDP or from the depletion of precursors that reside within the peritoneum.

7.2.3 Macrophage maturation might occur within the pancreas

Importantly, no obvious differences in the time-points of re-appearance of the $MMGL^+$ and $F4/80^+$ macrophages were observed in the pancreas. Therefore they may derive from the same source. Since the level of expression of F4/80 is the only phenotypical difference between the two subsets in the adult pancreas, it is possible that they do not represent distinct populations of cells, but rather different differentiation stages of the same type of macrophage. F4/80 expression may be down regulated by the $MMGL^+sialoadhesin^+CD45^+$ macrophages during their retention in the non-inflamed pancreas. Decreased F4/80 expression has been observed after the direct infection of macrophages [14,15] or upon activation and migration of Langerhans cells from the skin [16]. However, the mechanism responsible for the decreased F4/80 expression has not been studied further. A few other resident macrophage populations like splenic metallophilic marginal zone macrophages, macrophages in T-cell rich areas of lymph nodes and oral mucosal macrophages also lack the expression of F4/80. Yet, it is not clear whether these cells expressed the marker at an earlier stage.

Previous studies on the infiltration of macrophages in the pancreas report that the resident acid phosphatase-negative $MMGL^+sialoadhesin^+CD45^+$ macrophages are the first cells that accumulate around the pancreatic islets in NOD mice, directly followed by the acid phosphatase positive $F4/80^+$ cells [1]. I would like to suggest that the $MMGL^+sialoadhesin^+CD45^+$ macrophages acquire the expression of F4/80 and acid phosphatase during their further maturation within the pancreas, induced by local environmental conditions. Increased F4/80 expression correlates in general with enhanced macrophage maturation and F4/80 levels additionally increase upon stimulation with lipopolysaccharide (LPS) and interferon-γ (IFNγ) [17]. Moreover, like the increase in F4/80 expression, the expression of acid phosphatase is also considered an indicator of increased macrophage maturity. The $MMGL^+sialoadhesin^+CD45^+F4/80^-$ cells may represent responsive macrophages that mature and gain detectable F4/80 and acid phosphatase expression during their maturation in the NOD pancreas. The $F4/80^+$ macrophages therefore do not necessarily represent exudate macrophages recruited from the periphery, but may derive from cells that have matured locally.

This additionally implies that the activation of macrophages in NOD mice takes place within the tissue itself, before the presence of lymphocytes or other signs of inflammation are evident. It remains unresolved why the immune system targets the islets. The initial triggering of the islet autoimmune response may be related to the interaction of macrophages with the local pancreatic microenvironment as will be discussed below.

7.2.4 Pancreatic macrophages co-localize with fibronectin

In addition to their role as immune regulators, macrophages are also involved in the development and remodeling of tissues during fetal and adult life. In chapter 3 we show that fetal pancreatic macrophages support the development of β-cell growth *in vitro* and this was recently confirmed *in vivo* in macrophage-deficient mice [18].

In the NOD mouse the first accumulations of pancreatic macrophages are observed from birth to weaning, when the pancreas is still going through significant

developmental changes. $MMGL^+$ macrophages are mainly observed in the connective tissue and peri-vascular areas of the pancreas in co-localization with fibronectin (FN) (chapter 4). This component of the extracellular matrix (ECM) supports macrophage adhesion and spreading *in vitro*. Interestingly, higher levels of FN were found in the early pre-weaning pancreas of NOD mice (chapter 4), concomitantly with the presence of enlarged and irregular-shaped islets and, importantly, higher numbers of pancreatic macrophages and DC [4].

Excessive deposition of connective tissue proteins is often observed in parallel with the infiltration of macrophages [19]. Such macrophages are effective producers of FN that is even enhanced by the simultaneous release of fibrogenic cytokines and growth factors [20]. The haptotactic properties of FN (i.e. chemotactic activity of tissue-complexed proteins) may attract and retain more macrophages in the NOD pancreas, but whether the accumulation of macrophages is the cause or the consequence of the higher FN levels remains unclear.

We have not further investigated how macrophages can stimulate the expansion of the β-cells. The mechanism possibly involves the release of cytokines and growth factors. Disturbed cytokine production by NOD macrophages of those cytokines that influence β-cell proliferation and/or activity may affect endocrine development and some, like interleukin (IL)-6 and transforming growth factor-β, will additionally stimulate the deposition of ECM [19, 21]. In turn, the change in the microenvironment will directly affect the activation of the macrophages.

Various disturbances in cytokine production have been observed in macrophages of NOD mice in response to inflammatory cytokines or bacterial derivates [22, 23]. Little is known about the interaction of NOD macrophages with ECM components and this might be critically important considering their co-localization with FN in the pancreas.

7.2.5 The interaction of NOD macrophages with fibronectin is disturbed

In general macrophage function should be adapted to the tissue-specific needs and is dependent on the appropriate interaction of macrophages with their direct microenvironment. Cell-matrix interactions are regulated by integrins, a large family of heterodimeric transmembrane receptors comprised of α and β subunits that specifically recognize ECM-components or other cell surface receptors as counter ligands. The heterodimeric integrins α4β1 and α5β1 recognize the FN-sequences CS-1 and RGD, respectively. The regulation of expression and activation of these integrins is not only essential for the production of cytokines like IL-6 and IL-1β [24], but also for the efficient tissue trafficking of macrophages in inflammation.

