Adhesion and Migration of Monocytes and Dendritic Cells in Type 1 Diabetes

Hechting en migratie van monocyten en dendritische cellen in type 1 diabetes

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CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1.1

TYPE 1 DIABETES IN MEN AND MICE

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Diabetes mellitus - clinical characteristics and prevalence

Diabetes is a metabolic disorder characterized by chronic hyperglycaemia with disturbances of the metabolism of carbohydrates, fat and proteins resulting from defects in insulin secretion, insulin action or both. Two main types of diabetes exist of which type 2 diabetes is the most common. In type 2 diabetes, either the body cells have lost sensitivity for the insulin and/or the insulin production is insufficient for proper glucose maintenance. Type 1 diabetes (T1D) is characterized by an autoimmune response that destroys the insulin-producing β cells in the pancreas resulting in loss of insulin secretion. While type 2 diabetes is more common in the aged population, T1D is usually diagnosed in children and young adults, hence its previous characterization as juvenile diabetes.

The onset of clinically overt diabetes is characterized by various symptoms including: frequent urination, excessive thirst and hunger, unusual weight loss and fatigue. The disturbed glucose maintenance is the underlying cause for these symptoms. If not adequately taken care of, the greater the chance of severe complications, including increased risk of cardiovascular disease, retinopathy, neuropathy and nefropathy.

Type 1 diabetes has a large regional variation in incidence, varying from 3.2 cases per 100 000 per year in Macedonia to 40.2 cases per 100 000 per year in two regions of Finland (1). In the Netherlands the incidence of T1D is 18.6 per 100 000 per year (2). Over the past years a rapid increase in the incidence of T1D is observed, which is particular evident in children under 5 years of age (1).

Diabetes mellitus - genetic susceptibility

In T1D genetic associations play an important role, although it has become evident that a predisposition for the disease, but not the disease itself, is inheritable. Twin studies show a 25-50% concordance rate of developing diabetes in monozygotic twins (3, 4), indicating that apart from the genetic component environmental factors must play a role in the susceptibility for T1D. Several genetic susceptibility loci have been identified that are conventionally noted using the abbreviation IDDM (insulin-dependent diabetes mellitus) followed by a number, e.g. IDDM1, IDDM2 etc. To date up till 20 loci have been proposed: IDDM1-13, 15, 17, 18 and four other loci (5-8) of which IDDM1 and IDDM2 shows the strongest association with the risk of developing T1D.

The IDDM1 locus comprises several human leukocyte antigen (HLA) genes, which are located on the short arm of chromosome 6, and provides 40-50% of the inheritable risk (9). The HLA genes code a.o. for the major histocompatibility complex (MHC) class I and class II molecules, which present antigens to cells of the immune system. In humans the MHC class I consists of an α chain, of which in humans there are three genes, called HLA-A, -B, and -C, and the invariable β_2

microglobulin chain. The MHC class II consists of a paired α and β chain and there are three pairs of MHC class II α- and β-chain genes, called HLA-DR, -DP, and -DQ. The HLA-DR cluster contains an extra β-chain gene. This means that the three sets of class II genes can give rise to four types of MHC class II molecules. Although T1D initially was associated with some HLA class I alleles (10), later it was concluded that these associations were secondary to linkage disequilibrium with high-risk HLA-DR alleles (11). The HLA-DR and -DQ loci appeared to be the major constituents of the IDDM1 susceptibility locus (12). A strong association is found with HLA-DR3 (DRB1∗03) and HLA-DR4 (DRB1∗04), the DR3/DR4 heterozygous genotype showing the highest diabetes risk, followed by DR4 and DR3 homozygosity, respectively (13, 14). It is unclear, however, whether these alleles alone or whether a linkage disequilibrium with HLA-DQ alleles confer disease risk. The most important determinant of diabetes susceptibility was found to be the HLA-DQ locus. The HLA-DQA1∗0301-DQB1∗0302 (also referred to as DQ8) and HLA-DQA1∗0501-DQB1∗0201 (also referred to as DQ2) haplotypes are associated with a high risk to develop diabetes (5, 12, 15, 16). The DQB1*0302 allele that is risk-associated differs from the neutral DQB1*0301 allele by only one amino acid residue at position 57, where it lacks an aspartic acid residue resulting in the stronger susceptibility to develop diabetes (17). Approximately 90% of Caucasian individuals with T1D have either the DR3/DQ2 or the DR4/DQ8 haplotype (5, 18). Although large variation in risk association of HLA susceptibility loci exists between different ethnic groups, DR3/DR4 heterozygosity seems to confer strong susceptibility in most ethnic groups, although linkage disequilibrium of susceptible loci with protective loci may counteract susceptibility (reviewed in 16). Several loci that are protective for developing T1D have been found. The DQA1∗0102/DQB1∗0602 haplotype (also referred to as DQ6) found on the DRB1∗1501 genotype (also referred to as DR2) is associated with strong protection against T1D, while other DR2 haplotypes without DQ6 are not associated with protection (5, 19). A more extensive overview of high-risk and protective haplotypes is given in table 1 (for reviews see refs 5, 16, 18, 20).

The IDDM2 locus is determined by a variable number of tandem repeats (VNTR) region located 596 bp upstream of the insulin gene (21, 22). Alleles of the VNTR are divided into three classes based on their size: class I (26-63 repeats), class II (64-139 repeats) and class III (140-200 repeats). The class I alleles are associated with susceptibility to T1D, while class III alleles confer dominant protection (22, 23).

Other loci that are clearly associated with susceptibility to develop diabetes are the *CTLA-4* gene (IDDM12) and the *PTPN22* gene. Of these the *CTLA-4* (cytotoxic T lymphocyte associated-4) is a likely candidate gene for T1D because of its role in the regulation of T cell activation (24). The *CTLA-4* gene is located on chromosome 2 and its polymorphisms show association with diabetes in multiple ethnic groups, including the Belgian, Italian, Spanish, French, Mexican-

Table 1. HLA class II and diabetes susceptibility.

HLA-DR	DQA1	DQB1	DRB1	Susceptibility
DR2	0102	0602	1501	Protective
DR2	0102	0502	1601	Predisposing
DR2	0103	0601	1502	Neutral
DR3	0501	0201	0301	High Risk
DR4	0301	0302	0401	High Risk
DR4	0301	0302	0402	Predisposing
DR4	0301	0302	0403	Neutral
DR4	0301	0302	0404	Predisposing
DR4	0301	0302	0405	High Risk
DR4	0301	0301	0401	Neutral
DR4	0301	0303	0401	Neutral
DR6	0101	0503	1401	Protective
DR7	0201	0303	0701	Protective
DR8	0401	0402	0801	Predisposing

(from Pugliese A. and Eisenbarth G.S., Chapter 7, Type 1 Diabetes: Molecular, Cellular and Clinical Immunology, www.barbaradaviscenter.org)

American, Caucasian-American and Korean populations, but not in UK, Chinese or Sardinian families (25, 26). The *PTPN22* gene encodes the lymphoid protein tyrosine phosphatase, which is a suppressor of T cell activation (27). The presence of a polymorphism at nucleotide 1858 yielding a tryptophan (1858T) instead of an arginine confers strong predisposition to T1D (28). In two cohorts, one of 388 individuals from Sardinia and the other of 294 North American individuals, it was shown that the 1858T polymorphism was more frequent in T1D patients resulting in an odds ratio for the 1858T carriers of 2.31 and 1.83 for the Italian and North American individuals, respectively (28).

Diabetes mellitus - autoimmune involvement

Type 1 diabetes is considered to be an autoimmune disease, in which the immune system erroneously recognizes self-antigens and attacks 'self', in this case the β cells. One of the first hints of an autoimmune involvement was provided by the presence of autoantibodies in the serum of patients. These autoantibodies are directed against various islet cell antigens (ICA) including glutamic acid decarboxylase (GAD), insulin and the tyrosine phosphatase homologue IA-2. All these diabetes-associated autoantibodies have a high diagnostic sensitivity and specificity and double or triple positivity increases the diagnostic value even further (29, 30). The presence of autoantibodies may, in addition to the HLA typing, be used to characterize the relatives of patients that will have a high risk of progression to T1D (31). In the Diabetes Autoimmunity Study in the Young (DAISY) cohort of young first-degree relatives of T1D patients and infants identified through screening for diabetes-associated HLA-DR/DQ genotypes the presence of autoantibodies was detected in all 24 children who developed diabetes (32). In a large French cohort of

first-degree relatives the combination of antibodies against GAD and IA-2 showed a good predictive value. Of the 33 relatives who subsequently developed diabetes 29 were found positive for both antibodies (33). Also in the general population, screening for autoantibodies may successfully predict the development of T1D, as is shown by a study that followed 4505 schoolchildren. At an 8-year follow-up, 12 of these children had multiple diabetes-associated autoantibodies and six of them developed diabetes, representing 100% sensitivity and 50% positive predictive value (34). Also in a study by the Childhood Diabetes in Finland Study Group it was shown that the presence of diabetes-associated autoantibodies has a positive predictive value in the general population, but in first-degree relatives the positive predictive value is higher (35).

Stonger evidence for an autoimmune involvement in the pathogenesis of T1D comes from the presence of auto-reactive T cells. Preceding the β cell destruction in animal models infiltrations of leukocytes around the islets of Langerhans are detected, which include large numbers of T lymphocytes (36-40). These T cells are required for the destruction of the β cells, indicating that these T cells may be of auto-reactive nature (reviewed in 41, 42). In humans a major caveat is the difficulty of obtaining lymphocytes from the islet infiltrates for further study and characterization. Nevertheless, the presence of T cells among the infiltrating cells in human diabetic islets has been described in several reports in which the pancreas was studied from patients who had died (43-45). The presence of these T cells in the pancreas is suggestive of an auto-reactive nature, although formally a preceding viral infection cannot be excluded. In the circulation, however, auto-reactive T lymphocytes can be found against various islet antigens (46-48), including GAD (49-51) and IA-2 (52). The study and identification of such islet-reactive T cells will be of value for the diagnosis of T1D. For this purpose preparations of islet auto-antigens have been generated to improve the identification, characterization and quantification of such diabetogenic T cells (53). Depletion of diabetogenic T cells in T1D patients would be a good candidate therapy to stop the β cell destruction. Such therapies may become available in the future. Recently one study described a 14 days treatment with a non-activating humanized monoclonal antibody against CD3 - hOKT3γ1(Ala/Ala) – that stopped the deterioration of insulin production in nine out of twelve patients (54). Although such treatment does not result in a specific deletion of diabetogenic T lymphocytes it shows possibilities of intervention.

Animal models

Although a considerable body of knowledge about the pathogenesis of T1D has been gained from human studies, ethical and technical restrictions urged for animal models. Studying the development of diabetes in animals allows (I) study and manipulation of inheritance, (II) accessibility of the target organ and (III) new therapies to readily be tested. In fact, the use of animal models has enabled translational research (i.e. research directed at prediction and prevention) in T1D.

In 1978 the bio-breeding diabetes-prone (BB-DP) rat was bred from Wistar rats (55) and in 1980 the non-obese diabetic (NOD) mouse was established from inbreeding of CTS mice that were originally derived from the ICR strain (56). Both models develop diabetes spontaneously and are commonly used as animal models for the study of human T1D.

BB-DP rat

BB-DP rats are severely lymphopenic and almost completely lack the regulatory ART2$^+$ (previously referred to as RT6$^+$) T cell subset (57, 58). The regulatory role of ART2$^+$ T cells is shown when ART2$^+$ T cells are depleted in diabetes-resistant (BB-DR) rats. BB-DR rats are phenotypically normal and do not develop insultis or diabetes spontaneously. Depletion of the ART2$^+$ T cells in BB-DR rats renders these rats susceptible for diabetes development, which can be reversed again by adoptive transfer of ART2$^+$ T cells (59, 60). Environmental factors also play a role in the development of diabetes. Introducing BB-DP rats into a specified pathogen free (SPF) environment increased the incidence and accelerated the onset of diabetes (61). In contrast, infection of BB-DR rats with Kilham rat virus, an environmentally ubiquitous rat parvovirus, induced the development diabetes (62) as did administration of polyinosinic polycytidilic acid (poly I:C) (63). Interestingly, poly I:C administration in BB-DP rats accelerated the development of diabetes (64), but when administered in low doses it prevented the onset of diabetes (65). Similarly, long-term insulin treatment started at early age also prevented diabetes in BB-DP rat (66).

Genetic susceptibility

The gene conferring the MHC class II RT1^u allele is the strongest susceptibility gene in the BB rat and referred to as *Iddm1* (67). The second diabetes susceptibility gene, *iddm2*, is responsible for the severe lymphopenia (*lyp*) in BB-DP rats and is located on chromosome 4 (68). Positional cloning of the *lyp* locus revealed that the lymphopenia is caused by a frameshift mutation in a member of the immune-associated nucleotides (Ian genes), resulting in a truncated Ian4 (69) or Ian5 (70) protein. Additional susceptibility loci in BB rats have been mapped to chromosome 18 (*Iddm3*), chromosome 4 (*Iddm4*), chromosome 13 (*Iddm5*) and chromosome 3 (*Iddm6*) (71, 72). Although BB-DR rats are not lymphopenic they share all susceptibility genes with the BB-DP rats except for *Iddm2*.

Insulitis

Inbred BB-DP rats spontaneously develop insulitis and diabetes with a prevalence of up to 90% of the animals before 130 days of age with no gender differences (reviewed in 61, 73). Autoantibodies to several islet cell-surface markers are found in BB rats (74-77), although the significance of their presence and their contribution to the pathogenesis of diabetes remains unclear. Interestingly, no

antibodies against insulin are observed (78). BB rats develop insulitis in which macrophages (mφ) and dendritic cells (DC) are the first cells to accumulate around the islets, followed by lymphocytes and subsequent β cell destruction (36, 39).

NOD mouse

Since its discovery in 1980 (56), the NOD mouse has proved to be a highly informative animal model to study human T1D. Inbred colonies of NOD mice are maintained throughout the world, but differ in diabetes incidence. In contrast to humans and BB-DP rats, female NOD mice are more prone to develop diabetes than males. In the original NOD/Shi colony from the Shionogi Company 80% of females and 20% of males developed diabetes at 30 weeks of age (56), while in other colonies the diabetes incidence in males is usually higher; e.g. in the widely used NOD/Lt colony 40-60% of males develops diabetes (79). The incidence of diabetes in NOD mice is dependent on the specific pathogen-free (SPF) status of the colony. Abrogation of the SPF status by the introduction of bacteria or viruses stimulates the immune system and prevents diabetes development in NOD mice (80-82).

In addition to diabetes, the NOD mouse develops several other autoimmune diseases. Large perivascular and periductular leukocyte infiltrations can be found in the lacrimal and salivary glands (56). In NOD mice also the function of the salivary glands is impaired and saliva production decreased, which resembles human Sjögrens syndrome (83, 84). In NOD mice a high incidence of thyroiditis accompanied by the presence of anti-thyroid autoantibodies can be induced by the administration of high iodine dose to goitrous animals (85). Furthermore, NOD mice are susceptible to a variety of experimentally-induced autoimmune diseases including systemic lupus erythematosus (SLE) (86) and experimental autoimmune encephalomyelitis (EAE) (87).

NOD-related strains

Related mouse strains that are often used to study the development of diabetes or as controls for NOD mice include NOD/LtSz-scid (NOD/scid), NON/Lt and NOR/Lt mice. The NOD/scid was generated by backcrossing the *scid* (severe combined immunodeficiency) mutation for ten generations onto the NOD/Lt background (88). It lacks functional lymphocytes and as a result does not develop lymphocytic insulitis and diabetes (88). The NOD/scid mice are widely used for the adoptive transfer of diabetes. When T cells or T cell subsets are isolated from NOD mice and transferred to NOD/scid recipients they will rapidly develop insulitis and subsequently diabetes (89, 90). The NON/Lt (non-obese non-diabetic) mouse has been developed from the ICR strain, like the NOD mouse and they are therefore closely related (91). However, these NON/Lt mice do not develop diabetes, although small leukocyte infiltrations in the pancreas do develop (92). The NOR/Lt mice are the result of an outcross between NOD/Lt and C57BLKS/J followed by backcross to

the NOD background (93). These mice share 88% of the NOD genotype, inlcuding the $H2^{g7}$ MHC haplotype, but they very rarely develop diabetes (93). Several congenic NOD strains were also developed in which the NOD mice were crossed with other mice and the progeny selected for the NOD genetic background and the MHC genes of the other strain (reviewed in 94). NOD.$H2^{b}$ mice carry the MHC from C57BL/10 mice. These mice do not develop diabetes, but they do develop autoimmune infiltrations in the salivary glands (95, 96). The NOD.$H2^{h4}$ mice are highly susceptible to develop autoimmune thyroiditis (97), but they do not develop diabetes, although in 25% of the mice insulitis can be observed (96).

Genetic susceptibility

Like in humans and BB-rats the strongest susceptibility gene in NOD mice, *Idd1,* is formed by the MHC region that is located on mouse chromosome 17 (98-100). In mice the MHC class II genes are located in the H2-I region of the MHC multigene cluster. NOD mice do not express I-E of the MHC class II complex due to a deletion in the promotor region of the α chain of the I-E genotype (99). In addition, NOD mice have a rare I-A β chain molecule (101). This NOD I-A β chain, also referred to as $I\text{-}A^{g7}$, has a serine residue at position 57. Replacement of this serine residue by an aspartic acid residue reduces the diabetes incidence in these mice (102). Also in humans at position 57 of the corresponding HLA-DQ β chain, an aspartic acid residue is associated with relative protection to develop diabetes (17). Both the unique I-A molecule ($I\text{-}A^{g7}$) and the lack of I-E expression appear to be essential for the development of diabetes and both contribute to the diabetes susceptibility of the *Idd1* locus (94-96). In addition to *Idd1*, up to 18 *Idd* genes have been identified so far (for review see 94, 100, 103). Among these, *Idd3, Idd5* and *Idd10* show significant linkage to the development of leukocyte infiltrations around the islets (94). The *Idd3* locus is one of the strongest susceptibility genes and is located on chromosome 3 (103). A strong candidate for the *Idd3* susceptibility locus is the *interleukin (IL)-2* gene (104). Recently the *Idd5* locus has been shown to be associated with the regulatory gene *CTLA-4* on chromosome 1 (105). *Idd10* was mapped to chromosome 3. Originally it was thought to encode the *Fcgr1* gene, which encodes the high-affinity Fc receptor for IgG (103). However, recently it was demonstrated that *Idd10* was localized distally to *Fcgr1* and comprised three linked loci, *Idd10, Idd17* and *Idd18* (106, 107).

Insulitis

NOD mice develop spontaneously diabetes that is preceded by insulitis. At around three weeks of age the endothelium of the vessels adjacent to the islets of Langerhans becomes swollen. $CD11c^{+}$ DC and $ER\text{-}MP23^{+}$ (mMGL) and $MOMA\text{-}1^{+}$ mφ infiltrate the pancreas and are mainly found in the perivascular para-islet connective tissue (37, 38, 108). At later ages the infiltrating DC and mφ accumulate around the islets (108, 109) and $BM8^{+}$ mφ start to appear in the para-insular

infiltration (37, 110). As early as 7 weeks of age lymphocytes infiltrate the pancreas. The infiltration of lymphocytes occurs in a similar fashion as the mφ and around 10-17 weeks the lymphocytes as well as the DC and mφ have surrounded the islet completely (37, 38, 40, 109, 111). From 17 weeks of age onwards the lymphocytes and $BM8^+$ mφ are observed to infiltrate into the islets (37, 38, 40, 110) and β cell destruction is initiated, which finally results in massive loss of β cell mass and overt diabetes. Table 2 summarizes the different phases that can be distinguished in diabetes development in the NOD mouse.

Table 2. Insulitis in wild-type NOD mice.

stage 0:	Intact islet as observed in non-diabetes prone mice
stage I (wk 3):	Intact islet, but an increase in perivascular and periductular extracellular matrix and a perivascular increase of $CD11c^+$, ER-$MP23^+$, MOMA-1^+ and $BM8^+$ mφ and DC
stage II (wk 4-7):	Islet surrounded by DC and mφ with above-mentioned phenotypes; infiltration of islets by $CD11c^+$ DC; a major infiltration by lymphocytes is still absent
stage III (wk 7-10):	Parainsular recruitment of substantial numbers of $CD4^+$ and $CD8^+$ T lymphocytes and some B lymphocytes to the accumulation of DC and mφ
stage IV (wk 10-17):	Lymphocytes surround the islet (peri-insulitis)
stage V (> wk 17):	Lymphocytes and ER-$MP23^+$ and $BM8^+$ mφ infiltrate the islet
stage VI (>> wk 17):	Endstage; no insulin-producing cells detectable

(from Rosmalen et al. Subsets of macrophages and dendritic cells in nonobese diabetic mouse pancreatic inflammatory infiltrates: correlation with the development of diabetes. *Lab Invest* 80: 23-30, 2000)

Pathogenesis of type 1 diabetes

The first cells to be part of the insulitis in NOD mice are mφ en DC. Both cells are antigen-presenting cells (APC). Monocytes are important precursors for mφ and DC. After maturation in the bone marrow, monocytes enter the bloodstream. When the monocytes encounter the right trigger, they leave the circulation to enter the tissue where they differentiate into either mφ or DC (112, 113). At least a large part of the mφ and DC that are observed to accumulate around the islets of Langerhans are thought to be derived from circulating monocytes. These mφ and DC probably take up autoantigens and present these in the draining lymph node to naïve T lymphocytes. The lymphocytes that recognize the antigens become activated and home to the pancreas to mediate the destruction of the β cells (37, 38, 40). This emphasizes the important role that mφ and DC play in the development of diabetes.

Macrophages

Mφ are specialised in antigen uptake and removal of apoptotic cells and cell debris. NOD mφ, however, fail to phagocytose apoptotic cells, despite a fully competent phagocytosis of polystyrene beads, which may indicate a specific malfunction in the clearance of dead cells in NOD mice (114). A defective

clearance of apoptotic β cells may overburden the phagocytic system in NOD mice and possibly result in increased uptake and presentation of autoantigens and subsequent activation of autoreactive T lymphocytes. Apoptotic β cells have indeed been observed in NOD mice prior to the lymphocytic infiltration (115).

NOD mice show an intrinsic defect in mφ development, which is characterized by a low yield of mφ after culture from bone marrow precursors (116). Phenotypically NOD bone marrow-derived mφ are less mature in comparison to control strains (116) and they produce increased amounts of IL-12, IL-6 and IL-1 (117-119). In addition to a less mature phenotype, NOD mφ are less capable of T cell activation (116, 120). Culturing bone marrow precursor cells with GM-CSF, which typically is used to culture DC, yields elevated numbers of mφ in NOD mice (121). The importance of mφ in diabetes is further stressed by studies in which depletion of mφ by administration of silica (122) or liposomal dichloromethylene diphosphonate (lip-Cl_2MDP) (123) into NOD mice completely prevented the development of diabetes. Also when mφ were depleted and subsequently cyclophosphamide (CY) was administered to NOD mice, development of diabetes was prevented (122). Without mφ depeletion CY treatment results in a significant increase in the incidence of overt diabetes and severity of insulitis compared with that in untreated NOD mice.

Dendritic cells

In addition to mφ, an accumulation of DC in the pancreas is a hallmark of diabetes development. DC are professional APC that take up antigen in the tissue, home to the draining lymph node and activate naïve T lymphocytes to become effector T lymphocytes that then will home to the tissue to fight the source of the antigen (124). DC that reside in the tissue are in an immature state, which is characterized by low efficacy to stimulate T cells, low expression of MHC class II and co-stimulatory molecules like CD40, CD80 and CD86. Immature DC are well equipped to capture antigens (124). When immature DC are activated, maturation of the cells is induced. A variety of factors is able to induce such DC maturation, notably microbial and inflammatory products (125). Fully mature DC are characterized by potent T cell stimulatory capacity that is associated with high expression of MHC class II and co-stimulatory molecules and production of large amounts of pro-inflammatory cytokines. In vivo DC will act as sentinels that crawl through the tissue while taking up antigen, which will later be presented to T cells in the draining lymphoid tissues. What triggers the cells to migrate to the draining lymph nodes is still unclear. Upon inflammation the migration of DC to the lymph nodes via the lymphatic vessels is strongly increased (126, 127). Under inflammatory conditions local pro-inflammatory cytokines rapidly induce maturation of DC and induce a switch in their chemokine receptor expression. The cells down-regulate C-C chemokine receptor (CCR)1, CCR2 and CCR5 and up-regulate CCR7 (128, 129). CCR7 is a receptor for C-C chemokine ligand (CCL)19 and CCL21 that are expressed by the lymphatic vessels and in the lymph nodes to enable the migration of DC to the lymph node

(130, 131). At arrival in the lymph node, the DC have reached a fully mature state as determined by their MHC class II and co-stimulatory molecule expression (124, 132). The T cells that recognize the presented antigens are primed by the DC and induced to proliferate, resulting in an immune response. Homeostatic migration of DC to the draining lymph node will not result in an immune response. Such DC are also referred to as semi-mature or tolerogenic DC and despite the expression of MHC class II and co-stimulatory molecules they produce low to no pro-inflammatory cytokines (132, 133). An important feature of such tolerogenic DC is the induction of regulatory T cells. Regulatory T cells suppress the proliferation of antigen-activated T lymphocytes and modulate an immune reaction via IL-10 production and/or cell-cell contact (134). In addition to naturally occurring $CD4^+CD25^+$ regulatory T cells, they can be generated in vitro from naïve $CD4^+$ T cells by repeated stimulation with immature $CD83^-$ human DC (135) or in the absence of APC by stimulation with IL-10 and interferon (IFN)-α (136) or a combination of 1α,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and dexamethasone (137). Likewise, IL-10 is capable of rendering DC into a tolerogenic state. Stimulation of myeloid DC with IL-10 alone (138) or together with TGF-β (139) induces such tolerogenic DC that are capable of inducing $CD4^+$ regulatory T cells. Also immunomodulatory agents such as $1,25(OH)_2D_3$ can affect differentiation of human DC and induce a tolerogenic phenotype (140, 141).