Adhesion and migration are active processes that require dynamic changes of the cell shape and the coordinated attachment and detachment of cell surface proteins like integrins. Mechanisms that favor integrin-mediated migration and/or adhesion of inflammatory cells are in general thought to contribute to the development of autoimmunity (discussed in chapter 6). Accordingly, the inhibition of α4β1-mediated interaction with its ligand prevented the development of spontaneous diabetes in adult NOD mice [25]. Paradoxically, peritoneal, thymic and bone marrow-derived macrophages

(BMDMs) of NOD mice show an impaired interaction with FN, related to the decreased expression and function of the α-chain of α4β1, CD49d, whereas expression of the α-chain of α5β1, CD49e, appeared to be normal (chapter 5).

The balanced interaction of both α4β1 and α5β1 with FN is important for the efficient migration and adhesion of macrophages to FN. Alpha4β1 and α5β1-mediated interactions are regulated independently and influenced to a large extent by the composition of the ECM. While low FN-concentrations preferentially stimulate the usage of α5β1 for migration *in vitro*, the usage of α4β1 is favored on higher FN-concentrations [26]. If the FN levels are too high they will reduce the migration of cells by the inhibition of cellular polarization [27]. The high FN content of the pre-weaning pancreas of NOD mice may bias macrophages to the usage of α4β1. Unable to stabilize α4β1-mediated attachment due to the defective CD49d, NOD macrophages will not form cell protrusions efficiently, thus preventing migration. Migration will be further hindered by the high levels of FN in the NOD pancreas that may lead to the accumulation of the macrophages at sites where we observed dense FN labeling, like the islet-ductal pole and islet periphery.

Defective CD49d expression and function may thus be involved in the aberrant retention of macrophages in the pancreas. All mature NOD macrophage populations that we have examined, including those derived from precursors in the bone marrow, exhibited lower levels of CD49d. However, since we have not tested the resident pancreatic macrophages (due to technical difficulties to obtain the macrophages), it remains possible that this population does exhibit an adequate interaction with FN and normal levels of CD49d.

The regulation of CD49d expression was related to the genetic background of NOD mice. Interestingly, peritoneal macrophages from strains that develop diabetes like wildtype NOD and congenic NOD.CD45.1 mice exhibit low levels of surface CD49d, whereas peritoneal macrophages from diabetes-resistent F1(NODxC57BL/6) hybrids and congenic NOD.H-2b and NOR mice exhibited increased levels of CD49d expression as compared to the macrophages of parental NOD strains (chapter 5). There is, however, no direct evidence for a causative relationship between the lower expression level of macrophage CD49d with the development of diabetes. In this context, it must be noted that other mechanisms relevant to T1D pathogenesis can be disturbed due to the defective CD49d as well.

7.2.6 CD49d is functionally implicated in cellular communication

CD49d is classically known as an adhesion molecule. Importantly, α4β1 is the only member of the β1 integrin family that interacts with cellular ligands, like endothelial VCAM-1 and the disintegrin ADAM28 that is expressed by T-lymphocytes [28]. Hence the lower levels of CD49d and the defective CD49d function may impair cellular communication. Recently, the involvement of α4β1 in the priming of T-lymphocytes was demonstrated [29] and this is particularly interesting considering the low CD49d expression by thymic macrophages and the poor capacity of NOD APC to stimulate T-cells [30].

The usage of CD49d has also been described for the uptake of apoptotic leukocytes by endothelial cells [31]. Since macrophages are developmentally closely related to endothelial cells, it is conceivable that macrophage-CD49d has a similar function. The phagocytosis of apoptotic cells by NOD macrophages is defective [32] and perhaps related to the lower CD49d expression of NOD macrophages. Interestingly, apoptosis of pancreatic β-cells during the pre-weaning period is regarded as an important trigger for the initiation of islet-autoimmunity [33], and apoptotic cells should be cleared and processed efficiently to avoid local inflammation.

Thus, in addition to the observed FN-related adhesive and migratory problems, defective CD49d may contribute to T1D pathogenesis by affecting the cross-talk with T-cells and the uptake of apoptotic cells.

7.2.7 NOD macrophages exhibit disturbed LPS-responsiveness

The lower CD49d level is the consequence of an intrinsic problem possibly associated to the NOD genetic background. This problem may be responsible for several autoimmune phenomena that have been observed in this mouse. The defective CD49d expression is related specifically to the maturation of NOD-derived BMDMs induced by LPS (Chapter 5), whereas CD49d expression upon maturation induced by IFNγ or tumor-necrosis factor-α (unpublished observations) appeared to be normal. LPS-induces the phosphorylation of the mitogen-activated protein kinase (MAPK) extracellular regulated kinase (ERK)-1/2. We show that ERK-1/2 negatively regulates macrophage CD49d expression. High levels of this signaling molecule may suppress the increase of CD49d upon final maturation in NOD macrophages.

In human monocyte-derived DC, positive regulation of CD49d expression depends on the phosphorylation of the MAPK p38 [34]. Although ERK-1/2 and p38 MAPK are both phosphorylated upon LPS-stimulation, these signaling molecules usually do not operate in synergy. Direct “cross-talk” between pERK-1/2 and p38 MAPK can result in the selective mutual suppression of their activity [35, 36]. Thus, lower CD49d expression-levels might be explained by abnormally high expression levels of pERK-1/2 upon stimulation with LPS in NOD macrophages. This may be related to insufficient or delayed phosphorylation of p38 MAPK, leading to inefficient down-regulation of active ERK1/2 (Fig. 7.2.1).

In our studies we have examined the effects of this signaling pathway on the integrin expression profile of NOD macrophages. LPS-induced transcription of various relevant pro-inflammatory mediators like IL-1β, IL-6 and TNF-α is also regulated by the activation of either p38 or pERK1/2 [37-39]. It would be of value to examine whether NOD macrophages also show aberrancies in the regulation of gene expression of these cytokines, related to a disturbed LPS-responsiveness. Interestingly, the LPS-stimulated secretion of IL-1β, IL-6 and TNF-α by macrophages of type 1 diabetes patients was increased as compared to HLA-matched healthy controls [40]. The underlying defect of this increased production has, unfortunately, not been examined.

LPS is not the only ligand able to activate the LPS signaling cascade. Several interesting endogenous molecules like the heat shock protein-60 (hsp-60) and the type 3 repeat extra domain A of FN also activate this intracellular pathway [41, 42].

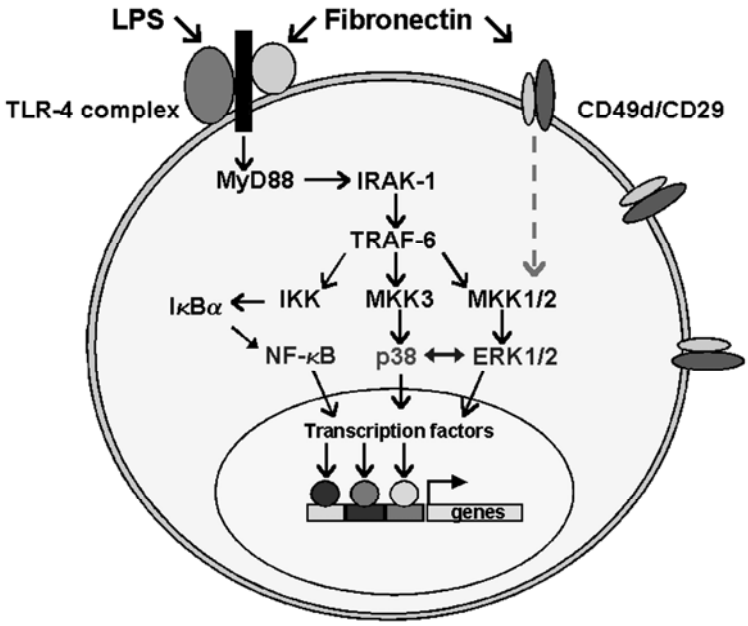

Figure 7.2.1
Lipopolysaccharide (LPS) and the type III repeat EDA domain of fibronectin both activate the membrane TLR-4 complex. This activation will lead to the activation of the intracellular signaling cascade downstream of the TLR-4 complex inducing the release of NF-κB and the phosphorylation of p38 and ERK1/2.

Fibronectin additionally triggers the CD49d/CD29 integrin, activating the signaling cascade downstream of this adhesion molecule. Simultaneous activation of TLRs and integrins may influence the activation mediated by TLR-4 since part of the signaling cascades overlap.

Disturbances in both NF-κB as well as ERK-1/2 expression (depicted in red) have been observed in NOD macrophages upon stimulation with LPS and this may alter the phenotype and cytokine profile of these cells.

7.2.8 Fibronectin is an endogenous activator of the Toll-like-receptor-4 signaling cascade

The defective upregulation of CD49d expression by NOD macrophages was observed exclusively upon stimulation with LPS. LPS is a well-studied exogenous activator of Toll-like-receptor-4 (TLR-4). TLRs are essentially implicated in the activation of macrophages and DC upon the recognition of bacterial cell wall components like LPS. TLR-activated APC will strongly activate naïve T-cells, thereby inducing a specific immune response against the responsible infectious microorganism. Inappropriate TLR-activation of APC may have profound effects on the development of autoimmunity by overruling autoreactive T-cell ignorance.

Our results suggest an additional role for TLR-4 signaling that is distinct from its classical role as innate receptor for gram-negative bacteria. The encounter of TLR-4 ligands during final macrophage maturation *in vivo* may enhance the levels of CD49d in macrophages of control mice (chapter 5). However, the activation of the TLR-4 pathway in NOD macrophages will not lead to enhanced CD49d expression. Fully mature peritoneal and thymic NOD macrophages indeed show lower levels of CD49d *in vivo* as compared to various controls strains, whereas levels on monocytes, the precursors of most macrophages, are not different. This may imply that TLR-4 signaling plays a role in the maturation of macrophages under non-infectious/non-inflammatory conditions.