NOD dendritic cells

NOD DC generated from bone marrow precursors show a defective phenotype, which is characterized by a low yield of cells after culture with GM-CSF (121, 142, 143), a low expression of MHC class II and co-stimulatory molecules (143-145) and poor T cell activation (121, 143-145). Culturing NOD DC from bone marrow precursors in the absence of IL-4 yields increased numbers of mφ (121). Furthermore, the DC that are present share many mφ phenotypic characteristics and perform poorly in T cell activation (121). DC isolated from spleens of NOD mice, also show reduced T cell proliferation (146), however, this effect is much less pronounced compared to in vitro generated DC. In contrast, there are also several reports showing that DC generated from NOD mice have an enhanced capacity to stimulate T cells and an increased expression of MHC class II and co-stimulatory molecules (142, 147, 148). The enhanced APC function of NOD DC is ascribed to a hyperactive IκB kinase resulting in an increased degradation of IκB and thus a hyperactivation of the transcription factor nuclear factor-κB (NF-κB) (149-151). Hyperactivation of NF-κB was also observed in B cells and in mφ of NOD mice and resulted in increased APC function (149, 152). Inhibition of NF-κB corrected the increased APC function of NOD mφ and DC (150-152) and administration of islet-pulsed NOD DC in which NF-κB function was inhibited, resulted in the prevention of diabetes and reduction of insulitis in NOD mice (153). In contrast, it has also been reported that lymphocytes and splenocytes of NOD mice are deficient in proteasome function, which results in a decreased activation of NF-κB (154).

Immature DC can be skewed by cytokines or vitamin D3 to a tolerogenic state in which they will induce regulatory T cells and give rise to tolerance (132, 138-141). In NOD mice there are indications that tolerogenic DC exist since transfer of DC from the draining lymph node of the pancreas of 8-20 wk old non-diabetic NOD mice, protected 4 wk old NOD mice from developing diabetes up to one year follow-up (155). Also regulatory T cells have been reported to be present in the NOD mouse, although in somewhat reduced numbers (156). Transfer of these regulatory T cells prevents the development of diabetes in adoptive transfer models, confirming the immunoregulatory potential of these cells (156). However, the mere existence of tolerogenic DC and regulatory T cells in the NOD mouse is apparently not sufficient to prevent spontaneous diabetes development. When vitamin D3 or analogues are administered to NOD mice, diabetes and insulitis is inhibited and regulatory T cells are induced, which is likely the result of stimulation by tolerogenic DC (157-159). In vitro, NOD tolerogenic DC can also be generated as is shown for ex vivo treatment of myeloid DC with IFN-γ that after transfer into NOD mice down-modulated oth insulitis and diabetes development (160). Although literature is not unambiguous about the APC function of NOD DC compared to control mice, it is clear that the differentiation and function of NOD DC is aberrant, which will have consequences for the break of tolerance that leads to the development of diabetes.

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CHAPTER 1.2

THE MULTISTEP PARADIGM OF LEUKOCYTE INFILTRATION

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Tethering and rolling

Many immune cells that reside in tissue are derived from the circulation. The mechanism, by which those cells enter the tissue, is referred to as the multistep paradigm (1) and comprises several sequential events (figure 1). Firstly the cells are slowed down from the bloodstream, by specific binding of selectins on the vascular endothelium to sialylated carbohydrate groups of the counter receptors on the leukocytes. This binding is rather weak, but mediates tethering of the circulating cell and subsequently rolling on the endothelium (1). The family of selectins is composed of three members: E-, L- and P-selectin (for review see 2). E- and P-selectin are located on the endothelium, while L-selectin is located on most leukocytes. L-selectin binds the sulfated sialyl Lewis^x moieties of members of the peripheral lymph node addressin (PNAd) mucins, like GlyCAM-1, CD34 and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (3, 4). The other selectin members, E- and P-selectin, recognize sialic acid residue-containing glycoproteins on the leukocyte membrane, of which P-selectin glycoprotein ligand-1 (PSGL-1) has been studied most extensively (5, 6).

Integrin activation

After the tethering, the cells firmly adhere to the vascular wall, as illustrated in figure 1. This binding is mediated via integrins on the leukocytes that bind to adhesion molecules on the endothelial cells. When the cells are slowed down from

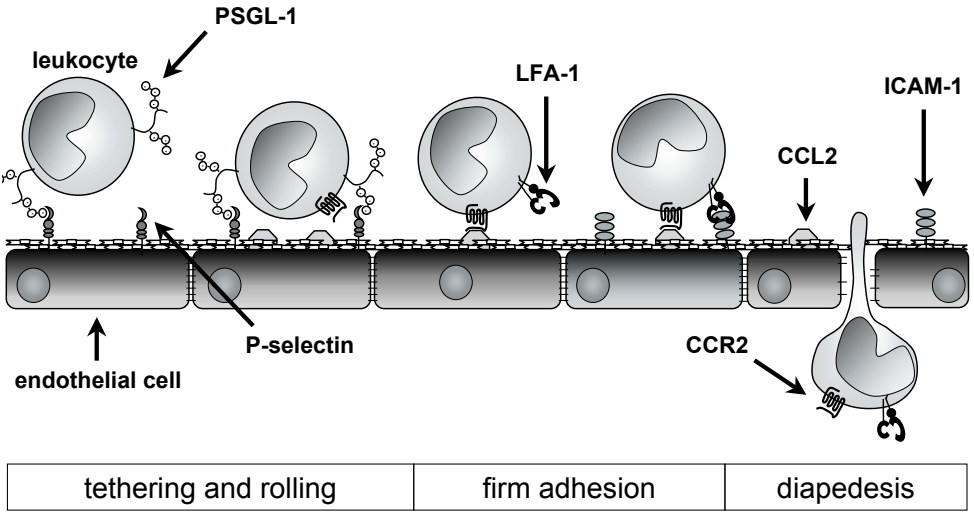

Figure 1.
Schematical overview of the distinct steps in the sequential model for leukocyte transmigration. See text for details.

the circulation, the specific recognition of chemokines by chemokine receptors on the leukocyte's membrane triggers an intracellular signalling that results in the activation of integrins and subsequently firm adhesion to the endothelial cells (7, 8).

Chemokines

About 40-50 chemokines have been identified in humans. Chemokines form a family of small (8-14 kDa) inducible proteins that cause chemotactic migration of leukocytes (9). Chemokines can be divided into two major structural subfamilies based on the arrangement of the two NH_2-terminal cysteine residues, which are in adjacent position (CC) or separated by a single amino acid (CXC). Two additional minor subfamilies can be distinguished of which the first family (C) consists of two members (XCL1 and XCL2), based on the single cysteine residue present in the NH_2-terminus, and the second family comprises one member (CX3CL1) defined by the presence of three amino acids that separate the two cysteine residues (9-11). Chemokines can bind to their receptors in a promiscuous way with both a high variety of chemokines that bind multiple receptors and also many receptors that bind multiple chemokines. Functionally, chemokines can be divided into two groups; the inflammatory and the homeostatic chemokines, although several chemokines show a dual function (10, 12). Chemokines bind to specific G-protein coupled receptors with seven transmembrane domains. Upon binding of the chemokine, the G-protein is modified intracellularly and dissociated into an α- and $\beta\gamma$-subunit that mediate further downstream signalling.

Integrins

The family of integrins is composed of a series of non-covalent heterodimeric complexes consisting of an α subunit and a β subunit. Currently, 18 α subunits and 8 β subunit are known that can associate in various combinations to form the 24 heterodimers (13, 14). The largest group consists of the β_1 family, of which each member contains a β_1 chain that is associated with one of the eleven α chains. These so-called very late antigen (VLA) proteins are widespread distributed and mediate binding of a cell to extracellular matrix (ECM) components (14). The β_2 subfamily contains four members that bind to members of the immunoglobulin (Ig) gene superfamily and their expression is restricted to leukocytes (13, 14). Integrins have a dual function in mediating signals from the cells' environment to the cells' cytoskeleton and vice versa. On the one hand, integrins form a physical link between the ECM outside the cell and the cytoskeleton inside the cell. Interaction of integrins with the ECM generates intracellular signals (outside-in signalling) resulting in a wide range of cellular functions including adaptation of motility and cell shape (15). On the other hand, chemokine recognition may trigger an intracellular cascade of signals that induces a conformational change of the integrin leading to an increased binding affinity (inside-out signalling). In addition to affinity changes,

an increased binding capacity can also be mediated by increased clustering of integrins on the membrane, causing an increased avidity for the ligand (7, 8).

Firm arrest and diapedesis

The activation of integrins occurs within seconds after the specific chemokine recognition and results in firm adhesion of the leukocyte to the endothelium. Leukocytes utilise β_2 integrins like leukocyte function antigen-1 (LFA-1) to bind to adhesion molecules of the Ig gene superfamily including intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) on the endothelium (16, 17). The leukocyte that is then tightly adhered, crawls along the luminal side of the endothelium (18) and migrates through a junction between adjacent endothelial cells into the tissue as is schematically shown in figure 1 (for reviews see 17, 19, 20). In addition, it has been shown that leukocytes can pass through the endothelial cells itself (21). During the migration through the endothelium, also referred to as transmigration or diapedesis, the leukocyte will polarize its cellular body to form an uropod at its tail and lammellipodia at the leading edge (22). This polarization is mediated by a synergism of integrins and the cytoskeleton. As a result of the polarization, a clustering of chemokine receptors and integrins is localized at the leading edge of the cell (23), by which the leukocyte is able to determine its route. At the endothelial junction, the intracellular Ca^{2+} concentration in the adjacent endothelial cells increases and as a result the cells retract to facilitate leukocyte transmigration (24). In the gap that is formed, the leukocyte binds sequentially to different adhesion molecules. The leukocyte that used VCAM-1 and ICAM-1 as a scaffold to crawl towards the junction (18), then binds to junctional adhesion molecule-A (JAM-A) (25). On resting endothelium JAM-A is located in the tight junctions that form the connection between endothelial cells, but when the endothelium is activated the junction is loosened and JAM-A shifted to the apical side of the endothelial cells (25). JAM-A can interact with the LFA-1 on leukocytes to direct the leukocyte to the endothelial junction (25). The downward migration to the abluminal side of the endothelial junction is mediated by homophilic binding of platelet-endothelial adhesion molecule-1 (PECAM-1) on the leukocyte with PECAM-1 located on the endothelial cells, followed by homophilic binding of CD99 (26, 27).

In order to initiate the insulitis that is a hallmark for diabetes development in the NOD mice, an influx of cells is a prerequisite. It is generally thought that at least part of the early mφ and DC that are observed in the NOD pancreas are derived from circulating monocytes. Those monocytes, but also the lymphocytes at a later stage, will enter the pancreas via the above described multistep mechanism. Targeting the influx of cells may provide a powerful tool in the prevention of diabetes. The expression of ICAM-1 and VCAM-1 on the endothelium was observed to increase with disease progression and found in close association with islet-infiltrating mφ, DC and lymphocytes (28-30). In addition, a specific induction of the addressins

MAdCAM-1 and PNAd was observed on vessels in inflamed islets in the NOD mouse and lymphocytes were shown to use these addressins to bind to the vessels (30-32). The increased expression of adhesion molecules on the endothelium near the islets facilitates the entry of more leukocytes into the pancreas, although prior to the influx of cells into the islets no strong expression of adhesion molecules was observed (table 3).

Table 3. Expression of endothelial adhesion molecules near islets of Langerhans in NOD mice during diabetes development.

	< wk 3	wk 3–5	wk 5–7	wk 7–10	wk 10–17	> wk 17	references
ICAM-1	+/-	+/-	++	++	++	++	(28-30)
VCAM-1	-	+/-	+/-	+	+	+	(29, 32, 33)
MAdCAM-1	-	+/-	+	++	++	++	(30-33)
PNAd	-	+/-	+/-	+	++	++	(30-33)

- = < 5% +/- = 5-15% + = 15-50% ++ > 50% % of islets with positive expression on endothelium

Many studies have shown that inhibition of the influx of cells into the pancreas prevents the development of diabetes. Table 4 summarizes the different adhesion molecules that were targeted in these studies and shows that inhibiting leukocyte transmigration at different steps in the multistep paradigm differ in effectiveness. Blocking L-selectin inhibited the spontaneous development of diabetes in NOD mice significantly and was most effective when administered at early age, prior to insulitis (34-36). In an adoptive transfer model for diabetes development, in which spleen cells from diabetic donors were given to irradiated NOD mice that as a result developed diabetes, blocking L-selectin in the recipients also inhibited diabetes, although with variable success, ranging from 40-100% of animals still developing diabetes (35-38). Interestingly, treatment of the diabetic donor with the blocking antibody against L-selectin increased the effectiveness of diabetes inhibition (36). When the leukocyte antigens Mac-1 and LFA-1 were blocked in adoptive transfer studies, disease development was significantly inhibited (38, 39). Furthermore, when the α_L integrin, which is the α subunit of LFA-1, was blocked diabetes development was completely inhibited (40), while blocking the β_2 integrin, which is part of both LFA-1 and Mac-1, resulted in only 70% inhibition in the adoptive transfer model and 50% inhibition of spontaneous diabetes development in NOD mice (40). Blocking of β_2 to prevent diabetes seems to be dependent on the antibody that was used, since blocking β_2 with antibody clone 2E6 did not prevent diabetes transfer (37), while the M18/2 clone showed 50% inhibition (40). The α_4 integrin dimerised with β_1 to form VLA-4 is involved in binding to fibronectin and VCAM-1. In addition, the α_4 integrin can dimerise with β_7 forming $\alpha_4\beta_7$, which not only binds fibronectin and VCAM-1, but also MAdCAM-1. Blocking the α_4 integrin resulted in almost complete inhibition of diabetes transfer (34, 35, 37, 38, 41, 42), with the exception of one report that found a prevalence of 63% after transfer (43). The spontaneous development of diabetes in NOD mice could also be reduced

Table 4. Inhibition of diabetes development in NOD mice by administration of inhibiting antibodies directed against adhesion molecules.

Inhibition of spontaneous diabetes development

		0-5 wks		5-12 wks		>12 wks		0 – 30 wks		
molecule	hybridoma	diabetes	insulitis	diabetes	insulitis	diabetes	insulitis	diabetes	insulitis	references
α_4	PS/2	++	++	+	+	+	+/-	nd	nd	(43)
	R1-2	+++	++	++	++	++	++	nd	nd	(34, 35)
β_7	Fib504	++	+++	++	+	nd	nd	+	nd	(45, 46)
α_L	KBA	nd	nd	+++	+++	nd	nd	+++	+++	(40)
β_2	M18/2	nd	nd	+	+	nd	nd	+	+	(40)
ICAM-1	YN/1	nd	nd	+++	++	nd	nd	+++	+++	(40)
L-selectin	MEL-14	++	+++	+	+/-	-	nd	nd	nd	(34-36)
MAdCAM-1	MECA-367	+++	+++	++	+	nd	nd	+++	+	(45, 46)

Inhibition of diabetes after adoptive transfer

molecule	hybridoma	recipient	diabetes	insulitis	references
α_4	PS/2	irradiated	+++	+	(41-43)
	R1-2	irradiated	+++	+++	(34, 35, 37, 38, 41)
β_2	M18/2	7 day old NOD	++	++	(40)
	2E6	irradiated	-	nd	(37)
β_7	Fib504	irradiated	-	nd	(45)
$\alpha_4\beta_7$	LPAM-1	irradiated	+++	+	(44)
LFA-1	M17/5.2	irradiated	++	+++	(38)
Mac-1	5C6	irradiated	+++	+++	(39)
ICAM-1	YN/1	irradiated	+	++	(38, 44)
VCAM-1	M/K2	irradiated	+++	++	(42)
L-selectin	MEL-14	irradiated	+/-	+/-	(35-38)
MAdCAM-1	MECA-367	NOD/SCID	-	nd	(45)

- = 0-20% +/- = 20-40% + = 40-60% ++ = 60-80% +++ = 80-100% inhibition nd = not determined

by blocking α_4, although less effective, since about 30-50% of the animals still developed diabetes (34, 43). Administration of the blocking antibody at early age was the most effective and only 0-33% of the animals developed diabetes (34, 43). The blockage of $\alpha_4\beta_7$ also was very effective, resulting in almost complete prevention of diabetes after transfer of T cells from diabetic donors (44). Blocking the β_7 integrin in the adoptive transfer model, however, did not prevent diabetes (45). In contrast, the spontaneous development of diabetes in NOD mice could be inhibited by blocking the β_7 integrin, with the highest effectiveness of treatment at early age (45, 46).

Inhibiting the entry of leukocytes into the pancreas and subsequent diabetes development can also be achieved by blocking the adhesion molecules on the endothelial cells (Table 4). When ICAM-1 was blocked, the spontaneous development of diabetes in NOD mice was almost completely inhibited (40), although ICAM-1 expression is not restricted to endothelium and is also expressed by DC that infiltrate into the islets (28, 30). Also blocking of MAdCAM-1 resulted in a strongly decreased diabetes development (45, 46). Blocking VCAM-1 has only been studied in an adoptive transfer model, in which only 5% of the recipients developed diabetes (42). When either ICAM-1 or MAdCAM-1 were blocked in such adoptive transfer studies only a mild reduction of diabetes was found for ICAM-1 (38, 44) and no effect of MAdCAM-1 blockage (45).

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CHAPTER 1.3

AIMS OF THE THESIS

AIMS OF THE THESIS

A hallmark of diabetes development in both humans and animal models is the accumulation of mφ and DC around the islets of Langerhans. These mφ and DC are at least in part derived from monocytes that circulate in the blood and subsequently enter the pancreas. It is thought that these mφ and DC initiate a T cell-mediated immune response that is directed against the β cells. Interference with the mechanisms underlying the transendothelial migration of monocytes into the pancreas affects the development of diabetes. When the adhesion molecules that are either present on the infiltrating cells or on the endothelium are blocked with antibodies, the influx of cells into the pancreas is inhibited and subsequently the development of diabetes can mostly be prevented.

In this thesis the role of the precursor cells for the mφ and DC, the monocytes, was studied in the development of diabetes. Adhesion and migration are key features in the multistep paradigm of transendothelial migration of monocytes and play an important role in the accumulation of mφ and DC in the pancreas. Therefore, this thesis aims to investigate the adhesive and migratory potential of monocytes and dendritic cells in T1D. To achieve this goal I studied:

- the adhesion of monocytes of patients with T1D to fibronectin. An increased fibronectin adhesion was observed and this was likely related to the increased presence of myeloid-related protein (MRP)8/14 in the serum of these patients (chapter 2.1). In addition, monocytes of T1D patients expressed higher MRP8/14 on their surface and secreted increased amounts of this protein when adhered to fibronectin, suggesting a positive feedback mechanism for MRP8/14 in monocyte adhesion to fibronectin in T1D (chapter 2.2).

- the migratory potential of monocytes of patients with T1D by using the Transwell system to study transmigration and the Boyden chamber to study chemotaxis. It was found that the transmigration as well as the chemotaxis to the pro-inflammatory chemokines CCL2 and CCL3 of the monocytes was reduced in T1D patients. In contrast, the migratory response towards the constitutively expressed CCL19 was increased in T1D monocytes (chapter 2.2).

- the apportioning between Ly-6C^{hi} immature and Ly-6C^{low} mature circulating monocytes in the NOD mouse model of T1D. Chapter 3.1 describes the increased frequency of Ly-6C^{low} mature monocytes and the preferred differentiation of both immature and mature circulating monocyte subpopulations into mφ over that of DC in the NOD mice.

- the in vivo migration of monocytes, macrophages and dendritic cells in response to inflammation using the artificially induced air pouch model. As described in chapter 3.2 the recruitment of NOD inflammatory cells is severely impaired in response to pro-inflammatory chemokines CCL2 and CCL3.

- the adhesion and migration of bone marrow-derived dendritic cells in vitro. NOD DC showed increased adhesion to fibronectin and decreased migration towards CCL19 that may play an important role in the early accumulation of dendritic cells in the pancreas of the NOD mouse (chapter 4.1). In addition the expression of CCL19 and CCL21 in the pancreas of NOD mice was found increased early in diabetes development. The pro-inflammatory chemokines CCL5 and CXCL10 were increased in the pancreas at a later stage of diabetes development and their expression correlated positively with the infiltration of lymphocytes into the pancreas, suggesting that the accumulation of mφ and DC is not the result of an expression of pro-inflammatory chemokines.

CHAPTER 2

ADHESION AND MIGRATION OF MONOCYTES IN HUMAN TYPE 1 DIABETES

CHAPTER 2.1

INCREASED SERUM LEVELS OF MRP8/14 IN TYPE 1 DIABETES INDUCE AN INCREASED EXPRESSION OF CD11b AND AN ENHANCED ADHESION OF CIRCULATING MONOCYTES TO FIBRONECTIN

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ABSTRACT

The recruitment of monocytes from the bloodstream is crucial in the accumulation of macrophages and dendritic cells in type 1 diabetic pancreases. Adhesion via integrins to endothelium and extracellular matrix proteins, such as fibronectin (FN), and the production of myeloid-related protein (MRP)8, -14, and -8/14 by recently transmigrated monocytes are thought to be instrumental in such recruitment. We determined the FN-adhesive capacity and integrin expression of monocytes of type 1 and type 2 diabetic patients and related them to the subjects' serum levels of MRP8, -14 and -8/14. Monocytes of type 1 diabetic patients displayed an increased adhesion to fibronectin in comparison with type 2 patients and healthy control subjects but had a normal expression of the FN binding integrins CD29, CD49a, CD49d, and CD49e (although CD11b and CD18 expression was increased). MRP8/14, which was increased in the sera of type 1 diabetic patients, induced healthy donor monocytes to adhere to FN and upregulate CD11b expression in a dosage-dependent manner. The observed MRP-induced increased adhesion of monocytes to FN and upregulation of CD11b most likely contributed to a facilitated accumulation of monocytes and monocyte-derived cells at the site of inflammation, in this case the pancreatic islets.

INTRODUCTION

Apart from lymphocytes, dendritic cells (DC) and various types of macrophages accumulate in the type 1 diabetes pancreas (1). In the NOD mouse and the BB-DP rat, animal models of the disease, DC and macrophages are among the first cells to accumulate in and around the islets (2–5), suggesting that these cells play a key role in the development of islet autoimmunity.

Various abnormalities have been described in the development and function of DC, macrophages, and monocytes (important precursors for DC and macrophages) in type 1 diabetes. In the NOD mouse, there is evidence that before insulitis onset, the development of DC and macrophages from bone marrow precursors is disturbed (6,7), resulting in cells with a poor antigen-presenting cell (APC) function (7,8), but an enhanced inflammatory phenotype (T. Nikolic and G.B., unpublished observations). DC have raised nuclear factor-κB (NF-κB) activity (9,10), and NOD macrophages produce higher quantities of proinflammatory compounds such as prostaglandin E_2 (11,12) and interleukin (IL)-12 (13). Also in the BB-DP rat, spleen DC are poor APCs (14), with macrophages exhibiting an elevated production of NO (15). Human studies have focused mainly on circulating monocytes. As in the animal models, cells show poor APC function and an enhanced proinflammatory cytokine production (16,17). There are, however, few studies on adhesion molecule expression and chemotactic ability of diabetic monocytes.