Several endogenous ligands for TLR-4, such as hsp60 and also FN, activate the TLR-4 signaling pathway [41, 42] (Fig. 7.2.1.). This is particularly interesting considering the co-localization of the pancreatic macrophages with FN and the higher local levels of FN preceding the appearance of macrophages with an activated/mature phenotype. Phosphorylated ERK1/2 is a negative regulator of CD49d expression. Since LPS-stimulated NOD macrophages show enhanced levels of pERK1/2, they will react to LPS

stimulation with a decrease in CD49d expression. However, NOD macrophages will show an increased expression of molecules that are positively regulated by pERK1/2 upon stimulation with LPS or other TLR-4 ligands. In support of such an abnormal TLR-4 responsiveness, NOD macrophages show a hyper-responsiveness upon stimulation with hsp60, resulting in the increased production of the Th1 cytokine IL-12 (p70) [43].

Since there is no evidence for the presence of an exogenous activator or infectious agent in the NOD pancreas, it is possible that the pancreas tissue itself triggers the activation of the macrophages. Hypothetically, FN may lead to TLR-4-mediated stimulation of macrophages. The high FN-levels in the NOD pancreas may specifically promote such activation. Importantly, TLR-4-mediated stimulation of NOD macrophages may lead to their inappropriate activation due to the abnormal TLR-4-responsiveness, possibly enforced by the defective CD49d function. The unfortunate presence of high FN levels combined with disturbed receptor-responsiveness might lead to the enhanced release of inflammatory mediators by the NOD macrophages that unintentionally damage the sensitive β-cells, thereby accidentally provoking an immune response. The suggested scenario is depicted in figure 7.2.2.

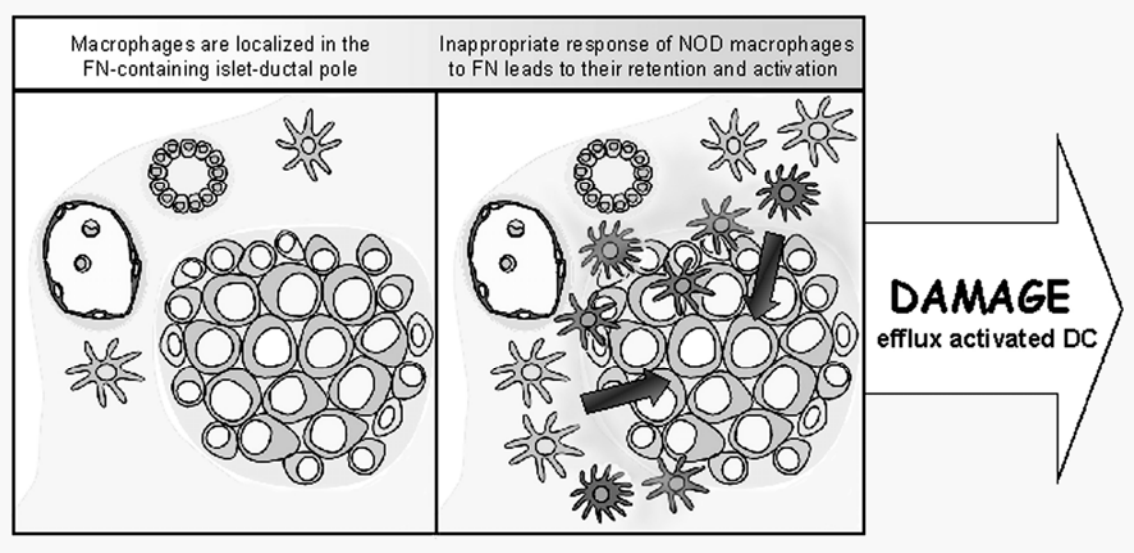

Figure 7.2.2.
Under non-inflammatory conditions, MMGL$^+$sialoadhesin$^+$F4/80$^-$ macrophages reside in the FN-containing connective tissue of the pancreas. High levels of FN, observed in the NOD pancreas during the early stages of weaning (i.e. 3 and 7 days of age), may attract and retain these macrophages in close proximity to the islets. This retention is possibly enforced by the functionally defective CD49d of NOD macrophages. The encounter with (high levels of) the TLR-4 ligand FN may hypothetically lead to the activation of macrophages. Since NOD macrophages show disturbances in the signaling molecules ERK-1/2 and NF-κB, they may respond to the TLR-4-mediated activation by the expression and release of pro-inflammatory cytokines like IL-1β, IL-6 and TNF-α. These cytokines are directly toxic for the β-cells, inducing β-cell damage and possibly the initiation of an inflammatory response against the islets.

7.3 Future directions

The findings described in this thesis may form the basis of some interesting new areas of research regarding autoimmunity in (NOD) mice and some of them were already postulated in the discussion. However, important data are still missing to support these suggestions.

In the discussion it was proposed that the activated F4/80$^+$ macrophages that were observed in the NOD pancreas are not recruited from the periphery, but rather derive from local pancreas-resident macrophages that are activated locally. Unfortunately, we have not used tracer dyes to track the conversion of the MMGL$^+$F4/80$^-$ pancreatic macrophages to the activated F4/80$^+$ acid phosphatase$^+$ macrophage, to obtain a more direct evidence for this suggestion. It may be difficult to label the macrophages in the pancreas with a tracer dye directly. This will be facilitated if we would be able to clearly establish the origin of the pancreatic macrophages. Precursors in the peritoneum may qualify as potential candidates and they can be labeled by the peritoneal injection of fluorescent liposomes. For the blood this procedure is difficult, since only a small proportion of the cells will be labeled and an even smaller proportion will reach the peripheral tissues. Furthermore, the injection of almost any reagent induces the disappearance of macrophages together with a certain level of activation. Macrophage activation will simultaneously induce a change in the receptor expression profile that may affect their trafficking pattern. This will therefore require a careful interpretation of the results.

Regarding the integrin expression profile and the trafficking potential of NOD macrophages, we showed that the adhesion and migration of NOD macrophages was hampered, related to the defective function of CD49d. However, impairment of the adhesion and migration of macrophages may be irrelevant for the development of T1D. To make a better assessment of the involvement of the defective macrophage CD49d in NOD autoimmunity, it may be worthwhile to test the involvement of CD49d in other relevant mechanisms like antigen presentation and the clearance of apoptotic cells. Since CD49d- and CD49e-mediated interaction with FN additionally induces the expression of various cytokines, it would be interesting to examine the cytokine profile of NOD macrophages upon interaction with FN and other ECM components, preferably under conditions that closely approach the *in vivo* situation.

The underlying reason for the defective CD49d expression in NOD macrophages was found in the TLR-4 signaling cascade. In our studies the inappropriate responsiveness of the TLR-4 pathway was examined by the analysis of the integrin expression profile. Considering the general involvement of ERK-1/2 in various signaling cascades, it would be of value to examine the cytokine profile of NOD macrophages upon stimulation with a variety of TLR-4 ligands, like hsp60, and as has been stated above, FN. Furthermore, considering the importance of TLRs in the initiation of specific immune responses, it would be interesting to examine the involvement of TLR-4 signaling in the maturation of macrophages in general, under non-inflammatory

conditions. This study may be extended with the investigation of the macrophage response after FN interaction via other FN-receptors like $\alpha4\beta1$ and $\alpha5\beta1$, or other receptor-ligand pairs that may attenuate the TLR-response. The usage of TLR-4 deficient or mutant mice would in particular be suited to examine such mechanisms.

Like macrophages, thymocytes and peripheral T-cells of NOD mice also show reduced FN-mediated adhesion and migration. These defects were not related to the expression of CD49d, but to the defective expression of the α-chain of $\alpha5\beta1$, CD49e [44]. Interestingly, both thymocytes and peripheral T-cells of NOD mice show disturbances in the levels of phosphorylated ERK1/2 upon TCR-mediated stimulation [45]. However, in NOD thymocytes the levels of pERK1/2 were not increased, but decreased upon stimulation. The lower levels of CD49e may be related to the decreased levels of pERK1/2. The levels of pERK1/2 positively correlated to the expression of CD49e in chondrogenesis [46]. Whether this correlation also exists for T-lymphocytes has yet to be determined, but theoretically the low levels of pERK1/2 in NOD thymocytes may account for the decreased levels of CD49e observed in these cells.

Interestingly, inhibition of ERK1/2 phosphorylation did not substantially affect the levels of CD49e in macrophages, and this may reflect the lineage-specific reprogramming of MAPK activities during cellular differentiation, resulting in distinct defects with a similar underlying cause. In this context it is noteworthy that the levels of active ERK1/2 are regulated by the activation of the upstream kinase, protein kinase C [46]. Defective PKC signaling has been reported for both lymphoid as well as myeloid lineages in NOD mice [22, 45] and this may indicate a possible defect of the PKC-RAS-MAPK cascade as early as the uncommitted hematopoietic precursor cell.

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Abbreviations

APC	antigen presenting cells
AGM	aorta-gonad-mesonephros
BB	biobreeding
BCG	Bacille Calmette-Guerin
BM	bone marrow
CCR2	common corresponding receptor-2
CSF-R	colony stimulating factor receptor
CXCR3	CXC-chemokine receptor 3
DC	dendritic cells
ECM	extracellular matrix
ERK	extracellular-regulated kinase
Fas-L	Fas-ligand
FN	fibronectin
GAD	glutamic acid decarboxylase
G-CFC	granulocyte colony-forming cells
G-CSF	granulocyte colony stimulating factor
GM-CFC	granulocyte-macrophage colony-forming cells
GM-CSF	granulocyte-macrophage colony stimulating factor
HEV	high endothelial venule
HSC	hematopoïetic stem cell
ICAM-1	intracellular adhesion molecule-1
IDDM	insulin-dependent diabetes mellitus
IFN-α	interferon-alpha/ gamma
IL	interleukin
IP-10	interferon-inducible protein-10
LFA-1	lymphocyte function-associated antigen-1
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
M-CFC	macrophage colony-forming cells
MCP-1	macrophage chemoattractant protein-1
M-CSF	macrophage colony stimulating factor
MHC	major histocompatibility class
MIF	migration-inhibiting factor
MIP-1	macrophage inflammatory protein-1
MMPs	matrix metalloproteinases
NK	natural killer
NO	nitric oxide
NOD	non-obese diabetic mouse
NON	non-obese normal
PECAM-1	platelet endothelial cell adhesion molecule-1