The recruitment of monocytes from the blood stream must play a crucial role in the accumulation of DC; macrophages in the pancreas and aberrances in this process may play an additional role in local abnormalities of the cells. Recruitment involves the expression of adhesion molecules on the vascular endothelium and activated monocytes, as well as the adhesion and transmigration of the monocytes (18,19). In the islets, monocytes will interact with extracellular matrix (ECM) proteins, such as fibronectin (FN), and will differentiate into macrophages and dendritic cells.

We first explored the adhesion of circulating monocytes of type 1 and type 2 diabetic patients to FN in the absence or presence of N-formyl-methionyl-leucyl-phenylalanine (fMLP), a cleavage product of bacterial and mitochondrial proteins that is generally used as a potent stimulatory agent for neutrophils and monocytes (20). After monocytes adhere to FN, a rearrangement of the cortical actomyosin cytoskeleton takes place, resulting in stretching and spreading of the monocytes on the FN-coated surface (21). We found an increased adhesion to and stretching on FN-coated surfaces of type 1 diabetes circulating monocytes in both the absence and presence of fMLP.

The increased adhesion and stretching of the total pool of circulating monocytes might be explained by a difference in the apportioning between different subsets of circulating monocytes. In humans, the circulating monocyte pool can be divided into $CD14^+CD16^-$ and $CD14^+CD16^+$ monocytes (22), which display distinct trafficking

and adhesion properties (23,24). Monocyte chemoattractant protein-1 upregulates CD11b expression on $CD14^+CD16^-$ monocytes, but not on $CD14^+CD16^+$ monocytes, as the latter are low in C-C chemokine receptor 2. This illustrates the different potential of the subpopulations to express adhesion molecules. It is notable that an expansion of the $CD14^+CD16^+$ monocyte subset has been observed in autoimmune rheumatoid arthritis and inflammatory diseases, such as HIV infection and sepsis (25–27). We therefore investigated whether the increased adhesion of the circulating monocyte pool of type 1 diabetic patients to FN could be ascribed to a difference in adhesion of the monocyte populations. We could not, however, detect any differences in the adhesion to FN of the two populations, nor could we find a difference in the size of these populations.

Myeloid-related protein (MRP)8 and -14 are calciumbinding proteins of the S100 family. These proteins form a heterodimeric complex in a calcium-dependent manner and are specifically expressed by recently transmigrated monocytes and granulocytes (28). MRP8- and MRP14-expressing cells dominate inflammatory reactions. Stimulation of monocytes with granulocyte-macrophage colonystimulating factor, IL-1β, or lipopolysaccharide induces the expression and secretion of the MRP8/14 heterodimer (29). When monocytes are stimulated with MRP14 or -8/14, a rapid increase in their CD11b surface expression is observed (30). Although the precise function of the MRPs is still unclear, they are thought to be involved in leukocyte-endothelium interactions and to play an important role in leukocyte trafficking (30,31).

We therefore also investigated the level of MRPs in the sera of type 1 and type 2 diabetic patients and explored whether an association exists between the serum levels of MRP proteins and the adhesion of circulating monocytes of type 1 and type 2 diabetic patients to FN. In addition, we studied the relation of the MRP levels in the serum with the surface expression of various adhesion molecules (CD11b, CD18, CD29, CD49a, CD49d, and CD49e) on monocytes of type 1 and type 2 diabetic patients. We showed that the enhanced adhesion of the type 1 diabetic circulating monocyte pool to FN is due to an increased level of MRP8/14 and that the increased serum levels of MRP8/14 in type 1 diabetic patients correlate with an increased CD11b expression on circulating monocytes.

RESEARCH DESIGN AND METHODS

Patients and controls

Heparinized blood was drawn from type 1 diabetic patients (30 men: average age 35.0 $\pm$ 3.1 years [$\pm$ SE], HbA_{1c} 8.5 $\pm$ 0.5%, duration of diabetes after initial diagnosis 15.0 $\pm$ 2.9 years; and 22 women: age 41.7 $\pm$ 2.5 years, HbA_{1c} 8.6 $\pm$ 0.4%, duration of diabetes after initial diagnosis 22.7 $\pm$ 3.2 years) and healthy control subjects matched for age and sex (12 men: age 38.8 $\pm$ 3.4 years; 9 women: age

33.8 ± 7.7 years). In addition, blood was drawn from type 2 diabetic patients as disease control subjects (8 men: age 52.9 ± 4.0 years, HbA_{1c} 7.8 ± 0.3%; and 14 women: age 56.9 ± 3.6 years, HbA_{1c} 8.0 ± 0.4%). Patients with obvious vascular complications and/or recent surgical interventions were excluded from this study.

Sera were collected and used for the detection of MRP8, -14, and -8/14. Monocytes were isolated using an adaptation of the protocol described by Fluks (32). Briefly, blood was diluted 1.5 times with PBS containing 0.1% BSA (Biowhittaker, Verviers, Belgium), layered on a Ficoll gradient (1.077 g/ml; Pharmacia, Uppsala, Sweden), and centrifuged (1000g, 15 min, room temperature). The cells at the interface were collected, washed, layered on a Percoll gradient (1.063 g/ml; Pharmacia), and centrifuged (400g, 40 min, room temperature). The monocytes were collected from the interface and washed to a final purity of 85–95%.

The Medical Ethics Committee of the Erasmus Medical Center (Rotterdam) approved this study on collected blood monocytes. All subjects gave their written informed consent.

Adhesion and stretching of patient and control monocytes

Monocytes were suspended in RPMI 1640 containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (Biowhittaker), plated on Chambertek glass slides (0.1 x 105 cells/chamber; Nalge Nunc, Naperville, IL), and coated with 10 μg/ml fibronectin (Sigma, Steinheim, Germany). After a 15-min incubation at 37°C in the absence or presence of the stimulatory agent fMLP, the cells were washed with PBS and fixed with 4% paraformaldehyde (Sigma) supplemented with 3% glucose. The cells were permeabilized using 0.5% Triton X-100 (Sigma) and stained with 0.1 μg/ml phalloidin-fluorescein isothiocyanate (Sigma) for 30–45 min. After cells were washed and mounted on slides, adhesion was determined as the number of cells in 10 high-power fields using a fluorescence microscope at 200x magnification. Stretching was recorded as the percentage of cells with a stretched morphology (Fig. 1C). Two individuals counted at least 200 cells independently.

Integrin expression and cell sorting

The expression of CD11b, CD18, CD29, CD49a, CD49d, and CD49e on $CD14^+$ monocytes in heparinized blood was determined using flow cytometry on a FACSscan (Becton Dickinson, San Jose, CA) and CellQuest software (BD Pharmingen). Whole blood samples were stained with the appropriate antibodies (BD Pharmingen, Alphen aan den Rijn, Netherlands), and then washed with PBS containing 0.1% BSA; the erythrocytes were lysed using BD lysing buffer (BD Pharmingen) for 10 min at room temperature. Samples were subsequently washed and analyzed.

For cell sorting, monocytes were labeled with CD14 and CD16 antibodies (BD Pharmingen). After sorting (FACS Vantage; Becton Dickinson, Amsterdam, Netherlands), the purity of the cell suspensions was >95%.

Myeloid-related protein detection

Levels of MRP8, -14, and -8/14 were measured in serum by a commercially available enzyme-linked immunosorbent assay (ELISA; Bachem, Heidelberg, Germany) according to the manufacturer's protocol.

Adhesion assay in the presence of subject or control serum

Monocytes were obtained from a buffy coat of a healthy control donor (Sanquin Blood Bank, Rotterdam, Netherlands) using Ficoll and Percoll gradients. Donor monocytes were exposed for 4 h at 37°C to dilutions of sera of either type 1 diabetic patients or healthy control subjects. After being exposed, cells were washed and allowed to adhere to FN-coated 96-well plates for 60 min at 37°C (note that this assay differs from the above-described adhesion assay in which adhesion was determined by individual cell counting). Cells were fixed using 20% formaldehyde (Sigma) and stained with 1% methylene blue (BDH Chemicals, Poole, England). Staining intensity was determined by measuring absorption at a wavelength of 650 nm using an ELISA reader (Thermo Lab Systems, Amersfoort, Netherlands) and a rate of cell adhesion. The assay was validated by comparing the staining intensities to direct cell counts (data not shown). In some cases, serum was depleted of MRP8/14 by immunoprecipitation with an antibody against MRP8/14 (Bachem); an unrelated monoclonal antibody with the same isotype was used as the control (Santa Cruz, Heerhugowaard, Netherlands). Sera were incubated with the antibodies for 60 min at 4°C and then protein A sepharose (Pharmacia) for 60 min at 4°C, and then centrifuged briefly at 12,000g. The supernatant was used to expose the cells, as described above.

Statistical analyses

Data were analyzed using one-way ANOVA with Bonferroni correction, unpaired Student's t test, or Mann-Whitney U test. Correlation was examined using Spearman's rho test. All data were tested for two-tailed significance. $P < 0.05$ was considered to be statistically significant.

RESULTS

Adhesion, stretching, and spreading of monocytes

Monocytes of type 1 and type 2 diabetic patients and healthy control subjects were isolated from the blood and allowed to adhere to an FN-coated surface in the absence or presence of fMLP. Adhesion was determined at several time points (5–120 min); monocytes reached maximal adhesion at 60–120 min. As shown in Fig. 1A and B, monocytes of type 1 diabetic patients displayed an increased adhesion to FN in comparison with monocytes of healthy control subjects at all time points studied and in the absence and presence of fMLP (although stimulation of

the monocytes with fMLP resulted in an increased adhesion to FN). Because the monocytes showed an increased adhesion at all time points studied, we concluded that the monocytes of type 1 diabetic patients display a higher, rather than a faster, adhesive capacity. Monocytes of type 2 diabetic patients were studied as disease

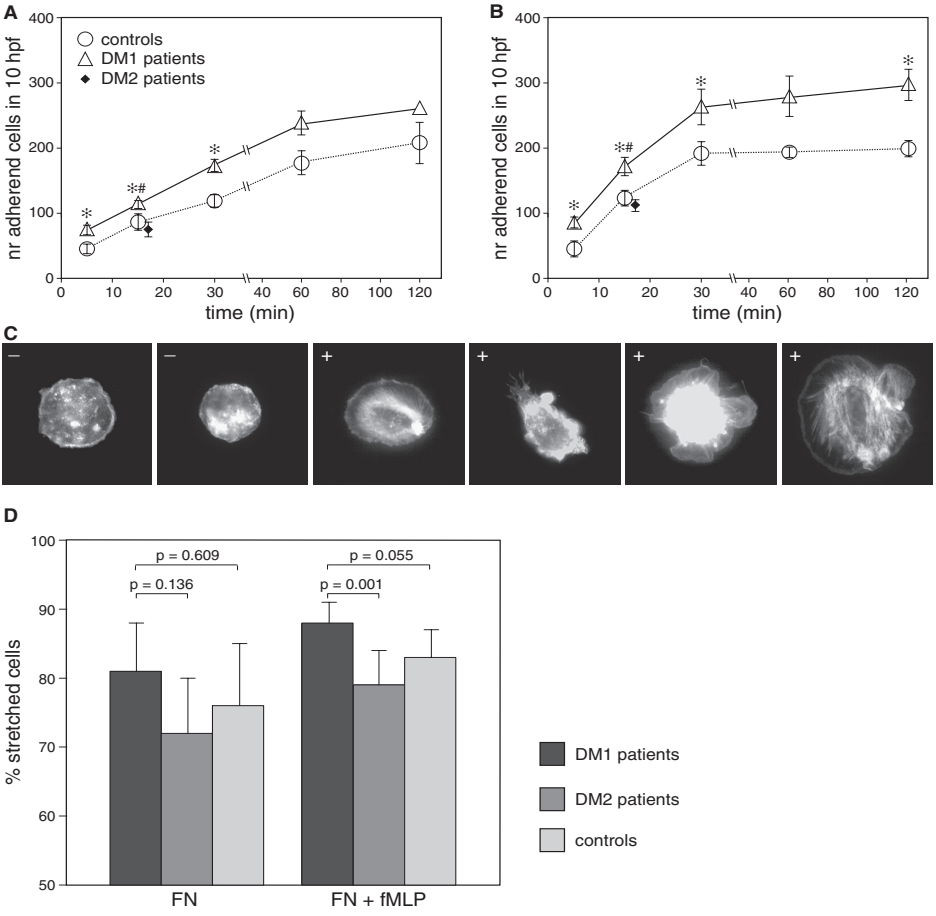

Figure 1.
Time-dependent adhesion of monocytes to fibronectin (FN) in the absence (A) and presence (B) of fMLP. At 15 min, data represent means ± SE of 10 type 1 patients (DM1), 6 type 2 diabetic patients (DM2) and 8 healthy control subjects; at the other time points, *n* = 3 (Students T-test). For 15 min data, one-way ANOVA with Bonferroni correction was used, **P* < 0.05 to controls, #*P* < 0.05 to type 2 patients). C: Examples of spreading and stretching (-, non-stretched; +, stretched or spread). The percentage of spread and stretched cells in the different test groups at 15 min is given in D (means ± SE, same *n* as in A and B, one-way ANOVA with Bonferroni correction).

controls at time point 15 min only; they had adhesion properties equal to those of healthy control monocytes (Fig. 1A and B).

After monocytes adhere to FN, a rearrangement of the cortical actomyosin cytoskeleton takes place, leading to a stretching of the cells. Figure 1C shows representative stretched monocytes to illustrate the changes in shape encountered. After 15 min of incubation, more type 1 diabetic monocytes displayed a stretched morphology than type 2 diabetic and healthy control monocytes, in both the absence and the presence of fMLP (Fig. 1D).

$CD14^+CD16^+$ and $CD14^+CD16^-$ monocytes and adhesion to FN

To study whether a putative shift in the apportioning between the $CD14^+CD16^+$ and $CD14^+CD16^-$ monocyte populations would be responsible for the above-described differences in FN adhesion of the total monocyte pool, the two monocyte populations were sorted (Fig. 2A) and their adhesion to an FN-coated surface studied. Differences could not be detected in the adhesion to FN between the two

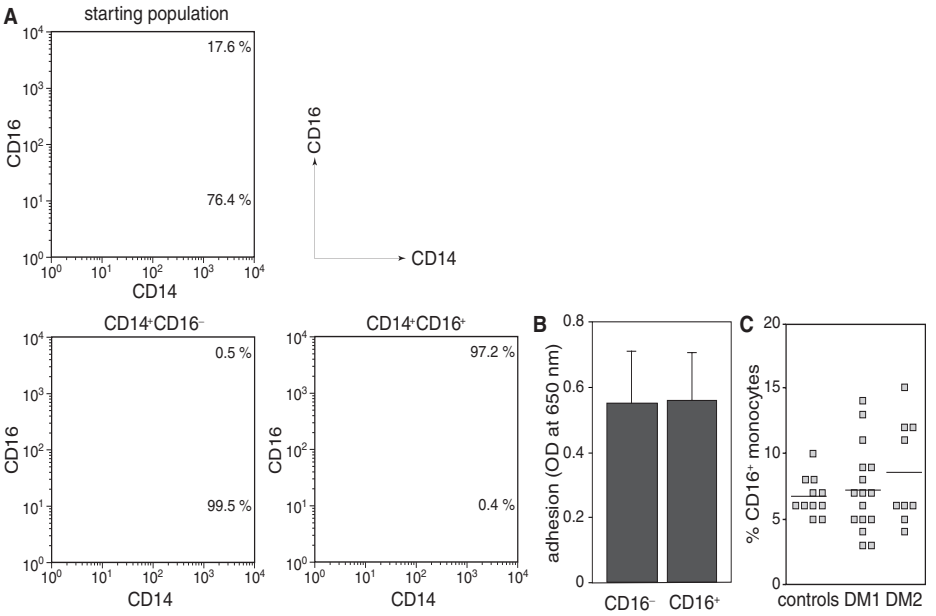

Figure 2.
$CD14^+CD16^+$ and $CD14^+CD16^-$ monocyte subpopulations were sorted and purities >95% were reached. A: Representative flow cyometric analyses of starting and sorted populations. B: Adhesion to FN of both subsets was determined; differences were not observed (means ± SD, *n* = 3). C: Differences were also not observed among type 1 diabetic patients, type 2 diabetic patients and healthy control subjects regarding the percentage of the $CD14^+CD16^+$ monocyte subpopulation in the peripheral blood.

populations (Fig. 2B). We also determined the percentages of both populations in the peripheral blood of type 1 and type 2 diabetic patients and healthy control subjects. Differences in the percentages of the $CD14^{+}CD16^{+}$ monocyte populations in relation to the total monocyte population also could not be detected (Fig. 2C).

Serum levels of MRP8, -8/14, and -14

To study the role of MRP8/14 and -14 in the increased adhesion of monocytes of type 1 diabetic patients to FN, we determined the serum levels of MRP8, -14, and -8/14. As shown in Fig. 3A–C, the serum levels of MRP14 and -8/14 were significantly higher in type 1 diabetic patients compared with the levels found in healthy control subjects (MRP8/14 was the most significant). MRP8 levels were normal in type 1 diabetic patients. A close and positive correlation was found between the levels of

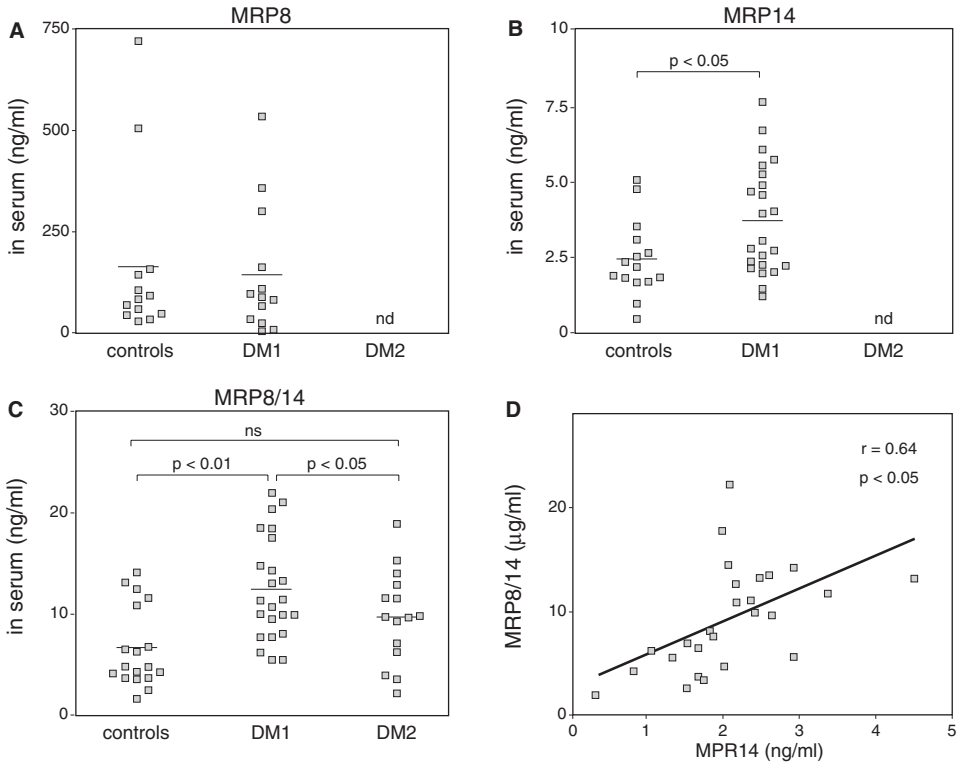

Figure 3.
Serum levels of MRP-8 of 13 control subjects and 13 type 1 patients (A; Student's t test), MRP-14 of 15 control subjects and 23 type 1 patients (B; Student's t test), and MRP-8/14 in 18 control subjects, 23 type 1 patients, and 15 type 2 patients (C: one-way ANOVA). nd, not determined. A positive correlation (Spearman's rho) was observed between MRP-14 and MRP-8/14 in the serum (D).

MRP14 and -8/14 (r = 0.64, P < 0.01) (Fig. 3D). Neither a correlation between the serum levels of both MRPs and HbA_{1c}, nor a correlation between serum levels of MRPs and patients' age or duration of type 1 diabetes could be found (data not shown). In type 2 diabetic patients, only the level of MRP8/14 was determined and was found to be normal (Fig. 3C).

Serum MRP-8/14 levels in relation to monocyte adhesion to FN

To investigate whether the increase in MRP8/14 in the serum of type 1 diabetic patients could play a direct role in the adhesion of monocytes to FN, monocytes of a healthy control donor were exposed to sera of type 1 diabetic patients or that of healthy control subjects. Figure 4 shows that a higher induction of adhesion to FN was observed using the sera of type 1 diabetic patients in comparison with sera of healthy control subjects. This higher induction of adhesion declined with the dilution of the serum (Fig. 4A).

To demonstrate a specific role for MRP8/14 in the induction of adhesion, sera of type 1 diabetic patients and control subjects were depleted of MRP8/14 using a specific monoclonal antibody. Depletion of the sera was confirmed using immunoblotting (data not shown). After exposing the monocytes of a healthy control donor to the depleted sera, it was found that the adhesion-inducing capacity of the

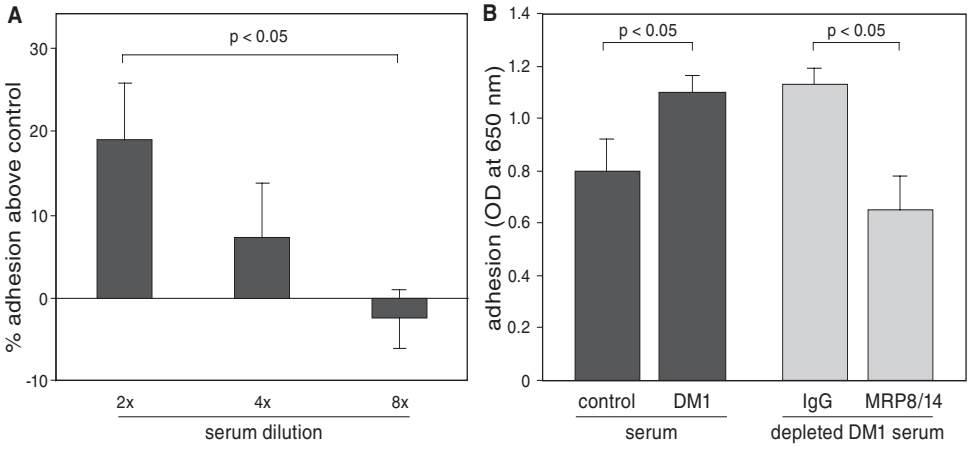

Figure 4.
A: Adhesion of healthy donor monocytes to FN after exposure to dilutions of serum samples of type 1 diabetic patients (tests were performed with 11 sera). The adhesion is expressed as a value relative to values of healthy control sera (*n* = 10, percentage above these controls). B: Increased adhesion of monocytes to FN by the serum samples of type 1 diabetes patients (■, means ± SD, *n* = 3) and the effect of serum depletion of MRP8/14 by monoclonal antibodies (□, means ± SD, *n* = 3). The experiment was carried out three times, always showing the same phenomenon by MRP8/14 depletion. Statistics were performed using Mann-Whitney test.

sera declined. With an irrelevant isotype control antibody, such an effect was not observed (Fig. 4B).

Serum MRP8/14 levels and integrin expression on monocytes

To investigate if the high serum MRP8/14 levels of type 1 diabetic patients could influence the expression of integrins, monocytes of healthy donors were exposed to sera that were depleted of MRP8/14 or depleted using an irrelevant isotype control antibody. Figure 5A shows that the presence of MRP8/14 in sera specifically upregulated the CD11b expression on healthy donor monocytes. The expression of CD18 was not influenced (Fig. 5B).