PLNs	pancreatic lymph nodes
P-sp	para-aortic splanchropleura
SCF	stem cell factor
SDF-1	stromal cell derived factor-1
SLE	systemic lupus erythematosus
SOCS-1	suppressor of cytokine signalling-1
STAT	signal transducer and activator of transcription
TCR	T-cell receptor
TE	thymic epithelium
T1D	type 1 diabetes
TF	transcription factor
TGF-β1	transforming growth factor beta-1
Th-1	T-helper-1
TLR	toll-like receptor
TNF-α	tumor necrosis factor-alpha
VCAM-1	vascular adhesion molecule-1
VLA-4	very late antigen-4

Summary

Type 1 diabetes is a disease that results from a disturbed glucose metabolism due to a deficiency in insulin production. This deficiency is the consequence of immune-mediated damage to the insulin-producing β-cells. The cause of type 1 diabetes is presently unknown and probably multifactorial. The initiation and progression of the inflammatory process that destroys the β-cells involves the interplay of environmental factors with an autoimmune-prone genetic background. Abnormal immune regulation explains the autoimmune phenomena observed in diabetic patients and in spontaneous animal models for the disease to a limited extent only. The precise reason for the immune system to target the pancreatic islets of Langerhans is still unclear.

The pathogenic process in the pancreas is characterized by the pathology-related intra-islet infiltration of T and B-lymphocytes that mediate islet destruction. This T and B-cell infiltration is preceded by an accumulation of macrophages and dendritic cells at the islet periphery. The early peri-islet accumulation of these antigen presenting cells probably reflects the first response of the immune system that is progressively heading for islet destruction.

Macrophages are involved in every step of the diabetogenic process. In the non-obese diabetic (NOD) mouse that spontaneously develops diabetes, various macrophage-abnormalities like defective maturation, reduced phagocytosis and increased production of IL-12, have been described previously. Moreover, macrophages are present in higher numbers in the pancreas of the NOD mouse from birth onwards, randomly distributed in the connective tissue and exocrine parenchyma.

In this thesis we present the results of our studies on the murine pancreatic macrophage compartment. We have questioned in particular the possible underlying causes for the abnormal early peri-islet accumulation of macrophages. Therefore, the studies were performed with an emphasis on the interactions of macrophages with the extracellular matrix of the pancreatic connective tissue.

In our first study we show that the depletion of macrophages and dendritic cells from the endocrine pancreas was accompanied by a total disappearance of lymphocytes from the pancreas. Hence, pancreatic macrophages and dendritic cells are critically involved in the local progression of islet inflammation in NOD mice by mediating the retention and possibly the recruitment of lymphocytes to the pancreas. Importantly, this depletion significantly postponed the onset of diabetes, leading to a strongly decreased incidence by 35 weeks of age.

In the second study described, we have phenotypically characterized the pancreatic macrophages. The majority of macrophages were characterized by the expression of MMGL and sialoadhesin. Only a minority of these pancreatic macrophages expressed the macrophage marker F4/80 under non-inflammatory conditions. By contrast, macrophages expressing F4/80 were observed massively before the onset of destructive insulitis in the NOD pancreas, and their presence is particularly associated with islet destruction. This suggests that F4/80 identifies a particular subset of inflammatory macrophages in the pancreas that are virtually absent under non-

inflammatory conditions. Interestingly, in the fetal pancreas mature macrophages were exclusively identified by their expression of F4/80 and these macrophages lacked the expression of MMGL and sialoadhesin.

In addition, we present data that support the conclusion that macrophages develop from pre-existing precursors that are present in the fetal pancreas at E12.5. Using an in vitro approach, we demonstrated that their numbers significantly increased in fetal pancreas explants cultured with M-CSF. This increase of F4/80-positive cells was paralleled by an increase in the number of insulin-producing cells, suggesting that macrophages support insulin-cell growth *in vitro.*

These results are in line with the results of the third study that is presented in this thesis. Early postnatal pancreases from NOD mice are characterized by an increase in the percentage of endocrine tissue and by enlarged and irregularly-shaped islets, concomitantly with the presence of increased numbers of macrophages. Importantly, the levels of the extracellular matrix protein fibronectin are significantly increased during this pre-weaning period as well. Fibronectin labeling was mostly localized at the islet-ductal pole, islet periphery and in intralobular septa. Interestingly, pancreatic macrophages mainly reside at sites with intense fibronectin-labeling.

In a fourth study we demonstrate that, paradoxically, NOD macrophages exhibit impaired fibronectin-mediated adhesion and migration due to the defective expression of the integrin-type fibronectin receptor α-chain, CD49d. In addition, we show that extracellular-regulated kinase-1/2 (ERK-1/2) is a negative regulator of CD49d expression in macrophages. NOD macrophages were characterized by increased levels of the activated form of this kinase when stimulated with the toll-like receptor-4 (TLR-4) ligand, lipopolysaccharide (LPS). LPS-stimulation resulted in the impaired upregulation of CD49d levels in NOD macrophages as compared to macrophages of other mouse strains. We believe that this specific defect in the macrophage compartment of NOD mice might play a role in the observed peri-islet accumulation.