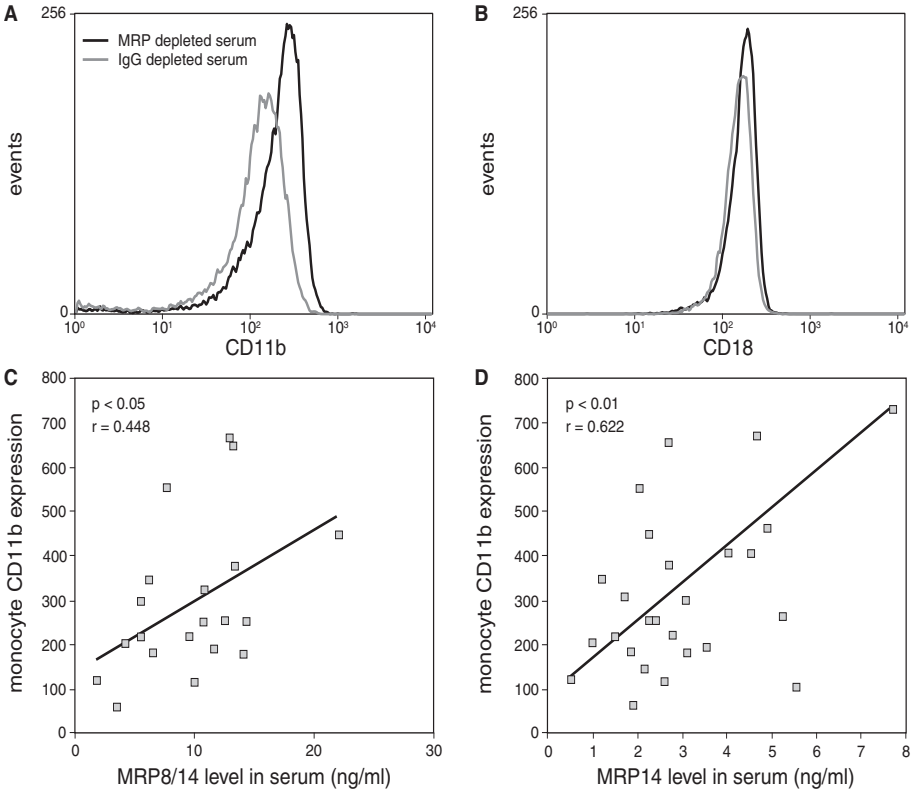

Figure 5.
A and B: Flow cytometric analysis of the effect of MRP8/14 in the serum of a type 1 diabetic patient on the surface expression of CD11b and CD118. The experiment was performed four times, always showing the same results by MRP8/14. C and D: CD11b surface expression on monocytes of type 1diabetic patients and healthy control subjects in correlation (Spearman's rho) to the serum level of MRP8/14 and -14.

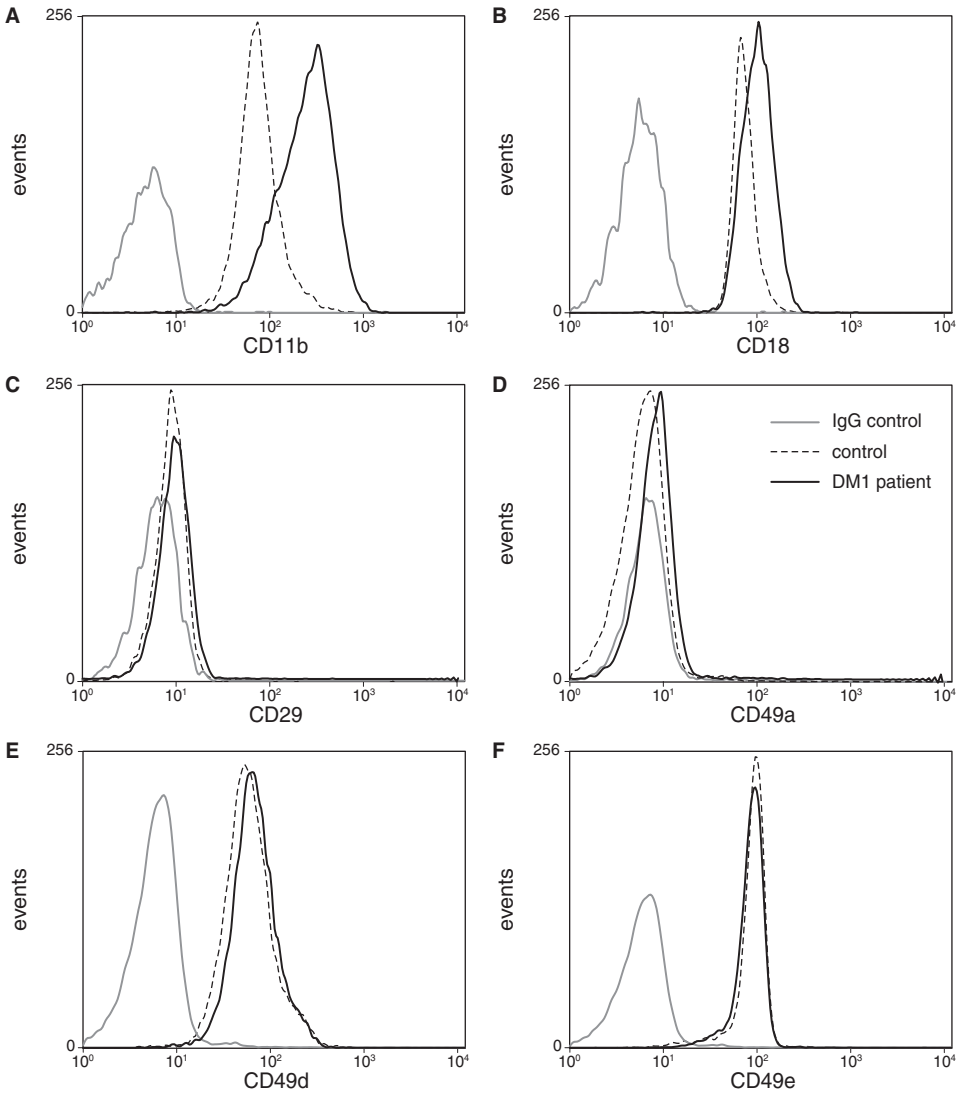

Figure 6.
A representative flow cytometric analysis of the surface expression of CD11b (A), CD18 (B), CD29 (C), CD49a (D), CD49d (E), and CD49e (F) on monocytes of a type 1 diabetic patient and an age- and sex-matched healthy control subject.

In addition, ex vivo monocytes of type 1 diabetic patients were studied to investigate whether an upregulation of CD11b could indeed be detected. Figure 6A and Table 1 show that monocytes of type 1 diabetic patients displayed a higher

expression level of CD11b. Furthermore, a positive and significant correlation was found between the serum levels of MRP8/14 ($r = 0.448$, $P < 0.05$) (Fig. 5C) and the CD11b expression on the monocytes. Such a positive correlation was also present between the MRP14 level and the CD11b expression ($r = 0.622$, $P < 0.01$) (Fig. 5D). However, it must be noted that the expression of CD18 was also (albeit just significantly) raised on ex vivo monocytes of type 1 diabetic patients as compared with those of healthy control subjects (Fig. 6B and Table 1). Moreover, monocytes of type 2 diabetic patients showed a higher expression level of the CD11b and CD18 integrins (CD11b: 569 ± 278 vs. 152 ± 78; CD18: 154 ± 59 vs. 76 ± 31).

With regard to the expression levels of the CD49d, CD49e, and CD29 integrins (combined forming the FN-binding adhesion molecules very late antigen [VLA]-4 and VLA-5), a statistically significant difference between monocytes of type 1 diabetic patients (Fig. 6C–F and Table 1), type 2 diabetic patients (data not shown), and healthy control monocytes could not be found.

Table 1. Integrin expression on circulating monocytes.

Integrin		fold expression#	MFI type 1 diabetic patients	MFI controls	n	p§
CD11b	α_M	3.24 ± 0.52	429.44 ± 93.09	218.11 ± 59.93	20	0.002
CD18	β_2	1.65 ± 0.13	122.93 ± 19.03	86.19 ± 11.45	20	0.046
CD29	β_1	1.10 ± 0.03	16.64 ± 2.11	16.03 ± 1.77	20	0.930
CD49a	α_1	1.16 ± 0.06	7.32 ± 0.51	7.19 ± 0.54	17	0.855
CD49d	α_4	1.25 ± 0.08	64.27 ± 5.25	57.93 ± 4.56	21	0.273
CD49e	α_5	1.05 ± 0.05	77.42 ± 4.29	79.2 ± 4.47	21	0.599

Data are means ± SE. MFI, mean fluorescence intensity. * Mean fold expression relative to healthy control subjects, §Statistical significance tested using the Mann-Whitney test.

DISCUSSION

In inflammatory conditions, circulating monocytes change from a quiescent, nonadhesive state into an adhesive state characterized by an increased expression of adhesion molecules and an increase in functional adhesion (18,19). This activation is associated with a higher secretion of MRP8, -14, and -8/14, which contributes to a further adhesion of the monocytes to the endothelium and a transmigration into the tissue (30). Contact of transmigrated monocytes with the ECM induces a further expression of MRP8/14 (33). MRP8/14 is bound by the vascular endothelium (28,34), and secretion of MRP8/14 by monocytes is inhibited by resting endothelial cells (35).

In this study we demonstrated that increased levels of MRP8/14 and -14 are present in the sera of type 1 diabetic patients and are specific for this subtype of diabetes (type 2 diabetic patients had normal levels). An effect of the hyperglycemic

state can be ruled out, as HbA_{1c} levels did not influence serum MRP8/14. High serum levels of MRP8/14 and -14 are also present in rheumatoid arthritis, in which the serum level of MRP8/14 correlates with the presence of MRP8/14 in the synovial fluid and with disease activity (35). Our data thus indicate that MRP8/14 levels in the peripheral blood of type 1 diabetic patients may be used as a marker for the activity of the islet inflammatory response.

Proinflammatory-activated monocytes in the circulation attach to the endothelium via an interaction between adhesion molecules like Mac-1 (the heterodimer of CD11b and CD18) and leukocyte function antigen-1 (LFA-1) (CD11a and CD18) and the endothelial adhesion molecules intracellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 (36). This interaction is the first in a sequence of events resulting in the transmigration of monocytes into the tissue (37). Blocking LFA-1 and Mac-1 on leukocytes and ICAM-1 on endothelial cells in NOD mice prevents the onset of diabetes (38–41), indicating the important role of these adhesion molecules in the development of diabetes. When monocytes have transmigrated through the endothelium, they encounter the ECM, of which FN is a major component. The monocytes attach to the ECM via integrins and a complex conjunctional action between their integrins and components of the ECM enables them to adhere, change shape, migrate, and differentiate into macrophages and DCs (42).

We have shown that circulating monocytes of type 1 diabetic patients display increased CD11b and CD18 surface expression, adhesion to FN-coated surfaces, and stretching on such surfaces. The latter observation suggests increased activity of the actomyosin cytoskeleton under circumstances of FN adhesion. Previously we reported an aberrant cytoskeletal function of FN-adherent monocytes of autoimmune thyroiditis patients when the cells are chemoattracted (43). Hence there is increasing evidence that integrin expression and function are abnormally regulated in “autoimmune” monocytes. In this study, the FN adhesion and spreading of the type 1 diabetic monocytes did not appear to be due to a difference in the size of the two important subsets in the circulating monocyte pool (i.e., the $CD14^+CD16^-$ and $CD14^+CD16^+$ monocyte subsets). Our data showed that these aberrances were most likely due to the raised serum MRP8/14 levels, because serum of type 1 diabetic patients was able to induce an increased adhesion of healthy donor monocytes to FN-coated surfaces and because this activity could be blocked by specific antibodies to MRP8/14. Although in our experiments only MRP8/14 was depleted from the serum, MRP14 might also contribute to the increased adhesion, as exposure of monocytes to MRP14 has been shown to increase the expression of adhesion molecules (i.e., CD11b) (30) and stimulation of neutrophils with MRP14 has been shown to increase both their adhesion to fibrinogen and their CD11b expression (44).

The increased adhesion of monocytes of type 1 diabetic patients to FN could be explained by a change in the affinity of the FN-specific VLA-4 and -5 integrins, given

that we did not observe an increase in the molecular expression levels of these integrins. Such an increase in affinity has been shown for VLA-4, of which a pool of low-affinity integrins is present that can change into a high-affinity conformation upon activation, thus mediating adhesion without changing the overall surface expression (45). Determining the surface expression of the activated forms of VLA-4 and VLA-5 using specific antibodies for such activated forms could provide support this idea and should be pursued.

An increased adhesion of type 1 diabetic monocytes to FN has been previously reported, but was restricted to monocytes of patients with poorly regulated type 1 diabetes and severe vascular complications (46). None of our patients had poorly regulated diabetes or had obvious signs of vascular complications. Moreover, monocytes of type 2 diabetic patients (with similar HbA_{1c} levels and a similar tendency to vascular complications) did not display an increased adhesion to FN.

It is interesting that the serum MRP8/14 of type 1 diabetic patients was able to not only increase the adhesion of healthy donor monocytes to FN, but also increase the expression level of CD11b on these monocytes. In accordance with such induction, we were able to detect an increase in CD11b expression on monocytes of type 1 diabetic patients. However, we also found the CD18 expression on such monocytes raised, whereas MRP8/14 in sera of type 1 diabetic patients was not able to enhance the expression of this integrin. Moreover, on monocytes of type 2 diabetic patients, the CD11b and CD18 surface expression was increased (and in this study in the absence of increased serum MRP8/14 levels). This showed that the increased CD11b and CD18 surface expression on monocytes of type 1 and type 2 diabetic patients cannot be due solely to MRPs and must at least be partially explained by consequences of the disease itself, most likely poor glycemic control.

An increased expression of Mac-1 (CD11b and CD18) on monocytes of type 1 diabetic patients has been previously reported (46). There has also been, however, a reported decreased expression of CD18 on isolated monocytes in type 1 diabetic patients (47). In this latter study, monocytes were tested in isolation by density gradient centrifugation. We tested monocytes in “whole blood” samples after lysis of the erythrocytes, thus reducing stimulation of cells by the isolation procedure to a minimum. Monocytes release Mac-1 from preexisting vesicles after stimulation, which results in a rapid increase in surface expression (48). Therefore, isolation procedures may influence expression levels of Mac-1 on monocytes.

If the level of MRP8/14 is indicative of the activity of the islet inflammatory response, it is of note that the type 1 diabetic patients used in this study had long-standing diabetes (>10 years). In most of these patients, the pancreatic inflammation must have subsided by the time of our study. Could it be that intrinsic (inborn) abnormalities in the monocytes of type 1 diabetic patients or in their endothelial cells force the cells to an intrinsic high production of MRPs and other proinflammatory substances? Such “intrinsic” abnormal “proinflammatory”

monocytes have indeed been detected in prediabetes (49) and in animal models of type 1 diabetes before islet infiltration (6–9). The determination of MRP production by monocytes of islet antibody-positive nondiabetic individuals and patients with established prediabetes needs to be done to answer this question (as would the simple determination of MRP8/14 in the sera of such individuals).

In conclusion, our results showed raised levels of MRP8/14 and -14 in the sera of type 1 diabetic patients. These factors are responsible for an increased adhesion of circulating monocytes of type 1 diabetic patients to FN and are at least partially responsible for an increased expression of CD11b on such monocytes. Such MRP-induced increased adhesion most likely contributes to a facilitated accumulation of monocytes and monocyte-derived cells at the site of inflammation, in this case, the pancreatic islets.

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CHAPTER 2.2

AN INCREASED MRP8/14 EXPRESSION AND ADHESION, BUT A DECREASED MIGRATION TOWARDS PRO-INFLAMMATORY CHEMOKINES OF TYPE 1 DIABETES MONOCYTES

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Submitted

ABSTRACT

In the early development of type 1 diabetes (DM1) and prior to the lymphocytic infiltration macrophages and dendritic cells accumulate close and around islets at sites of fibronectin (FN) expression (peri-islet edge and vaso-ductular pole). It is thought that these macrophages and dendritic cells are recruited from blood monocytes. Previously, we showed an increased level of MRP8/14 in the serum of DM1 patients. This serum MRP8/14 induced healthy monocytes to adhere more strongly to FN. Here we extended these studies and show that MRP8/14 is expressed and produced at a higher level by DM1 monocytes, particularly after adhesion to FN, creating a positive feedback mechanism for a high FN-adhesive capacity of DM1 monocytes. Not only adhesion to FN, but also adhesion to endothelial cells was increased in DM1 monocytes. Despite the increased adhesion to endothelium transendothelial migration (TEM) of monocytes of DM1 patients (as well as classical chemotaxis as measured in the Boyden chamber) was decreased towards the pro-inflammatory chemokines CCL2 and CCL3. Since NOD mouse monocytes show a similar defective in vivo and in vitro migration towards pro-inflammatory chemokines, we argue that an impaired monocyte migration towards pro-inflammatory chemokines might be a hallmark of autoimmune diabetes. This hampered monocyte response to pro-inflammatory chemokines questions whether the early macrophage and dendritic cell accumulation in the diabetic pancreas originates from an inflammatory-driven influx of monocytes. We here also show that the migration of DM1 monocytes towards the lymphoid tissue-related CCL19 was increased and correlated with an increased CCR7 surface expression on the monocytes. Since NOD mice show a high expression of these chemokines in the early pancreas it is more likely that the early macrophage and dendritic cell accumulation in the diabetic pancreas originates from an influx of monocytes due to an aberrant high expression of lymphoid-tissue related chemokines in the pancreas.

INTRODUCTION

Macrophages (mφ) and dendritic cells (DC) play an important role in the development of type 1 diabetes (DM1). Prior to the infiltration of autoreactive T cells around and into the islets a peri-islet accumulation of mφ and DC can be detected in animal models of DM1 (1-3). These mφ and DC act as antigen presenting cells and pick up auto-antigens to subsequently migrate to the draining lymph nodes where the cells present the auto-antigens to naïve T cells and to B cells. Later in the process mφ and DC are seen to infiltrate the islets together with the T cells, and particularly the mφ are capable to assist the T cells in the destruction of the β cells (1, 2).

Monocytes form an important precursor population for mφ and DC. Previously we published on a raised serum level of the myeloid related protein (MRP) 8/14 in the serum of DM1 patients (4). MRP8/14 is produced by recently transmigrated monocytes. We also showed that the serum-borne MRP8/14 was capable of inducing monocytes of healthy individuals to adhere more to the extra-cellular matrix (ECM) component fibronectin (FN). FN is abundantly present at the vaso-ductular poles of the islets of Langerhans (5). Here we extend these former studies and show that circulating monocytes of DM1 patients express higher surface levels and produce more of MRP8/14, particularly after FN adhesion. The cells also showed an increased adhesion to human endothelium.

Besides adhesion to endothelium and ECM components migration plays an important role in the accumulation of monocyte-derived cells at specific tissue sites. Chemokines direct such migration. Chemokines form a large superfamily of small (8-10 kDa) secreted proteins (6, 7). About 40-50 members are distributed into four structural families according to the relative positions of cysteine residues. In general, chemokines are functionally divided into inflammatory and constitutive chemokines, although several of the chemokines show a dual function (6, 7). Chemokines exert their biological functions through interaction with seven-transmembrane G protein-coupled specific receptors that are differentially expressed on leukocyte populations (8). Here we also show that FN-adhered monocytes of DM1 patients show an increased production of the pro-inflammatory chemokines CCL2 and CCL3 yet have a poor migratory response to these chemokines. Interestingly the chemotactic response of the monocytes to CCL19, a chemokine constitutively expressed in lymphoid tissue, was enhanced.

MATERIALS AND METHODS

Patients and controls

Heparinized blood was drawn from patients with type 1 diabetes (DM1 patients) and age and gender matched healthy control subjects. In addition, blood was drawn

from type 2 diabetic patients (DM2 patients) as disease controls. Patients with obvious vascular complications and/or recent surgical interventions were excluded from this study. Patients characteristics are summarized in table 1. Monocytes were isolated via Ficoll density (Pharmacia, Uppsala, Sweden; density 1.077 g/ml) gradient centrifugation, followed by Percoll density (Pharmacia; density 1.063 g/ml) gradient centrifugation and a final purity of 85-95% was reached. This study was approved by the ethics committee of the Erasmus MC, University Medical Center in Rotterdam and all subjects gave their written informed consent.

Chemotaxis

The in vitro migration towards CCL2, CCL3, CCL4, CCL19, CXCL12 (all from PeproTech, Rocky Hill NJ, USA) and fMLP of monocytes was evaluated using a Boyden chemotaxis chamber (Neuroprobe, Gaithersburg MD, USA) and polycarbonate membranes (5 μm pore size; Whatman, Clifton NJ, USA) as previously described (9). Monocyte (1.5×10^6/ml) migration was determined after 90 min and expressed as a migration index (chemokine-migrated cells divided by the medium-migrated cells). Each experiment was performed in triplicate and cells were counted in five high power fields (1000X magnification).

Adhesion

Human umbilical vein endothelial cells (HUVEC; kindly provided by dr. W. Sluiter) were grown to confluence in a flat-bottomed 96 wells plate in Ham's F-12, supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin (Cambrex, Verviers, Belgium), 20% fetal bovine serum, 50 μg/ml endothelial cell growth supplement (ECGS: BD Pharmingen, Alphen aan den Rijn, The Netherlands) and 100 μg/ml heparin (Sigma, St. Louis MO, USA). Monocytes were labeled with $Na_2{}^{51}CrO_4$ (Amersham BioSciences, Uppsala, Sweden) and co-incubated with the endothelial monolayer for 60 min at 37 °C. Non-adherent cells were washed away and adherent cells were lysed with 0.1 ml of 1% SDS, 0.05% NaOH. Radioactivity of non-adherent and adherent cells was measured and results are expressed as percentage of adherent cells.

Transmigration

HUVEC were grown to confluence on Transwell filters (5-μm pore; Costar, Acton MA, USA) that were pre-coated with 10 μg/ml fibronectin (Sigma) and 24 hrs stimulated with or without IL-1β (R&D Systems, Minneapolis MN, USA). In the lower compartment, the chemokines CCL2, CCL3 (both of PeproTech) or medium were added. In the upper compartment, a total of $1x10^5$ ^{51}Cr-labeled monocytes was seeded and coincubated with HUVEC monolayers for 1 hour at 37°C. The upper compartment was gently washed and together with the filter and the lower compartment measured for radioactivity. Transmigration was calculated as the radioactivity measured in the lower compartment and the downside of the filter,

Table 1. Patients' characteristics.

	DM1 patients				healthy control subjects				DM2 patients			
	male		female		male		female		male		female	
age	41.7 ± 2.5		35.0 ± 3.1		38.8 ± 3.4		41.0 ± 4.6		52.9 ± 4.0		56.9 ± 3.6	
HbA1c[#]	8.59 ± 0.4		8.45 ± 0.5		nd		nd		7.5 ± 0.3		8.0 ± 0.4	
	n	%	n	%	n	%	n	%	n	%	n	%
DR2	0	(0%)	1	(8%)	4	(36%)	5	(38%)	nd	-	nd	-
DR3/DR4[¶]	18	(82%)	10	(77%)	6	(55%)	6	(46%)	nd	-	nd	-
other DR	4	(18%)	2	(15%)	1	(9%)	2	(9%)	nd	-	nd	-
nd[$]	8		9		1		1		8		14	
total n	30		22		12		14		8		14	

[#] % in serum, [¶] individuals with HLA-DR3 and/or HLA-DR4 genotype, [$] not determined

divided by the total radioactivity. Transmigration is shown relatively to the migration towards medium.

Flow cytometry

The expression of CCR1, CCR2, CCR5, CCR6, CCR7, CXCR4 and MRP8/14 on monocytes, determined as $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{+}$ cells, within a PBMC fraction obtained after Ficoll density gradient purification was determined by flow cytometric analysis using a FACSCalibur (Becton Dickinson, San Jose, CA, USA) and CellQuest software (BD Pharmingen). The antibodies directed against CCR1, CCR2, CCR6, CCR7 and CXCR4 were obtained from R&D systems (R&D systems), CCR5, CD14 and CD16 from BD Pharmingen and MRP8/14 from BMA (BMA Biomedicals, Augst, Switzerland).

Calcium mobilisation

The mobilisation of intracellular calcium upon receptor activation was determined by flow cytometric measurement of calcium-bound Indo1-AM (Molecular Probes, Eugene OR, USA). Briefly, PBMCs were labelled with Indo1-AM in calcium-free medium, followed by washing and incubation with APC-labelled CD14 and PE-labelled CD16 (BD Pharmingen) in calcium-containing medium. After equilibrating to 37 °C, the cells were analysed on a FACSDiva (Becton Dickinson) and during measurement different stimuli added.

ELISA

The production of MRP8/14 (Bachem, Heidelberg, Germany), CCL2, CCL3 and CCL19 (R&D Systems) by monocytes (1×10^5) on fibronectin or unstimulated was measured in the supernatant after 24 hrs by commercially available ELISA according to the manufacturers protocol.

HLA-DRB1 typing

Genomic DNA was extracted from PBMC using a commercially available kit (QIAamp DNA Blood isolation kit; Qiagen, Hilden, Germany). Subsequently, HLA-DRB1 typing was performed at the two-digit level using a commercially available typing system in which the exon 2 of the HLA-DRB1 gene is amplified and analysed with allele-specific probes in a line probe assay (INNO-LiPA, Innogenetics, Ghent, Belgium), as described previously (10).

Statistical analyses

Data were analyzed using Student's t test. All data were tested for two-tailed significance. A p-value below 0.05 was considered to be statistically significant.