At the end of this thesis, we have postulated the working hypothesis that the observed presence of higher levels of the endogenous TLR-4 ligand fibronectin in the NOD pancreas in combination with the inappropriate TLR-4-responsiveness of NOD macrophages contributes to their local retention and activation. This unfortunate activation may damage the neighboring islets, unintentionally provoking an immune response eventually leading to the development of autoimmunity. We have uncovered several abnormalities that may trigger a mechanism that possibly contributes to the development of spontaneous autoimmune diabetes in the NOD mouse. Whether similar abnormalities are present in type 1 diabetic patients remains to be established.

Keywords: Pancreas; Macrophages; Autoimmune Diabetes; Integrins; Extracellular Matrix

Samenvatting voor niet-ingewijden

De pancreas, ook wel alvleesklier genoemd, is een in de buikholte gelegen orgaan met verschillende functies. Het grootste gedeelte van de pancreas bestaat uit exocrien weefsel dat verteringsenzymen uitscheidt in de dunne darm. Slechts iets meer dan één procent van de pancreas wordt gevormd door de eilandjes van Langerhans. Deze endocriene eilandjes produceren verschillende hormonen die worden uitgescheiden in het bloed. Het belangrijkste hormoon is insuline, gemaakt door de β-cellen die zijn gelegen in het midden van de eilandjes. Insuline verlaagt de glucose-spiegel en reguleert zo de opslag van energie uit ons voedsel.

Patiënten met type 1 diabetes hebben een gevaarlijk hoge concentratie van glucose in het bloed door een tekort aan insuline. Dit tekort wordt veroorzaakt door de vernietiging van de β-cellen door het eigen afweersysteem en resulteert in een ernstige verstoring van het metabolisme. Deze immuunreactie tegen deze eiland-cellen ontstaat vaak al op heel jonge leeftijd en is onomkeerbaar. Patiënten met type 1 diabetes zijn daarom genoodzaakt hun verdere leven insuline te gebruiken.

Er zijn verschillende afwijkingen gevonden in het immuunsysteem van type 1 diabetes patiënten, die gerelateerd zijn aan het onstaan van autoimmuniteit. Deze autoimmuniteit lijkt echter voornamelijk gericht te zijn tegen de endocriene eilandjes en daarom noemen we type 1 diabetes een orgaan-specifieke autoimmuunziekte. Waarom het immuunsysteem van type 1 diabetes-patiënten zich juist tegen de eigen β-cellen keert is niet duidelijk.

Het kan zijn dat de oorzaak van de immuunrespons tegen de eilandjes in de pancreas zelf ligt. Om te bestuderen wat er in de pancreas plaatsvindt voordat de specifieke afweerreactie ontstaat, zijn we genoodzaakt onderzoek te doen in proefdieren.

Niet-obese diabetische (NOD)-muizen ontwikkelen spontaan diabetes. Voorafgaand aan de specifieke immuunreactie tegen de β-cellen zien we in de pancreas van deze muizen veel niet-specifieke afweercellen rondom de eilandjes. Deze niet-specifieke immuuncellen worden macrofagen genoemd. Macrofagen zijn in alle organen aanwezig en vormen daar de eerste verdediging tegen pathogene indringers. Macrofagen ruimen bacteriën op en alarmeren andere cellen van het immuunsysteem om een afweerreactie tot stand te brengen. Deze afweerreactie moet specifiek gericht zijn tegen de pathogene indringers en niet tegen ons eigen weefsel. Dit laatste gebeurt helaas wel bij patiënten met type 1 diabetes. Er is echter onvoldoende bewijs dat de afweerreactie tegen de eilandjes samenhangt met een eerdere infectie.

Omdat macrofagen mede onze afweerreactie aansturen, kunnen ze een heel belangrijke rol spelen in het ontstaan van autoimmuunziekte. In het onderzoek dat is beschreven in dit proefschrift, hebben we de macrofagen in de pancreas nader bestudeerd en onderzocht wat de oorzaak kan zijn van de vroege accumulatie van macrofagen rondom de eilandjes.

We laten zien dat er verschillende typen macrofagen aanwezig zijn in de pancreas. In de volwassen muis bleek een bepaald type macrofaag specifiek geassocieerd met ontsteking en deze is in hoge mate aanwezig in de pancreas van de

NOD muis. De pancreas macrofagen bevinden zich in het bindweefsel van de pancreas, tussen het exocriene weefsel maar ook rondom de eilandjes waar veel fibronectine aanwezig is. Fibronectine is een matrix eiwit wat enerzijds een rol speelt bij pancreas-ontwikkeling en anderzijds bij de ontwikkeling en mobiliteit van macrofagen.