RESULTS

MRP8/14 expression and adhesion to fibronectin

We studied the surface expression of MRP8/14 on circulating monocytes in DM1. As shown in figure 1A, the expression of MRP8/14 on monocytes was slightly, but significantly, increased in DM1 patients in comparison to healthy controls and DM2 patients. We also determined the production of MRP8/14 by monocytes of DM1 patients and healthy control subjects. As shown in figure 1B, monocytes of DM1 patients produced increased amounts of MRP8/14 in comparison to healthy control

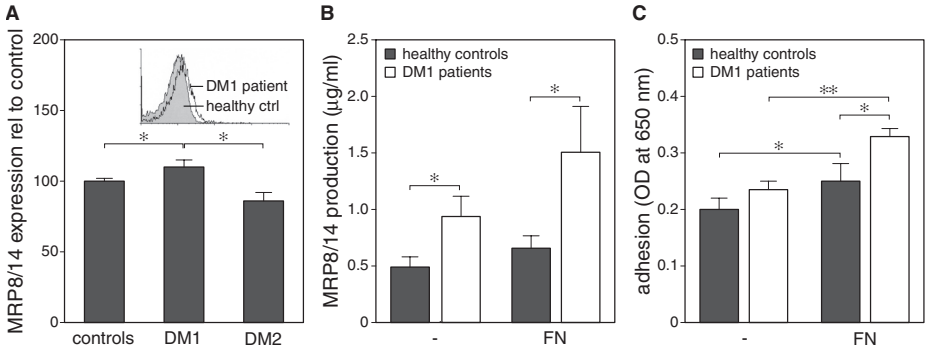

Figure 1. Adhesion and MRP8/14 expression of monocytes in DM1.
(A) Monocytes of DM1 patients (n=12) showed increased surface expression of MRP8/14 compared to healthy controls (n=13) and DM2 patients (n=3). The MRP8/14 expression is shown relative to that observed in healthy control subjects. (B) The production of MRP8/14 by monocytes after 24 hrs was increased in DM1 patients (n=9) in comparison to healthy controls (n=9), both with and without FN stimulation. (C) Monocytes of DM1 patients showed increased adhesion to FN in comparison to monocytes of healthy control subjects. Adhesion after 24 hrs is shown of nine DM1 patients and nine healthy control subjects. Data are presented as average ± SEM, * $p<0.05$ as determined with unpaired two-tailed Student's t test.

monocytes. MRP8/14 induces monocytes to adhere to fibronectin (FN) (4,11) and monocytes of DM1 patients indeed show an increased adhesion to FN (figure 1C and (4, 11)). When the monocytes were allowed to adhere to FN, a stimulus that is known to induce MRP8/14 monocyte surface expression (12), the monocytes of DM1 patients showed an increased MRP8/14 production (figure 1B) as compared to healthy control monocytes.

Production or pro-inflammatory CCL2 and CCL3

Apart from MRP8/14, a molecule instrumental in the adhesion of monocytes to endothelium and extracellular matrix components, we also studied the production of the pro-inflammatory chemokines CCL2 (MCP-1) and CCL3 (MIP-1α) by DM1 monocytes, since these chemokines are thought to play an important role in the inflammatory accumulation of monocytes and monocyte-derived cells in tissues. The production of CCL2 and CCL3 by monocytes of DM1 patients was slightly, but not significantly, increased compared to monocytes of healthy control subjects (figure 2A and B). Exposure to FN increased the production of CCL2 and CCL3 and monocytes of DM1 patients showed a significantly increased production of these chemokines in comparison to monocytes of healthy controls (figure 2A and B).

Chemokine-directed migratory responses

Previously we demonstrated that an increased level of MRP8/14 in the serum of DM1 patients was able to induce an increased adhesion of monocytes to fibronectin

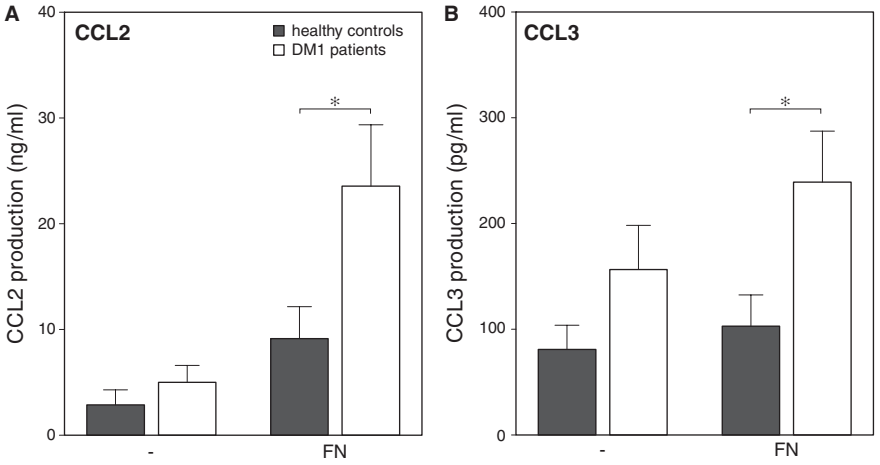

Figure 2. Expression of pro-inflammatory cytokines by monocytes under FN-adherent condition. (A) When stimulated with fibronectin, monocytes of DM1 patients showed an increased production of CCL2 compared to monocytes of healthy control subjects. (B) Also the production of CCL3 was increased by monocytes of DM1 patients after FN stimulation. Data are presented as average ± SEM, n=9, * p< 0.05, ** p<0.01 as determined with unpaired two-tailed Student's t test.

(FN) and an increased CD11b/CD18 expression (4). This increased adhesion and expression of CD11b/CD18 is a.o. involved in inflammation by participating in the trans endothelial migration (TEM) of monocytes (13, 14). Therefore, we studied the adhesive and migratory capacity of monocytes of DM1 patients in further detail. We allowed radio-labelled monocytes to adhere to a monolayer of human umbilical vein endothelial cells (HUVEC, kindly provided by dr. W. Sluiter). Monocytes of DM1 patients showed an increased potential to adhere to the endothelial monolayer compared to healthy control subjects and DM2 patients (figure 3A).

To study the actual TEM, transwell membranes were coated with HUVEC and the migration of radio-labelled monocytes towards the pro-inflammatory chemokines CCL2 and CCL3 studied. As shown in figure 3B, monocytes of DM1 patients showed a decreased TEM towards CCL2 and hardly any transmigration in response to CCL3 in comparison to monocytes of healthy control subjects. When the HUVEC were stimulated for 24 hours with IL-1β, which induces an up-regulation of adhesion molecules, the transmigration of monocytes to these chemokines stayed decreased in DM1 patients in comparison to healthy controls (data not shown).

Since the transwell assay is a laborious assay chemotaxis was also studied using the classical Boyden chamber, allowing us to test a larger array of monocyte attracting chemokines. The pro-inflammatory chemokines CCL2, CCL3 and CCL4

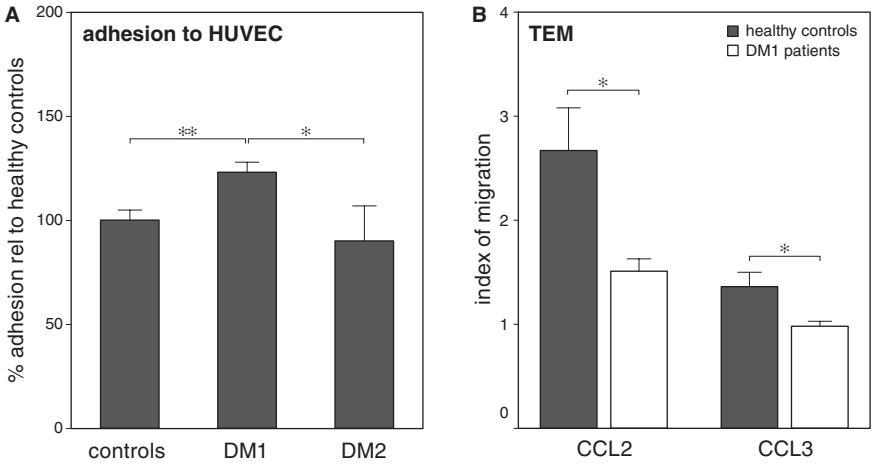

Figure 3. Monocyte adhesion and transmigration across HUVEC.
(A) Monocytes of type 1 diabetic patients (n=7) showed increased adhesion to HUVEC as compared to monocytes of healthy subjects (n=7) and type 2 diabetes patients (n=4). (B) Transmigration in response to CCL2 and CCL3 of type 1 diabetes monocytes (n=12) was strongly decreased compared to healthy control monocytes (n=12). Adhesion is shown relative to that observed with monocytes of healthy control subjects. Data is presented as average ± SEM. * $p < 0.05$, ** $p < 0.01$ as determined with unpaired two-tailed Student's t test.

(MIP-1β) and fMLP were used as chemoattractants in this assay, as were the constitutive chemokines CXCL12 (SDF-1) and CCL19 (MIP-3β). Migration of monocytes towards CCL3, CCL4 and CXCL12 proved to be of similar magnitude in DM1 patients and healthy control subjects, but the response to fMLP, CCL2 and CCL19 was not (figure 4 and data not shown). Monocytes of DM1 patients showed a decreased chemotactic response towards the pro-inflammatory compounds fMLP and to CCL2, but - surprisingly - the migratory response of DM1 monocytes to the constitutive chemokine CCL19 was increased in comparison to monocytes of healthy control subjects (figure 4). In this respect it is of interest to note that we could not detect a significant production of the lymphoid tissue-related CCL19 neither by monocytes of DM 1 patients nor of healthy controls (data not shown). To exclude the possibility that the monocytes of DM1 patients showed an altered chemotaxis due to alterations of the cells by exposure to high glucose levels in the blood, we performed a chemotaxis assay of healthy control monocytes towards CCL2 in the presence of increasing glucose levels. No significant effects of glucose were observed on the chemotactic response towards CCL2 of healthy control monocytes (data not shown).

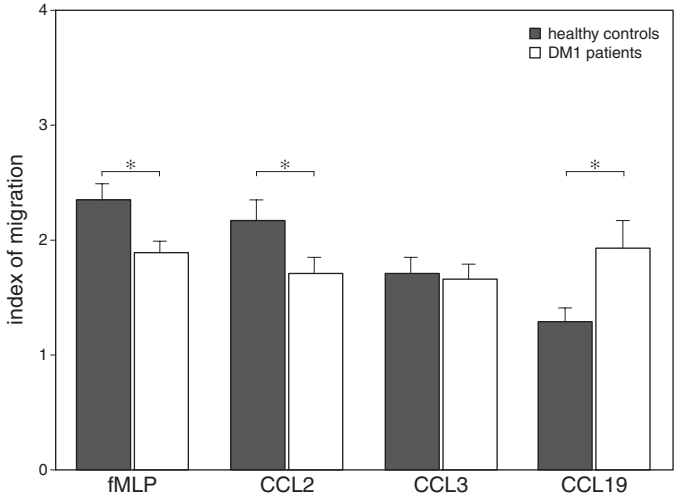

Figure 4. Disturbed chemotactic response of monocytes of type 1 diabetes patients.
Monocytes of type 1 diabetes patients displayed a decreased chemotactic response to fMLP and CCL2, while in contrast the response towards CCL19 was increased. Data is presented as average ± SEM, n=12, * $p < 0.05$, ** $p < 0.01$ as determined with unpaired two-tailed Student's t test.

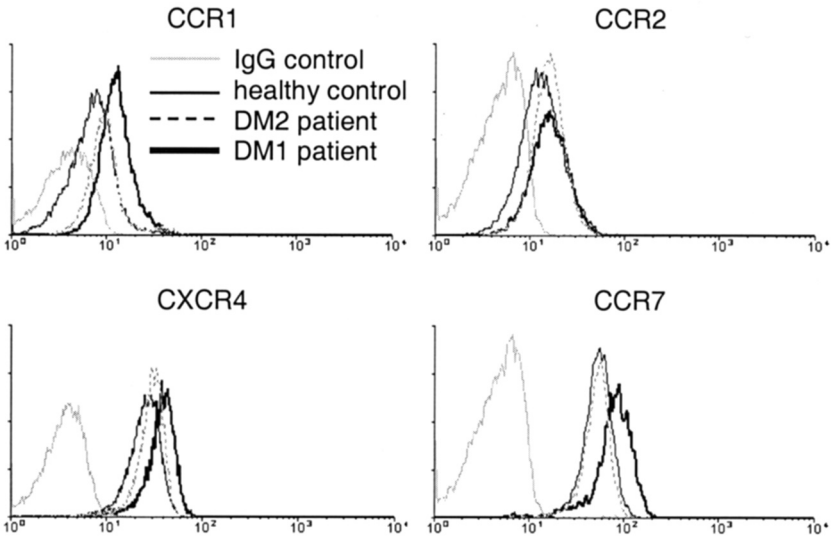

Figure 5. Chemokine receptor expression on monocytes.
Representative histograms of CCR1, CCR2, CXCR4 and CCR7 expression of 16 type 1 diabetes patients, 9 type 2 diabetes patients and 11 healthy control subjects show that the expression of CCR1, CXCR4 and CCR7 was increased in type 1 diabetes patients.

Chemokine receptor expression

To relate the decreased chemotactic response of the monocytes to pro-inflammatory chemokines to a lower expression level of receptors for such chemokines, we studied the surface expression of the chemokine receptors CCR2 (for CCL2) and CCR1 (for CCL3) on DM1 monocytes using flow cytometry. As shown in figure 5 and summarized in table 2, a reduced expression of CCR2 could not be detected on DM1 monocytes as compared to DM2 and healthy control monocytes. The surface expression of CCR1 was even increased. Since we observed a decreased TEM and chemotaxis of the monocytes of DM1 patients towards CCL2, but no differences in the expression of CCR2, we studied the ability of the CCR2 to mobilise intracellular calcium upon CCL2 stimulation, as a different functionality. No differences in the ability to mobilise calcium upon stimulation with CCL2 could be found between DM1 patients and healthy controls (data not shown).

Since we found an increased responsiveness of the DM1 monocytes to the constitutive chemokine CCL19, we also studied the surface expression of receptors for constitutive chemokines on DM1 monocytes and found CCR7 (for CCL19 and CCL21) and CXCR4 (for SDF-1) increased on monocytes of DM1 patients in comparison to healthy controls and DM2 patients (figure 5 and table 2).

Since it is known that the $CD14^{+}CD16^{+}$ subpopulation of monocytes shows a different expression of chemokine receptors (15), we also determined the

Table 2. Surface expression of chemokine receptors on monocytes.

	healthy controls (n = 11)			DM1 patients (n = 16)			DM2 patients (n = 9)		
CCR1	10.53	±	0.65	12.90	±	0.65 #$	10.06	±	1.31
CCR2	17.79	±	1.13	17.96	±	0.80	18.46	±	2.06
CCR7	50.38	±	7.04	65.16	±	2.87 #$	52.85	±	3.72
CXCR4	27.49	±	3.77	39.20	±	1.94 #$	31.58	±	2.81

# $p < 0.05$ to healthy control monocytes, $ $p < 0.05$ to DM2 patients' monocytes as determined with Student's t test. Mean fluorescent intensities are shown ± sem.

chemokine receptor expression on the $CD14^+CD16^+$ monocytes. No significant differences in chemokine receptor expression were observed on the $CD14^+CD16^+$ monocyte populations between DM1, DM2 patients and healthy controls, nor did we find differences in the percentage of $CD14^+CD16^+$ monocytes (data not shown).

Association with HLA-DR genotype

To investigate whether the aberrant adhesion to FN and the aberrant chemotactic migratory capacity of the circulating DM1 monocytes were associated with the HLA-DR genotype of the patients (see Table 1), we searched for possible associations between HLA-DR genotypes and the outcome of the different experiments. We were unable to find any statistically significant correlations.

DISCUSSION

MRP8/14, a product secreted by infiltrating monocytes, is known to increase the number of monocytes adhering to FN (4). Increased levels of MRP14 and MRP8/14 are present in the circulation of DM1 patients and indeed circulating monocytes show an increased adhesion to FN (4, 11). In this report we show that ex vivo circulating monocytes of DM1 patients express and secrete more of MRP8/14 as compared to monocytes of healthy control subjects, particularly after adhesion to a coating of FN. Our finding thus gives evidence for an enhanced positive feed-back mechanism in type 1 diabetes regarding the adhesion of monocytes to FN: type 1 diabetic monocytes are able to express and secrete more MRP8/14 as compared to healthy control monocytes inducing the cells to adhere more vigorously to FN as compared to healthy monocytes, which leads to an even larger expression and secretion of MRP8/14 as compared to healthy monocytes.

In NOD mice extensive deposits of FN are present at the vaso-ductular pole of the islets, the location of the mφ and DC accumulating prior to the extensive para- and peri-lymphocytic infiltration. Especially at pre-weaning age these deposits are larger as compared to control mice (5). Also NOD monocytes (16) and NOD DC (17) show an increased adhesion to FN - analogous to human monocytes - yet whether MRP8/14 is involved in this increased adhesion has not been studied. Nevertheless the parallel between NOD mice and humans regarding this enhanced FN adhesive capability of monocytes points to an important mechanism most likely

involved in the (early) accumulation of monocyte-derived cells, i.e. mφ and DC in the ducto-vascular poles in the pancreas.

We argued that such an enhanced adhesive phenotype of the circulating monocytes to FN (in both mice and men might also lead to a stronger adhesion to endothelial cells and an increased TEM (13, 18). Indeed here we show that monocytes of DM1 patients exhibited a stronger adhesion to HUVEC, but when we studied the migratory response of monocytes towards pro-inflammatory chemokines, such as CCL2 and CCL3, monocytes of DM1 patients showed in general a decreased TEM as well as a decreased membrane migration in the classical Boyden chamber assay. Such a decreased migration of T1D monocytes to pro-inflammatory stimuli has been described previously using zymosan-activated culture fluid (19) and C5a (20).

Here we also show that the in general impaired migratory response of DM1 monocytes is selective for pro-inflammatory chemokines: We observed an increased migration of monocytes of DM1 patients towards the constitutively expressed chemokine CCL19 that was paralleled by an increased expression on DM1 monocytes of the receptor for CCL19, i.e. CCR7.

Again an interesting parallel exists between the DM1 patient and the NOD mouse regarding this aberrant migratory function of monocytes. Also in the NOD mouse the chemotactic response of monocytes and DC towards pro-inflammatory chemokines is reduced both in vitro and in vivo, while there are signs of a role for constitutive chemokines in the diabetic process (9, 17). At the time of the early mφ and DC accumulation, which occurs close to and around the islets of Langerhans prior to the lymphocyte infiltration, CCL19 and CCL21 were found to be expressed at a higher level as compared to control mice (17). The pro-inflammatory chemokines CCL5 and CXLC10 only came to a noteworthy expression in the NOD pancreas after the initial mφ and DC accumulation and at the time of excessive lymphocytic infiltration (17) and we therefore assume that the early increased expression of CCL19 and CCL21 in the NOD pancreas plays a role in the early peri- and para-islet mφ and DC accumulation.

Since the aberrant monocyte adhesive and chemotactic properties are present in the NOD mouse prior to the mφ and DC accumulation (5-7 wks of age) and since the here studied patients all had longstanding type 1 diabetes (hence the acute islet inflammation must have been subsided), we assume that the aberrant adhesive and migratory properties of the monocytes are intrinsic to cells of individuals prone to develop autoimmune insulitis. Other monocyte-specific defects have been reported in DM1 patients that may be involved in the decreased migration towards pro-inflammatory chemokines. Monocytes of DM1 patients show an aberrant prostaglandin synthase 2 (PGS2) expression (21, 22). Activated monocytes and mφ express high levels of PGS2, resulting in abundant expression of prostaglandins, including prostaglandin E_2 (PGE_2) (23). In monocytes, PGE_2 induces a down-regulation of the pro-inflammatory chemokine receptor CCR5 (24). Furthermore,

PGE_2 is involved in migration of DC towards CCL19 and CCL21 (25, 26). Possibly, the constitutive active PGS2 in monocytes of DM1 patients yields high PGE_2 levels that down regulate the responsiveness of monocytes to pro-inflammatory chemokines and up regulate CCR7 expression and responsiveness.

The actual contribution of the increased FN adhesion and aberrant chemotactic responsiveness of monocytes to the development of islet autoimmunity remains to be identified. Also in other autoimmune diseases like rheumatoid arthritis (27) and systemic lupus erythematosus (28) an impaired monocyte migration has been observed, while patients with the Wiskott Aldrich syndrome (WAS) show a defective monocyte migration due to an inherited defect in the cytoskeletal-associated WAS protein (29) and are prone to develop autoimmune disorders (30). Normal homeostatic trafficking of DC is thought to be important for the maintenance of peripheral tolerance (31-33). However, little is known about the kinetics of trafficking of monocytes, mφ and DC in the pre-diabetic pancreas. When APC are retained in the tissue they may acquire an immunocompetent rather than a tolerogenic phenotype. Indeed contact of DC with ECM induces maturation of the cells (34, 35). Therefore a prolonged contact with ECM may induce complete maturation of the DC in NOD mice resulting in the induction of an immune response in the draining lymph node rather than the induction or maintenance of tolerance.

In conclusion, we here show an increased expression and secretion of MRP8/14 of DM1 monocytes that is closely linked to their enhanced adhesive capability to FN. In addition, monocytes of DM1 patients exhibited a decreased migration towards pro-inflammatory chemokines, but an increased migration to the constitutively in lymphoid tissue expressed chemokine CCL19. Given the fact that similar aberrancies have been found in the NOD mouse prior to disease development, it is likely that these aberrations are associated with the development of islet autoimmunity.

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CHAPTER 3

ADHESION, MIGRATION AND DIFFERENTIATION OF MONOCYTES IN THE NON-OBESE DIABETIC MOUSE

CHAPTER 3.1

DIABETES-PRONE NOD MICE SHOW AN EXPANDED SUBPOPULATION OF MATURE CIRCULATING MONOCYTES, WHICH PREFERENTIALLY DEVELOP INTO MACROPHAGE-LIKE CELLS *IN VITRO*

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Submitted

ABSTRACT

In the non-obese diabetic (NOD) mouse, a model of autoimmune diabetes, dendritic cells (DC) and macrophages (mφ) are important for the initiation and progression of autoimmunity and the final destruction of β-cells. Previous studies suggested that an aberrant development of DC and mφ is related to their pathogenic function. To study this in vivo, we investigated NOD mouse monocytes, the direct precursors of DC and mφ. The recently described discrimination between immature (Ly-6C^{high}) and mature (Ly-6C^{low}) monocytes enabled us to investigate the apportioning between blood monocyte populations in the NOD mouse.

The NOD mice had an abnormally high number of mature monocytes in circulation and this phenomenon appeared to be intrinsic to the NOD background, since NOR and NOD-H2b mice also showed this altered balance. After depletion by apoptosis-inducing liposomes, the reappearance and transition of immature to mature monocytes had similar kinetics as control mice, but led again to the presence of a larger mature monocyte compartment in the blood. In addition, while monocytes from C57BL mice down regulated their capability to adhere to fibronectin and ICAM-1 upon maturation, the mature NOD monocytes retained their high adhesion capacity, characteristic of immature cells. Furthermore, both monocyte subpopulations of NOD mice showed enhanced differentiation into mφ-like F4/80^{high} cells, *in vitro*.

In conclusion, mice with the NOD background have raised numbers of mature monocytes in the circulation and a pro-inflammatory, mφ-directed monocyte development.

INTRODUCTION

Type I diabetes (T1D) 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) are closely related to the initiation and progression of autoimmune diabetes. Significant accumulation of DC and mφ around islets at 4-5 weeks of age precedes the peri-insular concentration of lymphocytes and their subsequent infiltration in the islets and their subsequent infiltration in the islets [1, 2].

Monocytes are direct precursors of DC and mφ [3-5]. It has been generally accepted that the majority of DC and mφ in non-lymphoid tissues originate from blood monocytes, especially in inflammation [6, 7]. Mouse blood monocytes can be phenotypically and functionally separated into at least two subsets [8, 9]. Monocytes that have recently emigrated from the bone marrow (BM) and express high levels of the Ly-6C molecule represent the first subset. These Ly-6C^{high}, immature monocytes, are readily attracted to sites of inflammation, have the potential to develop into DC or F4/80^{+} inflammatory mφ [5, 9] and co-express CD62L/L-selectin and CCR2, which enables the cells to be attracted towards inflammation-induced CCL2/MCP-1 signals [8, 10]. The second subset of blood monocytes is generated through a maturation step from the immature monocytes, which is marked by a reduction of the surface expression of Ly-6C and an increase in expression of CD43 [9]. These Ly-6C^{low} monocytes, which express a low level of CD11c and a high level of CX_3CR1 (fractalkine receptor), migrate to non-lymphoid organs in the absence of inflammation and have been proposed to be the precursors for the steady state pool of peripheral DC and mφ [8].