In de pancreas van NOD muizen werden allereerst verhoogde concentraties fibronectine gevonden in de periode voorafgaand aan het ontstaan van de afweerreactie tegen de eilandjes. Verder laten wij zien dat de eilandjes in de pancreas van de NOD muis groter en grilliger van vorm zijn dan die in gezonde muizen. Deze afwijkingen kunnen samenhangen met het verhoogde aantal macrofagen dat in de NOD pancreas aanwezig is.

In tegenstelling tot wat wij hadden verwacht, vertonen macrofagen van de NOD muis een verzwakte interactie met fibronectine. Ze hebben een verstoorde expressie van een van de eiwitten waarmee macrofagen aan fibronectine binden, het CD49d integrine. Dit leidt tot een defect in de beweeglijkheid van de NOD macrofagen over fibronectine en kan daardoor bijdragen aan de vroege accumulatie van de macrofagen rondom de eilandjes. De oorzaak van de defecte expressie van dit integrine ligt in een verstoorde aansturing van de expressie binnenin de cel. Deze verkeerde aansturing is ook betrokken bij de activatie van macrofagen door moleculen uit de bacteriewand. Stimulatie van de NOD macrofagen door deze moleculen of eiwitten die daar op lijken, leidt tot een verstoorde expressie van CD49d maar ook tot een verstoorde activatie van de macrofaag. Eén van de eiwitten die tot deze verstoorde activatie kan leiden is fibronectine, wat in verhoogde mate aanwezig is in de NOD pancreas. Deze factoren tezamen kunnen bepalend zijn voor de ongewenste beschadiging van de eilandjes door de macrofagen in de NOD muis.

De orgaan-specifieke afwijkingen in combinatie met de door ons gevonden defecten in de NOD macrofaag kunnen het lokale evenwicht in de pancreas verstoren, mogelijk gevolgd door schade aan de eilandjes en de initiatie van een specifieke immuunrespons.

De beschreven resultaten bieden mogelijk de basis voor een nieuwe hypothese voor het onstaan van lokale activatie van pancreas macrofagen. Ook macrofagen van patiënten met type 1 diabetes vertonen een verstoorde respons op hetzelfde ligand als door ons is getest in de NOD muis. Wij hopen dat met onze bevindingen in de NOD muis een nieuwe weg kan worden geopend in het onderzoek naar het ontstaan van type 1 diabetes in de mens.

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To accomplish this thesis, and especially to put it in the context of a 'cotutelle de these' was not the easiest pursuit, not for anyone that was involved (it is not double the amount of paperwork, but an exponential increase!).

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I was never completely without contact with the Netherlands and that was mainly because of Tanja. It was on the kick-off of the MONODIAB project that we first met, and we directly got along very well. Our contact has continued ever since and whenever I came over from Paris we had so much to discuss that time always came in short. In between we also managed to talk a bit about work and even doing some experiments! Thank you for all your support. I'm really glad we had the (stressfull) last year of working side by side. Unfortunately we have separated ourselves now, but I dare say that we will continue collaborating in some way. Što želim kazati, Lieve Tanja, Zahvaljujem vam mnogo. Good luck on the third!

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Sadia

Curriculum vitae

The author of this thesis was born in Alkmaar, the Netherlands, on the 4th of June 1973. From 1985 to 1990 she attended the Jac. P. Thysse College in Castricum. In 1991 she began her studies at the "Hoge laboratorium opleiding" in Utrecht and graduated in January 1996 by receiving Bachelor of Science degrees in both Zoology and Medical Biotechnology with majors in Immunology and Molecular biology.

For the following period she worked with great enthusiasm as a research technician in the field of Multiple Sclerosis in the group of Dr. J.M. van Noort, at the Division of Immunologic- and Infectious-diseases of the TNO institute of Prevention and Health in Leiden.

In September 1996 she continued her study at the Free University of Amsterdam and received a Master of Science degree in Medical Biology in September 1998 with majors in Oncology and Gene therapy. From October 1998 till October 1999 she worked with great pleasure as a research assistant in the field of immune-mediated gene therapy for the treatment of colon carcinomas in the group of Prof.dr. R.C. Hoeben at the former Department of Molecular Carcinogenesis at the Sylvius laboratory, University of Leiden.

After refreshing her knowledge in immunology by attending the ALIFI course in Immunology, she began her Ph.D. as "cotutelle de these" in March 2000 at Necker Hospital, Paris, France, under the supervision of Dr. F. Homo-Delarche, Dr. W. Savino and Prof.Dr. H.A. Drexhage (Department of Immunology, Erasmus MC, Rotterdam, the Netherlands). During her thesis she spent a month in the laboratory of Dr. T. Otonkoski at the Department of Developmental and Reproductive Biology, Biomedicum in Helsinki, Finland, to gain experience in the field of pancreas development. To finalize her thesis in the context of a "cotutelle de these" she has spent the last year of her thesis in the group of Dr. P.J.M. Leenen at the Department of Immunology, Erasmus MC in Rotterdam.

In October 2004 she has started her first post-doctoral research on the role of chemorepellent-receptor interactions in the homing and mobilization of hematopoietic stem cells in the group of Dr. P.B. van Hennik, at the Department of Experimental Immunohematology, Sanquin Research at CLB in Amsterdam.

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