In the study that described two circulating monocyte subsets in mouse [8], authors have suggested that mouse CX_3CR1^{low} (Ly-6C^{high}) monocytes correspond to human CD14^{++}CD16^{-}monocytes, while the CX_3CR1^{hi} (Ly-6C^{low}) monocytes correspond to the CD14^{+}CD16^{+} human monocyte population, based on the cell phenotype as a standard. Others have used different criteria to subdivide circulating human monocytes as well, such as the ability to adhere to fibronectin [11]. The fibronectin-adherent, so-called pro-inflammatory “P-monocytes”, constitute 20-30% of the circulating monocyte population, express high levels of adhesion molecules and have an enhanced chemotactic responsiveness, phagocytosis capacity and pro-inflammatory cytokine production capability [12, 13]. We found that fibronectin-adherent monocytes are equally present in both the CD14^{+}CD16^{+} and the CD14^{++}CD16^{-} population [14], indicating that the divisions based on the fibronectin-adherence and on the CD16 expression are probably not identical.

Different pathological conditions appear to correspond with changes in the composition of the monocyte pool. In humans, the CD14^{+}CD16^{+} monocyte

population expands greatly in acute and chronic infections, systemic inflammatory syndromes, AIDS and renal failure [15-20], while other conditions stimulate the prevalence of the $CD14^{++}CD16^{neg}$ population [21]. Also in mice, acute and chronic infection models revealed a shift in the monocyte balance in the circulation [9, 22]. However, normal numbers of CD14/CD16-defined monocyte populations were found in T1D patients and in multiple sclerosis (MS) patients [14, 23], while we found raised number of fibronectin-adherent monocytes in T1D patients as compared to healthy controls [14]. Furthermore, we previously reported a hampered capability of monocytes of T1D patients to develop during an overnight culture into DC-like cells [24].

To investigate a putative reflection of the autoimmune-prone status of the NOD mice in their blood monocyte profile and compare this with published data for human T1D monocytes, we applied recently developed methodology [9], to study NOD mouse monocytes. Here we describe an excess of mature Ly-$6C^{low}$ monocytes in the blood of mice with the NOD background, which have abnormal high fibronectin adhesive capacity. In addition, we found that NOD monocytes have an enhanced capability to mature and preferentially acquire a mφ-like phenotype.

MATERIAL AND METHODS

Animals

Mice used in this study were between 5-16 weeks of age with the exception of diabetic NOD mice, which were 25 weeks old. Mice were age-matched between strains in all experiments. Female C57BL/6j and BALB/c mice were purchased from Harlan (Horst, The Netherlands) and female NOR mice were purchased from Jacksons Laboratory (Bar Harbor ME, USA). NOD/Ltj, NOD.B10H2^{b} (further referred to as NODH2b) and C3Heb/Fej mice were bred in the animal care facility at Erasmus MC, Rotterdam. All mice were specific pathogen-free and kept with free access to food and water, under the institutional guidelines for usage of experimental animals approved by the Erasmus University Animal Welfare Committee. Glycosuria in NOD mice was tested with the Gluketur test (Roche Diagnostics GmbH, Mannheim, Germany).

Antibodies

Specifications of monoclonal antibodies and fluorescent conjugates against surface markers used in this study are listed in Table 1. Directly conjugated isotype control mAb were purchased from BD Pharmingen (San Diego, CA). Anti-rat IgG conjugated with FITC- or R-phycoerythrin-(R-PE)- (mouse-absorbed; GaRa-FITC or GaRa-PE) were purchased from Caltag Laboratories, San Francisco, CA. Biotinylated Ab were detected by allophycocyanin- (APC) conjugated streptavidin (SAV-APC) purchased from BD Pharmingen.

Table 1. Monoclonal antibodies used in this study.

marker	monoclonal antibody	Used form (conjugate)	origin
CD3	145-2C11	FITC, APC	BD Pharmingen, San Diego, CA, USA
CD11b	M1/70	PerCP-Cy5.5, APC	BD Pharmingen, San Diego, CA, USA
CD11c	HL3	FITC, PE	BD Pharmingen, San Diego, CA, USA
CD16/32	2.4G2	∅[a]	ATCC, Rockville, MD, USA
CD19	1D3	PE	BD Pharmingen, San Diego, CA, USA
CD43	S7	FITC, PE	BD Pharmingen, San Diego, CA, USA
CD54	YN1/1.7	∅	ATCC, Rockville, MD, USA
CD62L	MEL-14	PE	BD Pharmingen, San Diego, CA, USA
CD115	AFS98	∅	Dr. Nishikawa, Kyoto, Japan
ER-MP58	ER-MP58	∅	own laboratory
F4/80	F4/80	FITC	Caltag, San Francisco, CA, USA
Ly-6C	ER-MP20	FITC	own laboratory
MBR	MIV 38	∅	Dr. Falkenberg, Bochum, Germany
NK1.1	PK136	PE	BD Pharmingen, San Diego, CA, USA

[a] unconjugated hybridoma supernatant

In vivo elimination of mononuclear phagocytes

Multilamellar liposomes containing clodronate (dichloromethylene bisphosphonate, a gift from Roche Diagnostics) (lip-CL_2MDP) in the aqueous phase were prepared as described previously [25, 26]. Liposomes consisted of phosphatidyl choline and cholesterol in 6:1 molar ratio. After washing, the liposomes were resuspended in PBS. Mononuclear phagocytes were eliminated *in vivo* by intravenous (i.v.) injection of 0.2 ml clodronate-loaded liposomes into the lateral tail vein, as described before [27].

Preparation of leukocytes, flow cytometry or cell sorting

Mice were euthanized by CO_2 exposure. Blood was obtained by heart puncture and collected in heparin-coated tubes. For flow cytometry, erythrocytes were eliminated using BD lysing buffer (San Diego, CA). For sorting, blood samples were treated with a sterile ammonium chloride lysing solution. Leukocytes were subsequently washed by centrifugation at 1500rpm for 5min in PBS pH 7.8 containing 0.5% BSA (Biowhittaker, Verviers, Belgium), resuspended and counted in a Bürker hemocytometer.

For phenotypic analysis aliquots of 0.5-1x10^6 cells were pipetted into 96-microwell plates (round bottom, Nunc, Denmark) and incubated with the prepared mix of monoclonal antibodies. Each incubation step was performed at RT for 10min. Cells were analyzed using a FACSCalibur equipped for 4-color flow cytometry and up to 5x10^5 events were obtained. Data were analyzed using CellQuest software (Becton Dickinson, Sunnyvale, CA).

Cell sorting was performed on a FACSDiva by applying a previously described protocol [3]. Briefly, lysed blood samples (pooled from 5 to 10 mice) were washed in sterile PBS supplemented with 5% heat inactivated FCS (Biowhittaker) and

antibiotics (60mg/ml penicillin and 100 mg/ml streptomycin) (further referred to as sorting buffer) and incubated for 30min on ice with the mix of CD11b, Ly-6C and Ly-6G antibodies (Table 1). Subsequently, cells were washed with sorting buffer and filtered over a 30μm sieve (Polymon PES, Kabel, Amsterdam, The Netherlands) to avoid clogging of the nozzle. After sorting, the purity of the cell suspensions was analyzed by re-running sorted samples. Purity exceeded 95%, unless stated otherwise. Cells were kept at 4°C throughout the staining and sorting procedure.

Adhesion test

The adhesion capacity test was performed as previously described [28]. Briefly, sorted monocytes were suspended in RPMI-1640 culture medium (Biowhittaker) containing antibiotics supplemented with 1% heat-inactivated FCS and then plated at a density of $0.2x10^5$ cells per chamber, on coated chambertek glass slides (Nalge Nunc International, Naperville, USA) previously coated either with 10μg/ml fibronectin or 10μg/ml ICAM-1 (Sigma, Steinheim, Germany). After 60min incubation at 37°C, cells were washed with PBS and fixed with 4% paraformaldehyde (PFA; Sigma), supplemented with 3% glucose. Cells were permeabilized using 0.5% Triton X-100 (Sigma Chemical, Saint Louis, USA) and stained with 0.1 μg/ml FITC-labeled phalloidin (Sigma Chemical) for 30-45 min. After washing and mounting the slides, the cells were counted using a fluorescence microscope. Adhesion was expressed as the number of cells in 10 high power fields (hpf) at 200x magnification.

Culture of sorted cells

For analysis of *in vitro* differentiation capacity of monocytes, sorted cells were seeded into 96-well culture plates at a concentration of $1x10^6$/ml in RPMI-1640 medium supplemented with antibiotics and 10% FCS. Cells were incubated at 37°C with 5% CO_2, either or not stimulated with 100 ng/ml LPS (Sigma). After 24h incubation, cells were used for phenotypic analysis.

Statistical analysis

To determine differences between the groups, data were compared by a two-tailed Student's t test using the SPSS software package. Results are presented as the mean ± SEM, unless differently indicated.

RESULTS

Both Ly-6C^{high} and Ly-6C^{low} monocytes are found in the blood of NOD mice

In the NOD mouse blood, the standard definition of the monocytes [9, 29], could be applied (Fig. 1A). Similarly to the C57BL mouse, more than 97% of SSC^{lo}CD11b^{hi} cells in the NOD mouse blood were M-CSF-receptor positive (CD115 in Fig. 1C) and could be separated into two populations defined by Ly-6C

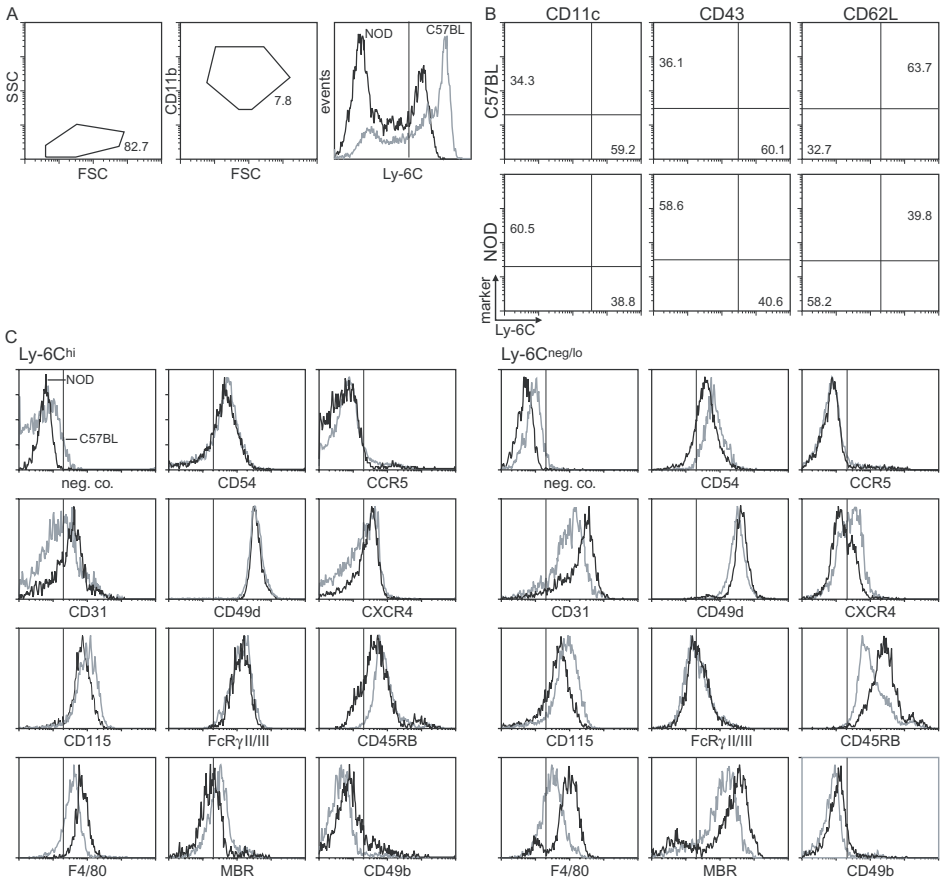


Figure 1. Definition and phenotypic analysis of blood monocytes in the NOD mouse.
A. Gating of SSC^{lo} and CD11b^{hi} cells in peripheral blood leukocytes separates two monocyte populations as seen from the bi-phasic profile of the Ly-6C histogram. B. Although Ly-6C levels are lower on NOD monocytes, Ly-6C separation identifies the same two monocyte populations as in the C57BL mice, as judged by CD11c, CD43 and CD62L expression. C. Additional markers tested, differentially or similarly expressed between two monocyte subsets, show similar expression between NOD and C57BL mice within a particular population. Difference in CD31 is strain related while F4/80 and CD45RB are not statistically significant (Student's t test). Dot-plots and histograms show a representative staining of a minimum of 3 independent stainings for each marker.

expression (Fig. 1A). However, Ly-6C expression in the positive population was significantly lower in NOD mice than in other mouse strains (Fig. 1C and Table 2). Therefore we also analyzed CD11c, CD43 and CD62L expression. These three molecules are selectively expressed either on the Ly-6C^{high} or Ly-6C^{low} monocytes [8, 9] (Fig. 1B). A similar distribution of all three markers was found in NOD as in

Table 2. Phenotype of monocyte subpopulations.

	Ly-6C^{hi}		Ly-6C^{lo}	
	NOD	C57BL	NOD	C57BL
neg. co.[a]	2 ± 1	2 ± 0	2 ± 0	3 ± 0
Ly-6G	5 ± 1	6 ± 0	9 ± 1	9 ± 0
NK1.1	7 ± 0	5 ± 1	8 ± 1	7 ± 2
CD115	71 ± 24	45 ± 17	52 ± 15	40 ± 11
F4/80	68 ± 16	81 ± 26	72 ± 21	62 ± 13
Mac-2	61 ± 1	28 ± 12	49 ± 2	27 ± 11
MBR	17 ± 7	15 ± 5	172 ± 43	102 ± 14
ER-MP58	541 ± 297	264 ± 145	90 ± 41	312 ± 223
Scav.R	93 ± 29	30 ± 15	102 ± 46	44 ± 20
FcRγII/III	77 ± 29	42 ± 20	16 ± 3	13 ± 2
CD1d	20 ± 0	16 ± 6	18 ± 0	19 ± 5
Ly-6C	357 ± 22**	463 ± 120	8 ± 0	14 ± 2
CD43	28 ± 6	63 ± 29	524 ± 139	614 ± 130
CD11c	7 ± 1	9 ± 2	36 ± 5	45 ± 8
CD62L	20 ± 6	21 ± 9	7 ± 1	8 ± 2
CD31	31 ± 1**	15 ± 1	195 ± 40*	61 ± 12
CD54	51 ± 9	38 ± 0	49 ± 11	65 ± 0
CD49d	397 ± 24	391 ± 21	501 ± 35	500 ± 75
CD49b	10 ± 2	6 ± 1	4 ± 1	4 ± 0
CD45RB	78 ± 34	93 ± 21	177 ± 19	129 ± 16
CCR3	18 ± 14	7 ± 1	6 ± 4	4 ± 2
CCR5	4 ± 1	5 ± 1	8 ± 0	7 ± 0
CXCR4	28 ± 0	18 ± 0	16 ± 0	27 ± 0

[a] data represent average mean fluorescence intensity (MFI) ± SEM calculated from 3-8 independent exp. for each marker.

** $p<0.01$ NOD vs. C57BL; * $p<0.05$ NOD vs. C57BL

C57BL mice. Furthermore, the lower expression of Ly-6C molecule did not prevent clear separation of the monocyte subsets in the NOD mouse (Fig. 1B).

Further phenotyping of Ly-6C^{high} and Ly-6C^{low} monocytes did not reveal substantial differences between the two mouse strains (Fig. 1C and Table 2.). Although not statistically significant, the macrophage marker F4/80 had a reproducibly higher surface expression on both subsets of NOD monocytes. In addition, CD31 expression was significantly higher in NOD mice when compared to C57BL (Table 2.) but not when compared to BALB/c mice (not shown). Both

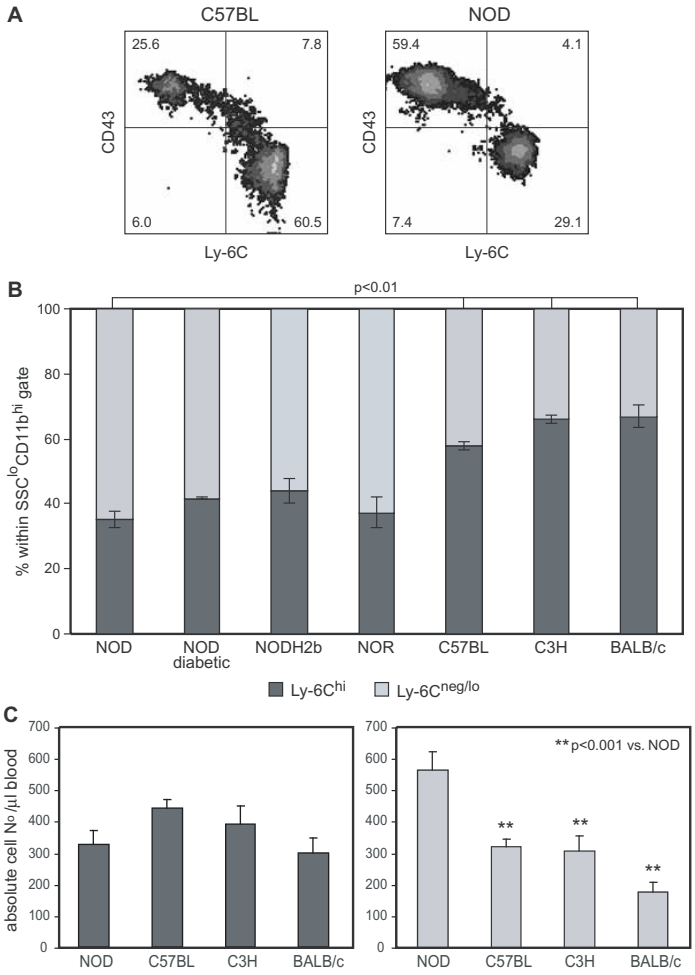


Figure 2. Different balance between Ly-6C^{hi} and Ly-6C^{lo} monocytes in the blood of mice with a NOD genetic background.

A. A representative dot-plot of the Ly-6C/CD43 profile of NOD and C57BL monocytes shows a higher frequency of Ly-6C^{lo} monocytes in NOD mice. B. Analysis of the frequency of blood monocytes in different mouse strains revealed a specific shift towards Ly-6C^{lo} monocytes in all mouse strains with the NOD genetic background. C. Calculation of the absolute cell number showed that similar number of Ly-6C^{hi} monocytes is found in the NOD as in the C57BL, the C3H and the BALB/c mice. In contrast, the number of Ly-6C^{lo} monocytes in the NOD mouse blood is significantly higher than in any control mouse strain tested. Graphs represent average values ± SEM calculated from 26 C57BL mice, 20 NOD mice and 6-9 mice of other mouse strains.

monocyte subtypes similarly expressed all other markers tested in different mouse strains (Fig. 1C and Table 2.).

Mice with the NOD background have more mature (Ly-6C^{low}) monocytes in the blood

Although the phenotype of the NOD monocyte subsets did not differ from that of C57BL mice, we noticed that the frequency of the Ly-6C^{high} monocytes was repeatedly lower in NOD mouse blood when compared to C57BL blood (Fig. 1B and 2A). Correspondingly, Ly-6C^{low} monocytes in the NOD blood formed the major monocyte population.

Is this overrepresentation of mature monocytes in the blood specific to the NOD mouse? We determined the frequency of monocyte subsets in several mouse strains (Fig. 2B). Between 58-67% monocytes were of the Ly-6C^{high} type in the strains of mice unrelated to the NOD. In contrast, in the strains with the NOD background, Ly-6C^{high} monocytes never exceeded 44% of the total monocyte pool. Correspondingly, the higher frequency of Ly-6C^{low} monocytes was found in both healthy and diabetic NOD mice, as well as NOR and NOD-H2b mice (55-65% for the NOD-related strains vs. 30-35% for the NOD-unrelated strains) (Fig. 2B). Hence, all mice with the NOD background had a reversed ratio of monocyte subsets in favor of the Ly-6C^{low} cells.

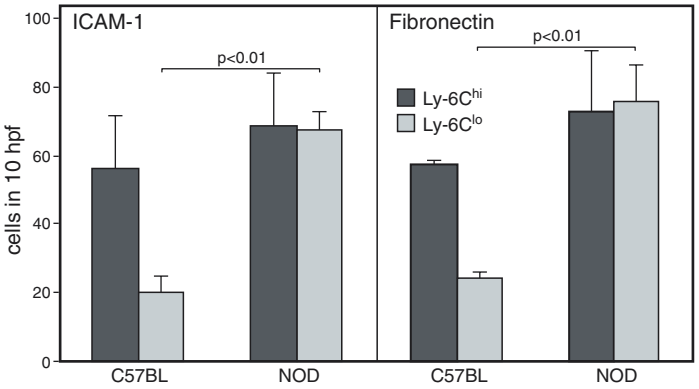


Figure 3. High in vitro adherence of sorted Ly-6C^{lo} monocytes from the NOD mouse.
Blood monocytes were sorted and put to adhere to ICAM-1 or fibronectin for 60min. Subsequently cells were washed, stained with phalloidine and quantified on the fluorescence microscope. A high number of Ly-6C^{hi} monocytes from both mouse strains adhered to both substrates. In contrast, while Ly-6C^{lo} monocytes from the C57BL mice downregulated the adhesion capacity upon maturation, the Ly-6C^{lo} monocytes from the NOD mice failed to do so. Graphs represent an average ± SEM from three independent pools of a minimum of 5 mice per pool, obtained in two independent experiments.

Next, we analyzed whether this reversed monocyte ratio in the NOD mouse was caused by the shift in the absolute number of one or both monocyte populations. The number of immature (Ly-6C^{high}) monocytes per μl blood appeared to be similar in the NOD mouse blood and in the C57BL, C3H and BALB/c mice (Fig. 2C). In contrast, the absolute number of Ly-6C^{low} monocytes (Fig. 2C) was significantly higher in NOD mice than in all other tested control strains ($p<0.001$ for all three control strains). Therefore, we concluded that a clear excess of Ly-6C^{low} monocytes typifies the disturbed monocyte subset-ratio in the blood of mice with the NOD background.

Ly-6C^{low} monocytes from NOD mice display unusually high adhesion to fibronectin and ICAM-1, typical for Ly-6C^{high} monocytes

Adhesion capacity is also used as a discriminating property of different monocyte populations [11]. To evaluate the ability of the two NOD monocyte subsets to adhere to fibronectin or ICAM-1, we sorted Ly-6C^{high} and Ly-6C^{low} monocytes from the mouse blood and tested their adhesiveness to these compounds *in vitro*.

As shown in figure 3, Ly-6C^{high} monocytes isolated from both NOD and C57BL equally adhered to fibronectin and ICAM-1. In contrast, a much lower number of Ly6C^{low} monocytes from C57BL mice adhered to these compounds; hence the maturation of monocytes in the circulation leads to a decline in the ability to attach

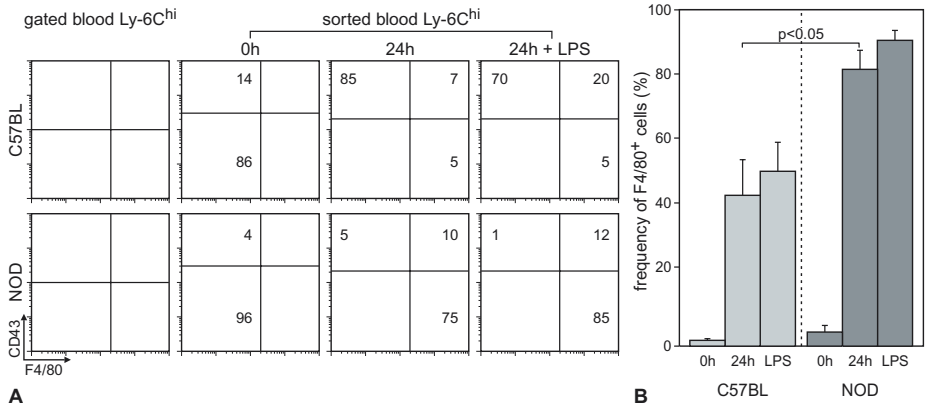


Figure 4. Ly-6C^{hi} monocytes from NOD mice display a different spontaneous maturation.
A. Spontaneous maturation of Ly-6C^{hi} monocytes upon *in vitro* cultivation for 24h with or without LPS proceeds via different phenotypic stages in NOD mice when compared to C57BL. Dot-plots show a representative staining of Ly-6C^{hi} monocytes prior and after the sorting as well as after 24h incubation alone or with LPS. Numbers represent percent of cells in the quadrant. B. Frequency (±SEM) of F4/80^{hi} cells derived from Ly-6C^{hi} monocytes, *in vitro*. Data are derived from 4 independent samples per mouse strain obtained from a pooled blood of 5-6 mice per strain, in three independent experiments.

to these integrin ligands. Interestingly, although expression of several integrins did not differ between corresponding NOD and C57BL monocyte populations (Fig. 1C and Table 2.), Ly-6C^{low} monocytes in NOD mice preserved the high adhesion capacity to both fibronectin and ICAM-1, comparable to that of Ly-6C^{high} monocytes (Fig. 3).

NOD monocytes show an enhanced spontaneous differentiation *in vitro,* predominantly in the direction of macrophage-like cells

Monocytes are direct precursors of both DC and mφ [3-5]. To assess the capacity of the two subpopulations of monocytes to differentiate spontaneously *in vitro*, we sorted and incubated them in culture fluid for 24h, without adding additional cytokines.

The overnight culture of sorted immature Ly-6C^{high} monocytes from the C57BL mouse induced an upregulation of the CD43 molecule – characteristic of mature monocytes - on the majority of the cells (Fig. 4A). In addition, a fraction of the cells expressed F4/80 at high levels, typical for mφ. Unlike C57BL cells, NOD Ly-6C^{high} immature monocytes did not acquire CD43 in vitro, but all cells had strongly upregulated the F4/80 molecule (note that the NOD mouse fresh blood monocytes had somewhat higher F4/80 expression). Hence, a significantly higher percent of Ly-6C^{high} monocytes of NOD mice spontaneously became F4/80^{high} mφ-like cells ($p<0.05$) (Fig. 4B). Addition of LPS increased the percentage of F4/80^{high} cells in both cultures.

Overnight culture of sorted C57BL and NOD mature Ly-6C^{low} monocytes yielded three cell populations (Fig. 5A): CD43^{+}F4/80^{low}- undifferentiated mature monocytes, CD43^{low}F4/80^{med}- cells with a DC-like phenotype and CD43^{high}F4/80^{high}- cells with a mφ-like phenotype. The DC-like cells were also CD11c^{+}MHCII^{hi} while mφ-like cells were CD11c^{low}MHCII^{low}, as previously reported [4, 5]. Although all three populations were present in NOD as well as C57BL samples, they had different frequencies (Fig. 5B). More than half of the Ly-6C^{low} monocytes remained unchanged in the cultures from C57BL mice while in the NOD cultures, only less than 20% of cells preserved the phenotype of Ly-6C^{low} monocytes ($p<0.001$, NOD vs. C57BL). The percentages of DC-like cells were similar in NOD and C57BL cultures, in both non-stimulated and the LPS stimulated samples, so the NOD monocytes did not excessively mature into DC. Instead, the NOD monocytes differentiated more readily and predominantly into mφ-like cells ($p<0.05$) (Fig. 5B). With LPS as an additional stimulus, the enhanced differentiation into mφ-like cells was evident in cultures of both mouse strains.

Taken together, both Ly-6C^{high} monocytes and Ly-6C^{low} monocytes from the NOD mouse show an enhanced spontaneous differentiation, predominantly into cells with a mφ-like phenotype.

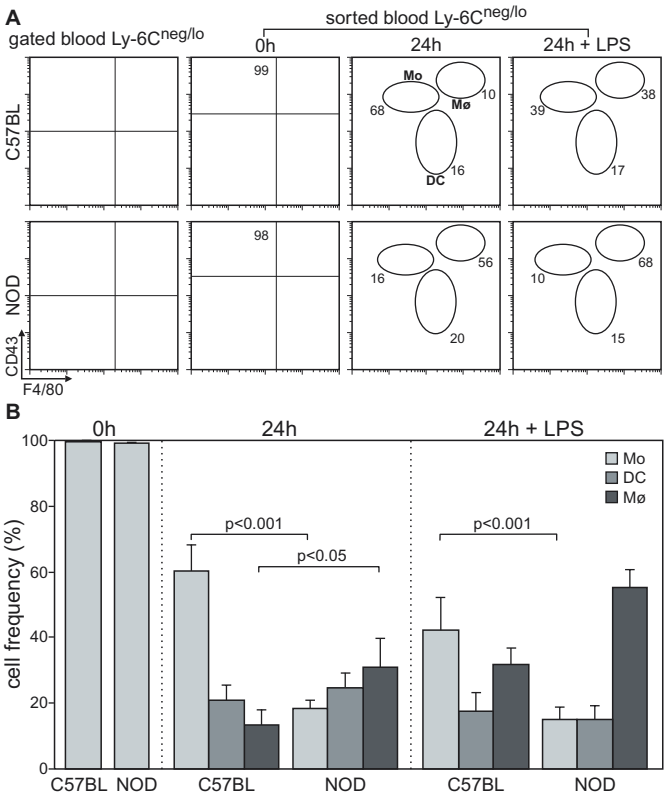


Figure 5. Predominant maturation of Ly-6C^{lo} monocytes from NOD mice into mφ.
A. Culture of isolated mature Ly-6C^{lo} monocytes for 24h in the presence or absence of LPS results in three distinct populations of cells: monocytes (CD43^{med}F4/80^{lo}), DC (CD43^{lo}F4/80^{med}) and mφ (CD43^{hi}F4/80^{hi}). These populations are phenotypically similar in cultures from NOD and C57BL mice but occur in different frequencies, as marked on representative dot-plots by ellipses ("gates"). B. The average frequency ± SEM of cells phenotypically defined as monocytes (light grey bar), DC (dark grey bar) and mφ (black bar), points to an increased spontaneous maturation of the NOD Ly-6C^{lo} monocytes into F4/80^{hi} mφ *in vitro*. Data are calculated from the samples obtained in the experiments described in the legend of the figure 4. P-values are probabilities derived from the Student's t test.

Normal restoration and transition time of monocytes in the NOD mice upon *in vivo* challenge

To follow the monocyte maturation *in vivo*, we made use of clodronate-loaded liposomes (lip-CL_2MDP). A single injection of lip-CL_2MDP caused an almost complete depletion of monocytes (SSC^{lo}CD11b^{hi} cells) from the blood within the first 18h in both NOD and C57BL mice (Table 3). Apparently, the NOD monocytes were equally able to phagocytose and fragment lip-CL_2MDP as the C57BL monocytes. Around 48h post-injection, monocytes started to re-appear in the blood of both

Table 3. Return of blood monocytes after i.v. depletion with lip-CL_2MDP.

	Time after depletion with lip-CL_2MDP (hours)					
	0	18	24	48	96	192
NOD [a]	987 ± 85	122 ± 34	92 ± 22	471 ± 228	982 ± 205	2482 ± 578
C57BL	799 ± 169	205 ± 113	158 ± 57	320 ± 48	1036 ± 229	1126 ± 296

[a] absolute number of monocytes/μl blood ± SEM

NOD and C57BL mice and the total number of monocytes did not differ significantly among these two mouse strains (Table 3).

Also the kinetics of the return of Ly-6C^{high} and Ly-6C^{low} monocytes in the circulation was similar between NOD and C57BL mice (Fig. 6). Like previously established for the C57BL mice, the first monocytes found in the blood of NOD mice at 48h were exclusively immature Ly-6C^{high} cells (Fig. 6A). The number of these cells in the circulation kept rising until the 96h time point after which the frequency of Ly-6C^{high} monocytes started to decline in both mouse strains and returned to the normal level at 192h (Fig. 6A). Similarly, the return of Ly-6C^{low} monocytes had identical kinetics in both NOD and C57BL mice when related to their respective steady state values. In both mouse strains, Ly-6C^{low} monocytes started to appear in the circulation from 96h and higher numbers of Ly-6C^{low} monocytes (like before depletion) were found in the blood of NOD mice till the end of the observation period (Fig. 6B). Therefore, normal release and transition into mature monocytes enabled prompt restoration of the enlarged Ly-6C^{low} monocyte pool in the NOD circulation.

Normal steady-state efflux of Ly-6C^{low} monocytes from the blood of the NOD mice

A reduced efflux of mature blood monocytes to the periphery could explain the elevated number of Ly-6C^{low} monocytes in the NOD mouse blood. We therefore determined the percent of Ly-6C^{low} monocytes in the peritoneal cavity and the spleen before and after the application of lip-CL_2MDP.

Before application of lip-CL_2MDP, the frequency of Ly-6C^{low} monocytes in the spleen of NOD mice was lower than in the C57BL mice ($p<0.02$) (Table 4 and Fig.

Table 4. Frequency of Ly-6C^{lo} monocytes in different compartments before and after depletion.

	blood		spleen		peritoneum	
	NOD	C57BL	NOD	C57BL	NOD	C57BL
untreated [a]	8.56 ± 1.05*	5.55 ± 0.65	0.59 ± 0.05**	0.96 ± 0.08	6.81 ± 1.24	6.32 ± 1.50
treated (d7)	4.90 ± 0.74	3.57 ± 0.43	0.71 ± 0.05*	0.97 ± 0.07	6.07 ± 0.77	3.35 ± 1.56

[a] percent of all leukocytes/ml blood ± SEM

** $p<0.01$ NOD vs. C57BL; * $p<0.05$ NOD vs. C57BL

7A-B) but similar to other tested mouse strains. In the peritoneal cavity, NOD mice and C57BL mice had similar monocyte frequency before depletion (Table 4). One week after depletion, Ly-6C^{low} monocytes returned to the steady state point in both the spleen and peritoneal cavity (Table 4), and importantly with similar kinetics in NOD and C57BL mice (not shown). Hence in absence of inflammation, we found

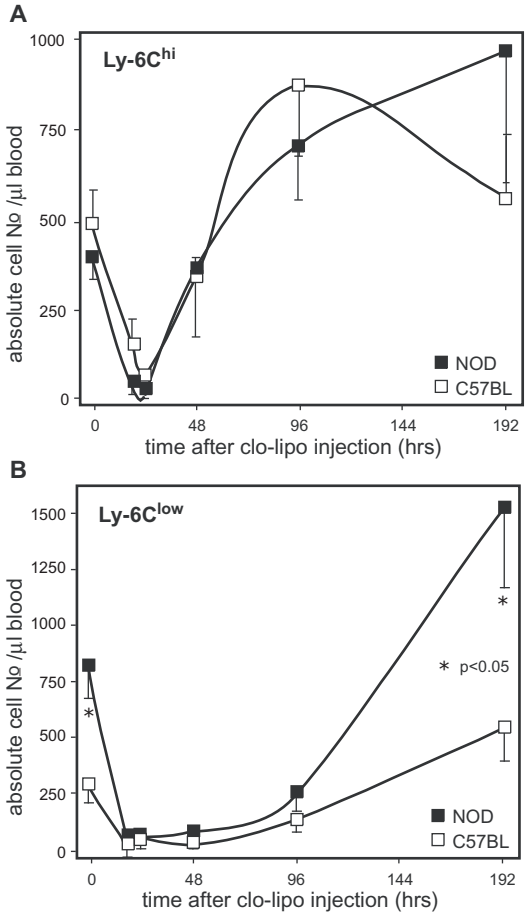
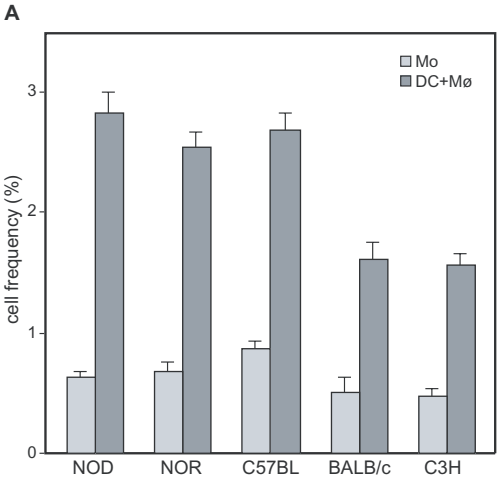


Figure 6. Normal restoration and similar kinetics of the monocyte-return after depletion with lip-CL_2MDP.
Numbers of Ly-6C^{hi} (A) and Ly-6C^{lo} monocytes (B) in the NOD (black squares) and the C57BL (empty squares) circulation are shown, at different time points after an i.v. injection of lip-CL_2MDP. Upon depletion of all monocytes, C57BL and NOD mice showed similar kinetics in restoration of both Ly-6C^{hi} and Ly-6C^{lo} monocyte populations. Data represent the average frequency ± SD of a minimum of 5 mice for each time point, for each mouse strain



B

Mo	NOR	C57BL	BALB/c	C3H
NOD	0.589*	**0.002**	0.234	0.102
NOR		0.055	0.242	0.093
C57BL			**0.009**	**0.002**
BALB/c				0.506

DC+Mø	NOR	C57BL	BALB/c	C3H
NOD	0.309	0.562	**0.002**	**0.002**
NOR		0.481	**0.001**	**0.000**
C57BL			**0.001**	**0.000**
BALB/c				0.925

* data represent p values

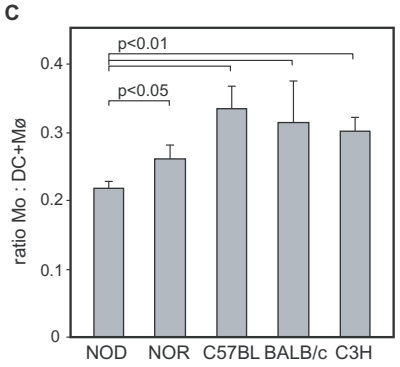


Figure 7. Different balance between mature (Ly-6C^{lo}) monocytes and mature myeloid cells (DC + mφ) in the spleen of NOD mice.

(A) The average frequency ± SEM of monocytes (Mo - light gray bars) and mature myeloid cells (DC+mφ - dark grey bars) within the total leukocyte pool of the spleen from different mouse strains were determined by phenotypic analysis (FACS). (B) P values derived from the statistical comparison (Student's t test) among all mouse strains for Mo or DC+mφ (C) To compensate for the large inter-strain variation, the monocyte : mature cell ratio was calculated. In this case, a significantly lower ratio was found in NOD mice as compared to all other mouse strains. Comparison of ratios between other mouse strains did not result in P values lower than 0.1. Data are calculated from 13 C57BL mice, 19 NOD mice and 5-8 mice of other mouse strains.

no sign of a reduced efflux of monocytes from the circulation to the periphery in NOD mice.

Next, we determined the frequency of the monocytes and of their progeny in the spleen (mφ and DC) in different mouse strains (Fig. 7A), and performed a detailed statistical analysis. As already mentioned, C57BL mice had higher monocyte frequency than any other mouse strain (Fig. 7B). In the case of mφ+DC, C57BL mice but also NOD and NOR mice had a significantly higher mφ+DC frequency than BALB/c or C3H mice (Fig. 7B). Hence, the strains unrelated to the NOD had the monocytes and mφ+DC values in balance; both were either high or low). In contrast, there was a disproportion in the monocytes and mφ+DC values in the NOD and partially in NOR mice (Fig. 7A). Therefore we calculated the monocyte : mφ+DC ratio in the spleen (Fig 7C). Indeed, the ratios were similar in C57BL, C3H and BALB/c mice. The NOR mice were in between other mouse strains and NOD mice had a significantly lower ratio (more mature cells than monocytes) than any other mouse strain. This indicated that the balance between monocytes and their progeny in the spleen is in NOD mice also tipped towards the more differentiated cells.

DISCUSSION

The recent phenotypic definition of two monocyte subsets in the normal mouse blood, i.e. the Ly-6C^{high} immature monocytes and the Ly-6C^{low} mature monocytes, enabled us to analyze blood monocytes in diabetes-prone NOD mice and compare with our previously reported data on monocyte subsets in human T1D [14].

In this study we found that NOD mice display an altered balance between Ly-6C^{high} immature monocytes and Ly-6C^{low} mature monocytes, i.e. the mice contained an abnormally high number of circulating mature monocytes. This phenomenon appeared to be intrinsic to the NOD background, since NOR and NODH2b mice also showed this altered balance. In addition, we found more DC and mφ in the NOD spleen relative to monocytes, when compared to other mouse strains. When isolated from the blood, NOD monocytes demonstrated an increased tendency to mature into mφ spontaneously, a feature found in control monocytes only after stimulation with LPS. In addition, although *in vivo* depletion of the blood monocytes by lip-CL_2MDP did not reveal significant differences in the release from the bone marrow or the transition time of immature to mature NOD monocytes, the shifted monocyte ratio was rapidly restored in the NOD mice after depletion. These observations support a view that mice with a NOD background show a skewing of cells of the monocyte lineage towards more differentiated cells in both the circulation and the periphery.

The here reported finding of an enlarged subpopulation of mature Ly-6C^{low} monocytes in the NOD mouse circulation raises the question of its functional significance for the aberrant development of islet autoimmunity in the NOD mouse

and the mechanism(s) that lead to the shift towards the more mature forms.

The true nature and function of the mature Ly-6C^{low} monocytes in the circulation is not yet clear. The cells correspond to the Gr1^{-}CCR2^{-}CX$_3$CR1^{hi} cells, that have been proposed to represent precursors for resident mφ and DC [8]. Indeed, mouse Ly-6C^{med} monocytes (included in the Ly-6C^{low} population in our gating) have been found to contain direct precursors for DC that upon antigen uptake migrate to LN [29]. Interestingly, the CCR2$^{-/-}$ mice used in the latter study show a shifted monocyte ratio towards mature cells similar to that found by us in the NOD mouse. Moreover, MCP-1/CCL2 - deficient mice have a similarly shifted monocyte ratio (D. Drevets, personal communication). These observations point to a defective CCR2/MCP-1 signaling as a potential cause for the shifted monocyte ratio in the NOD mouse. Indeed, we recently observed a deficient chemotactic response of NOD leukocytes toward CCL2/MCP-1 [28]. However, even if true, CCR-2 signaling probably is not responsible for all functional aberrations of the NOD monocytes reported here, like the increased adherence to fibronectin or the poor migratory response to inflammatory stimuli (including MCP-1) that we reported. Moreover, there are no reports of autoimmune processes in the CCR2$^{-/-}$ and MCP-1 (CCL2) deficient mice. However, since both NOD and autoimmunity-prone SJL mice (not shown) have a shifted ratio towards mature monocytes, autoimmunity and the shifted ratio in circulating monocyte subpopulations might be indirectly linked.

The Ly-6C^{low} monocyte population has been proposed to represent the mouse equivalents of human CD14^{+}CD16^{+} monocytes [8]. In humans, these monocytes are generally considered to act as an important pro-inflammatory effector-subset based on their inflammation-related amplification in the blood and high level production of pro-inflammatory cytokines upon stimulation [30]. Hence, a parallel can be drawn between the enlarged CD16^{+} pool during acute and chronic inflammations in the human [15-20] and the enlarged pool of Ly-6C^{low} monocytes in the NOD mouse that suffers from various (autoimmune) chronic inflammations. This further implies that the imbalance towards mature forms of circulating monocytes may relate to the proneness to develop (autoimmune) chronic inflammations rather than to the presence of such inflammations per se.

Is the higher percentage of fibronectin-adherent blood monocytes in the NOD mouse relevant for the autoimmune process? If taken irrespectively of degree of Ly-6C expression, we also found a raised adhesion to fibronectin of monocytes in patients with T1D [14], as reported here for the NOD mouse. However, like in the case of the increased Ly-6C^{low} monocyte pool, our data do not provide a formal proof of a causal link between fibronectin adherence and the autoimmune pathogenesis.

Also the reasons for the increased adherence of the NOD monocytes to fibronectin are not obvious from our data. We found a similar expression of adhesion molecules and several chemokine receptors in NOD as compared to C57BL mice. Perhaps the modified Ly-6C molecule in myeloid cells of the NOD

mouse plays a role in this abnormality [31]. Cross-linking of Ly-6C molecules on the surface of T cells induces integrin expression and has been associated with cell adhesion [32]. Expression of integrins involved in fibronectin and ICAM-1 binding were not modified in the NOD monocytes. Still, the recombination in the Ly-6C gene might have changed the function of this molecule in such a way that it aberrantly influences the activation of integrins and not their expression.

Direct comparison of our findings in the mouse with the human monocytes point to the possibility that the Ly-6C^{high}/Ly-6C^{low} subdivision of mouse monocytes might not completely correspond to the CD14/CD16 based division in the human.

The Ly-6C^{high} monocytes from C57BL mice have a selective capability to adhere to fibronectin and ICAM-1. In contrast, both CD16^{-} and CD16^{+} human monocytes similarly adhere to fibronectin [14]. Discrepancy between the populations is also present in autoimmune situation: the frequency of CD16^{+} circulating mature monocytes in human cases was not raised [14], while we here show an increased presence of Ly-6C^{low} monocytes in the NOD mouse blood. Alternatively, homology can be proposed between the mouse Ly-6C^{high} monocytes and the “P-monocytes” in humans [11], since they both have a clearly raised ability to adhere to fibronectin. However, the fibronectin-adhering monocytes in the NOD mouse blood were not only in the Ly-6C^{high} population. Therefore, the overlap between the Ly-6C^{high} and the “P-monocytes” also might not be absolute. Taken together, a word of caution is necessary in trying to define and compare monocyte subpopulations among species; the functional flexibility of the cells might make such definition troublesome.

With regard to the capability of blood monocytes of the NOD mouse to differentiate into cells with a DC- or mφ-like phenotype, we observed a tendency of both immature and mature monocytes to differentiate *in vitro* preferentially into cells with a mφ-like phenotype, i.e. F4/80^{high}CD11c^{low}MHCII^{low} cells. This strengthens our previously expressed view – based on in vitro development of BM precursors - that a differentiation into the DC direction is hampered in NOD mice and skewed into the mφ direction [33]. This abnormally skewed production of mφ-like cells could be due to dysfunctional signaling pathways, such as the hyperactivity of the NF-κB and ERK-1/2 pathways, that have been found previously in NOD mouse DC and mφ [34-36].

In conclusion, mice with the NOD background show larger numbers of mature monocytes in the circulation and a preferential development of mφ-like cells from both immature and mature monocytes. We can only speculate on the contribution of these described results to the proneness of NOD mice to develop various autoimmune conditions. We excluded the possibility that they are the consequence of the autoimmune inflammations, since they were also present in pre-diabetic NOD mice and inflammation-protected NOD-H2b and NOR mice. Conversely, if they are causally related to autoimmunity, we can envisage several mechanisms. First, the larger number of Ly-6C^{low} monocytes that preferentially mature into

mφ might support the destructive character of the spontaneous autoimmune inflammation. In addition, the imbalance between the generation of DC vs. mφ from monocytes in favor of the mφ lineage may play a role, particularly since DC are essential in tolerance induction and maintenance. Indeed, transfers with optimally functioning DC have proven to prevent the development of autoimmune diabetes in the NOD mouse [37-39]. Therefore, an insufficient production of high-quality DC, due to developmental aberrancies in their direct precursors, could form a key to the initiation of autoimmunity.

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CHAPTER 3.2

NON-OBESE DIABETIC MICE HAVE A SEVERELY IMPAIRED ABILITY TO RECRUIT LEUKOCYTES INTO SITES OF INFLAMMATION

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ABSTRACT

The accumulation of macrophages (mφ) and dendritic cells (DC) in the pancreas plays a crucial role in the pathogenesis of autoimmune diabetes. We studied the recruitment of monocytes, mφ and DC to sites of inflammation, i.e. the peritoneal cavity and a subcutaneously-elicited air pouch in the NOD mouse model of autoimmune diabetes.

The leukocyte recruitment was studied from 1 through 7 days after injection of thioglycollate (peritoneum), C5a (peritoneum, air pouch), CCL2 and CCL3 (air pouch). C57BL/6 and BALB/c mice served as controls. Morphological and flowcytometric analysis of the recruited cells was performed, IL-1β, TNF-α, IL-6, IL-12 and IL-10 in exudates measured, and in vitro CCL2-chemotaxis of exudate mφ (Boyden chamber) determined.

NOD mice were strongly impaired in the recruitment of mφ, DC and monocytes, as well as that of granulocytes. Chemokine-injected air pouches of NOD mice showed an increased IL-10 and a decreased IL-1β level, while the other cytokines were normally or very low expressed. In addition, NOD exudate Mø displayed an impaired in vitro CCL2-induced migration.

Our data show that NOD mice have an impaired ability to recruit leukocytes into sites of inflammation elicited in the peritoneum and the air pouch. A raised IL-10/IL-1β ratio at these sites and a deficient migratory capacity of NOD monocytes are important determinants in this impairment.

INTRODUCTION

Macrophages (mφ) and dendritic cells (DC) play a crucial role in the pathogenesis of type-1 diabetes. In rodent models, mφ and DC are the first cells to accumulate in the pancreas and are predominantly situated at the edges of the islets (1, 2). The cells play a role in the initiation and perpetuation of the islet autoimmune response by taking up islet antigens and by presenting these antigens to naïve islet-specific T cells in the draining lymph nodes (3). In later phases of the disease DC and mφ form an integral part of the lymphocytic insulitis. The mφ assist in these phases the lymphocytes in the destruction of the β cells. It is assumed that the islet-associated mφ and DC are mainly derived from monocytes recruited from the blood stream.

The recruitment of monocytes into sites of inflammation involves a complex series of steps. After margination in the circulation, the cells start to role on the endothelium to firmly adhering to it (4). Thereafter the cells squeeze through the endothelial layer and diapedese into the tissues. Selectins, integrins and chemokines play prominent roles in these processes (4-6). The early infiltrating monocytes induce endothelial cells to secrete chemokines and to up regulate the expression of vascular adhesion molecules by the production of inflammatory compounds, such as interleukin (IL)-1β, IL-6, tumour necrosis factor (TNF)-α and MRPs (myeloid related proteins) (7-10). This facilitates a further infiltration of monocytes. The recruited monocytes will differentiate into inflammatory mφ or DC that produce cytokines like IL-12 and TNF-α to maintain the inflammatory environment and to initiate an immune response (11, 12). Anti-inflammatory cytokines produced in the inflammation, such as IL-10 and transforming growth factor (TGF)-β, may exert suppressive effects by a down-regulation of the production of pro-inflammatory cytokines and will thus limit the inflammatory reaction (13, 14).

The non-obese diabetic (NOD) mouse is a widely used animal model to study type-1 diabetes. Here we report a study on the recruitment of inflammatory cells, including monocytes, into two separate sites of inflammation in the NOD mouse, i.e. into the peritoneal cavity (15) and into an air pouch elicited subcutaneously (16, 17). Inflammatory cells were attracted to these sites by injecting thioglycollate (TG) (peritoneum), the potent chemo-attractant C5a (peritoneum, air pouch) or the chemokines CCL2 (MCP-1) or CCL3 (MIP-1α) (air pouch). Interestingly, NOD mice were in all instances strongly impaired in the recruitment of monocytes (as well as that of granulocytes) into these sites of inflammation. IL-10 was clearly raised in the air pouch of NOD mice, while IL-1bβ levels were decreased. Anti-IL-10 treatment did only partially restore the severely hampered monocyte recruitment, indicating that the role of this cytokine is limited in the recruitment disturbances.

Mouse blood monocytes can be subdivided into two populations, based on the differential expression of the Ly-6C molecule (18); the immature monocytes (Ly-6C^{hi}) and the mature monocytes (Ly-6C^{low}). Immature monocytes preferentially migrate to sites of inflammation (18, 19). Here we will also report on the effects of

the induction of the inflammatory reaction in the air pouch on the frequency of Ly-6C^{hi} cells in the circulation of NOD mice. The outcomes urged us to study the in vitro (Boyden-chamber) chemokine-induced migratory capability of NOD exudate mφ.

MATERIALS AND METHODS

Animals

Female NOD/LTj mice were bred in our facilities under specified pathogen-free conditions. Female C57BL/6 and BALB/c mice were obtained form Harlan (Horst, The Netherlands). All mice were fed standard pellets and received water ad libitum. The cumulative incidence of diabetes at 30 weeks of age in our NOD mouse colony was 90% in females and 60% in males. All animal procedures were carried out with the approval of the Erasmus University Animal Welfare Committee.

Peritonitis

Peritonitis was induced by intraperitoneal injection of sterile thioglycollate (TG) (3% w/v in 0.5ml of sterile saline; Sigma, Steinheim, Germany). At various time points, the mice were killed by carbon dioxide exposure and peritoneal cavities were washed with 5 ml of PBS containing 0.02% EDTA (ethylenediaminetetraacetic acid; Sigma). Cells were subsequently centrifuged (5 min/500 g), resuspended in 1ml of RPMI supplemented with 10% FCS (Biowhittaker, Verviers, Belgium) and kept on ice for further analysis.

Air pouch model

The air pouch model was applied as described elsewhere (17). Briefly, pre-diabetic (5–7 wks) NOD and control C57BL/6 and BALB/c mice were subcutaneously injected on the back with sterile air (day 0: 5ml; day 3: 3ml). On day six, 0.5 mg of C5a (Sigma), CCL3 or CCL2 (Peprotech, London, UK) dissolved in 1ml of carboxymethylcellulose (Sigma; 0.5% in PBS, containing 10 ng/ml lipopolysaccharide) was injected into the air pouch. After 24 hours, the animals were killed and the pouches were washed with 1ml of PBS. Volume and cell number of the lavage fluid was recorded and 100 ml aliquots were used for flow cytometry. The remnant was centrifuged and the supernatant stored at -80°C for cytokine measurement.

To study the effect of IL-10, the air pouch was injected either with 1ml CCL2 in combination with a monoclonal antibody against IL-10 (0.5 mg/ml; JES-2A5.1, produced in our own laboratory) or with CCL2 in combination with recombinant mouse IL-10 (0.25 mg/ml; R&D systems).

Flow cytometry

Flow cytometric analysis was performed using a FACSCalibur apparatus (Becton Dickinson, Amsterdam, The Netherlands). The antibodies used were: biotinylated Ly-6C (ER-MP20; produced in our own laboratory), Ly-6G-PE, CD11b-PerCP-Cy5.5, CD11c-FITC, biotinylated CD11c (BD Pharmingen, Alphen aan den Rijn, The Netherlands) and F4/80-FITC (Caltag, San Francisco, USA). Streptavidin-APC (BD Pharmingen) was used as conjugate. For intra-cellular detection of IL-10, a biotinylated monoclonal antibody against IL-10 (JES-2A5.1) and the isotype control GL113 were used (both obtained from BD Pharmingen).

In vitro migration assay

The in vitro migration towards CCL2 (Peprotech) of inflammatory peritoneal Mø (thioglycollate-elicited, after 4 days) was evaluated using a Boyden chemotaxis chamber (Neuroprobe, Gaithersburg, MD, USA) and polycarbonate membranes (5 mm pore size; Whatman, Clifton, NJ, USA) as previously described. Mφ (3×106/ml) migration was determined after 4 hrs and expressed as a migration index (CCL2-migrated cells divided by the medium-migrated cells). Each experiment was performed in triplicate and cells were counted in five high power fields (1000X).

ELISA

The levels of IL-1β, IL-6 (R&D Systems, Minneapolis MN, USA), IL-10, IL-12 and TNF-α (BioSource, Camarillo CA, USA) were measured in air pouch exudates using ELISA, according to the manufacturers protocol.

Statistics

Data were analysed using Student's t test or Mann-Whitney. When more than two groups were compared, Kruskall-Wallis test was performed, followed by Mann-Whitney test to determine the significance of the differences between the individual groups. All data were analysed for two-tailed significance. A p-value below 0.05 was considered statistically significant.

RESULTS

Impaired influx of inflammatory cells into the peritoneal cavity in NOD mice

To study the in vivo recruitment of inflammatory cells, a sterile inflammation was induced by injecting thioglycollate (TG) intra-peritoneally (i.p.). In control C57BL/6 mice, inflammatory cells including granulocytes and monocytes were recruited early and in large numbers in the peritoneal cavity after TG injection as determined by morphological analysis using differential cell count (figure 1B and C, see insets for the morphological identification). The recruitment of monocytes and granulocytes was followed by an increase in the numbers of mφ with a maximum

at 4 days (figure 1D).

NOD mice showed a strongly decreased recruitment of inflammatory cells after TG injection as shown in figures 1A-D. Since the autoimmune-related cellular infiltrates in the pancreas, salivary glands and other autoimmune inflammations of the NOD might be responsible for the reduced recruitment into the peritoneum after TG administration (via f.i. redistribution or the cells), we also injected TG into very young mice (3 wks) prior to the development of such cellular autoimmune infiltrates. It is important to note, that such young NOD mice also showed a decreased recruitment of inflammatory cells into the peritoneum after TG injection (data not shown). In addition, the low influx of cells could not be explained by a delayed inflammatory response of NOD mice, since the kinetics of inflammation onset were similar in NOD and C57BL mice (figure 1A).

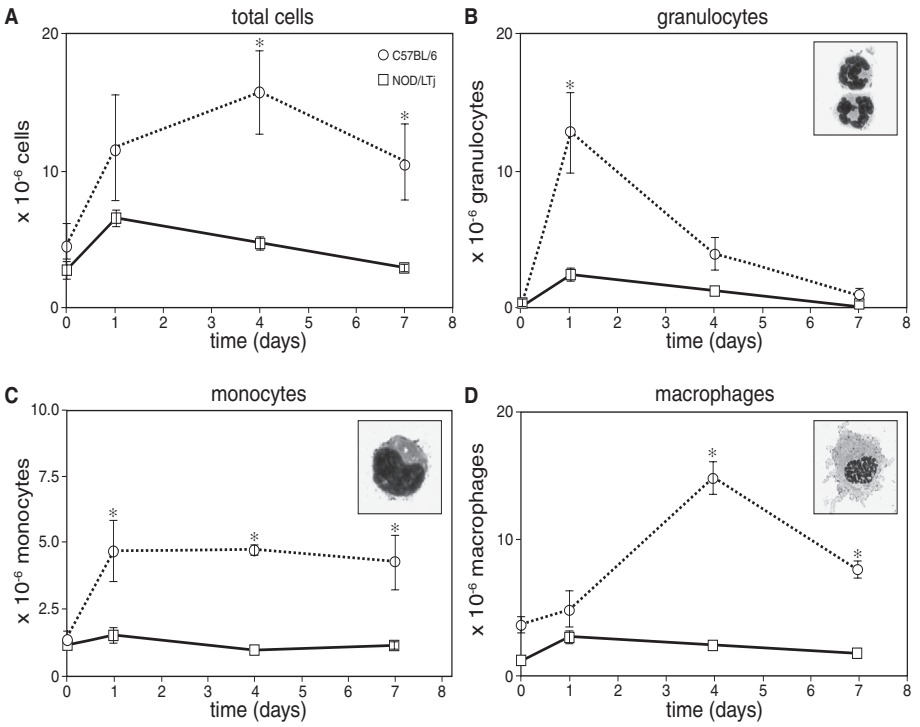

Figure 1.
NOD mice (5-7 wks) showed a strongly decreased influx of inflammatory cells after thioglycollate injection (A). Combined result of two experiments is shown (n=5/group, mean ± SEM). Morphological analysis (see insets for representative example) showed a decreased influx of granulocytes (B), monocytes (C) and mφ (D) in NOD mice. Data shown are representative of one experiment with three mice per group (mean ± SD). * means $p<0.05$ as determined with Student's t test.

With regard to the various subsets of inflammatory cells, the influx of granulocytes in the peritoneal cavity of NOD mice was strongly decreased as compared to that of the C57BL/6 mice (figure 1B), while the number of monocytes did not increase at all (figure 1C). There was a small increase in the number of mφ at day 1, but much reduced compared to C57BL/6 (figure 1D). Upon injection of another inflammatory substance, i.e. the chemoattractant C5a into the peritoneum, NOD mice also displayed a strongly reduced recruitment of granulocytes, monocytes and mφ in the peritoneal cavity in comparison to the control C57BL/6 mice (data not shown).

Severely reduced recruitment of inflammatory cells in the air pouch in NOD mice

To investigate whether reduced recruitment of inflammatory cells was a peritoneum-specific or generalized phenomenon in NOD mice, we also studied another controlled environment for inducing an inflammation by creating a pouch filled with sterile air on the back of the mouse (16, 17). The chemoattractant C5a was injected into the air pouch, which after 8 hrs in control C57BL/6 mice resulted in a 3-fold higher influx of inflammatory cells compared to animals that were injected with the vehicle only (figure 2A). Again granulocytes were the early infiltrating cells, while monocytes and mφ were the predominant cells after 48 hours as determined by morphological analysis using differential cell count (figure 2B-D). In NOD mice a lower number of granulocytes and monocytes were recruited in the air pouch after C5a injection (figure 2B and C); the number of mφ was only slightly and not significantly reduced (figure 2D).

We tested another potent granulocyte and monocyte chemoattractant in the air pouch model and injected CCL3. This experiment was carried out with an additional control strain, i.e. the BALB/c mouse, to exclude the possibility that the effect was specific for the C57BL/6 mouse. Again NOD mice showed a strongly reduced influx of inflammatory cells in the air pouch 24 hours after injection in comparison to these two control strains (figure 3A). This reduced recruitment again involved both the granulocytes and monocytes (figure 3B-C and Table 1).

Finally CCL2, a specific monocyte chemoattractant, was tested in the air pouch model. Again NOD mice showed a strongly diminished influx of cells into the air

Table 1. Recruitment of cells in absolute numbers in response to CCL3.

	total cells ($x10^{-6}$ cells)[a]		granulocytes ($x10^{-6}$ cells)		monocytes ($x10^{-6}$ cells)	
	control	CCL3	control	CCL3	control	CCL3
NOD/LTj	1.56 ± 0.34	1.53 ± 0.42	0.92 ± 0.14	0.84 ± 0.28	0.27 ± 0.05	0.25 ± 0.08
C57BL/6	2.21 ± 0.56	4.42 ± 0.93[b]	0.59 ± 0.01	1.93 ± 0.40[b]	0.06 ± 0.01[c]	0.16 ± 0.03
BALB/c	3.55 ± 0.51[c]	9.89 ± 1.13[b]	1.87 ± 0.54	3.69 ± 0.66[b]	0.12 ± 0.05	0.39 ± 0.09

[a] Data are shown as averages ± SE and each group represents at least six animals (5-7 wks of age)

[b] $p < 0.05$ (Mann-Whitney) relatively to NOD CCL3-injected animals

[c] $p < 0.05$ (Mann-Whitney) relatively to NOD control (vehicle injected) animals

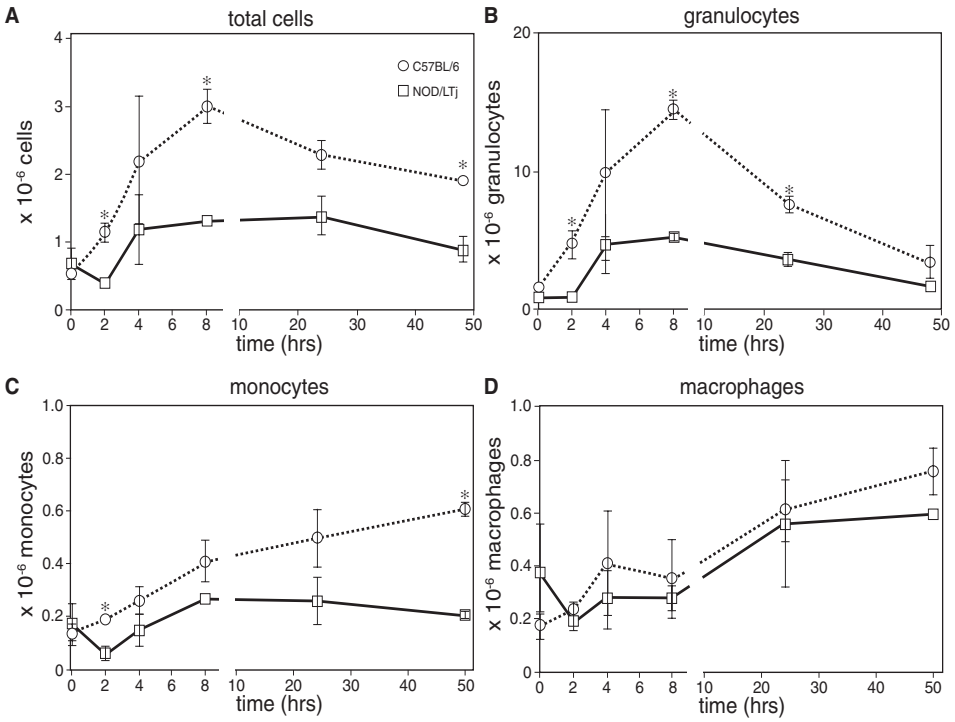

Figure 2.
NOD mice (5-7 wks) showed a strongly impaired recruitment of inflammatory cells into the air pouch 24 hrs after C5a injection (A). Morphological analyses showed less granulocytes (B) and monocytes (C) in NOD compared to C57BL/6. Mφ (D) increased similarly in time in C57BL/6 and NOD mice. Data are represented as mean ± SD (n=2/time point). Dashed line represents C57Bl/6, solid line represents NOD, * means $p<0.05$ relatively to NOD as determined with Student's t test.

pouch after 24 hours in comparison to C57BL/6 and BALB/c mice and this reduction mainly involved the influx of monocytes, which was very low in NOD after CCL2 injection (figure 3D and E, respectively). A significant recruitment of granulocytes could not be detected in response to CCL2 injection neither in control mice nor in NOD mice (data not shown).

Inflammation in the air pouch lowers the number of inflammatory circulating monocytes, but not in NOD

The monocytes that were recruited into the peritoneal cavity and into the air pouch derive from monocytes that circulate in the blood. These cells are direct precursors of mφ and DC (18). Therefore we studied whether the lower cell numbers found at the inflammatory site in the NOD mouse originated in lower recruitment

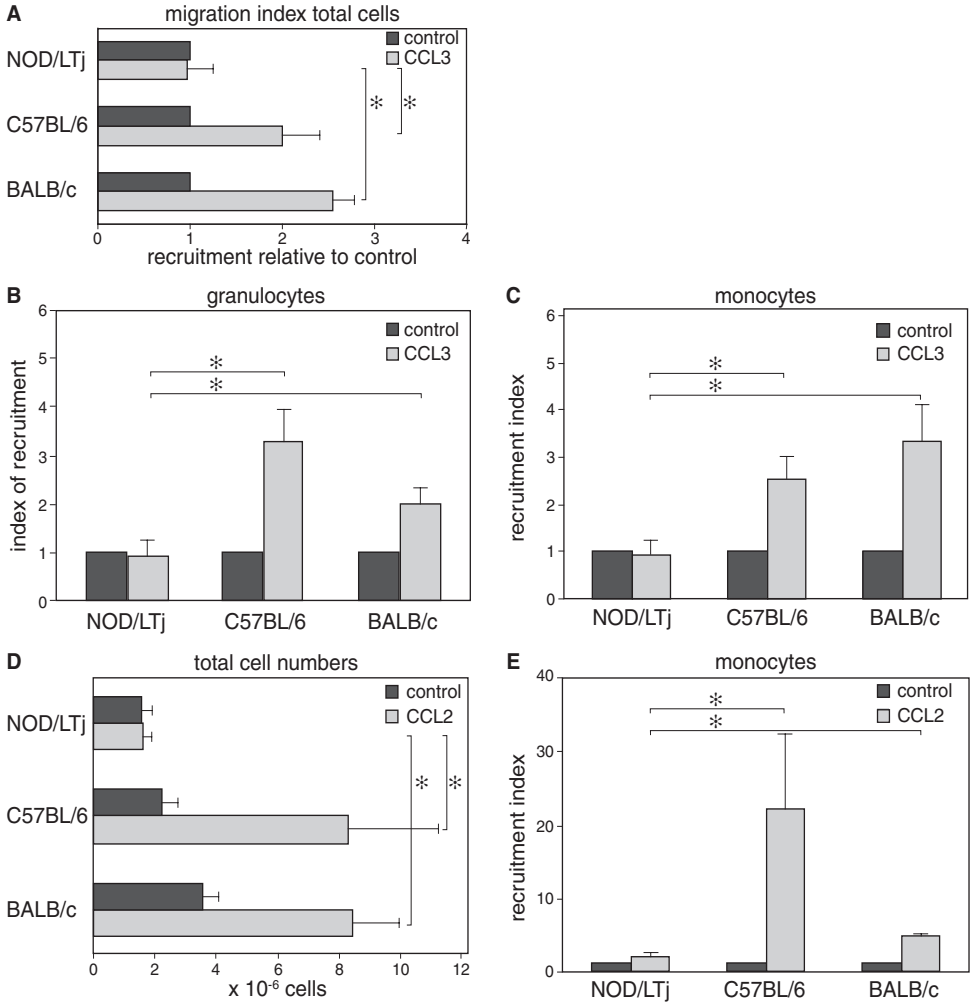

Figure 3.
The accumulation of cells 24 hrs after CCL3 injection was hampered in NOD (5-7 wks) mice (A). NOD mice failed to attract granulocytes (B) and monocytes (C). In response to CCL2, NOD mice showed a deficient accumulation of cells into the air pouch after 24 hours (D). The accumulation of monocytes to CCL2 was almost absent in NOD (E). Data are cumulative of two individual experiments and represented as mean ± SEM. N=6/group in figure A and C and n=4/group in figures B, C and E. * means $p<0.05$ relatively to NOD as determined with Mann-Whitney (A and D) or Student's t test (B, C and E).

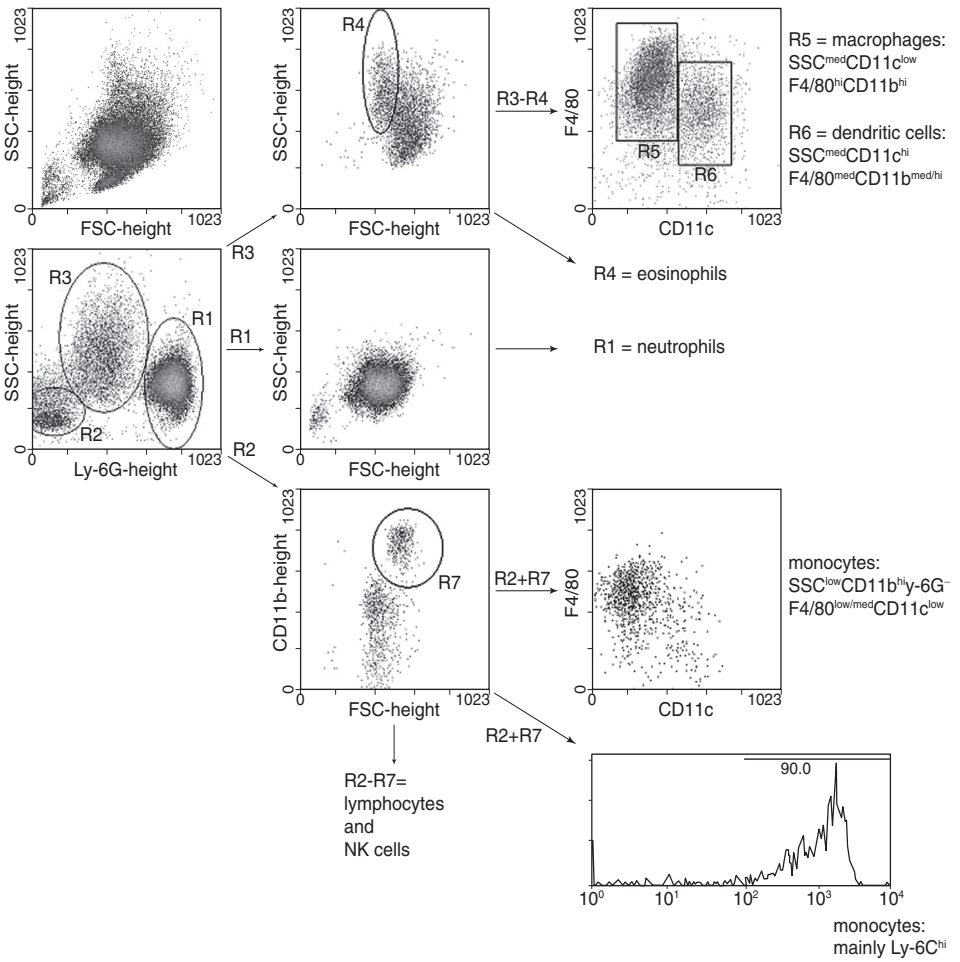

Figure 4.
Flow cytometric analysis of different cell types in air pouch exudates. Based on SSC and Ly-6G profile, three populations were defined (R1, R2 and R3). Monocytes were defined as $CD11b^{hi}Ly\text{-}6G^{-}F4/80^{low/med}CD11c^{low}$ in the R2 gate and based on their Ly-6C expression, two subpopulations are identified. Granulocytes were defined in the R1 gate and by exclusion from gate R3 on their high SSC profile. Mφ and DC could be defined using the gate R3 after exclusion of the granulocytes in gate R4. Mφ are $F4/80^{hi}CD11c^{low}$ (R5) and DC $F4/80^{med}CD11c^{hi}$ (R6). Representative figures are shown of a BALB/c mouse 24 hours after CCL3 injection.

of the cells from the circulation. Monocytes were defined in FACS analysis as $SSC^{low}CD11b^{hi}Ly\text{-}6G^{-}$ cells and were divided into two populations based on the expression of Ly-6C (18). Of these, the Ly-$6C^{hi}$ monocytes represent the recently bone marrow-emigrated, immature monocytes that are predominantly recruited into sites of inflammation (18). Figure 4 presents the gating method that we used

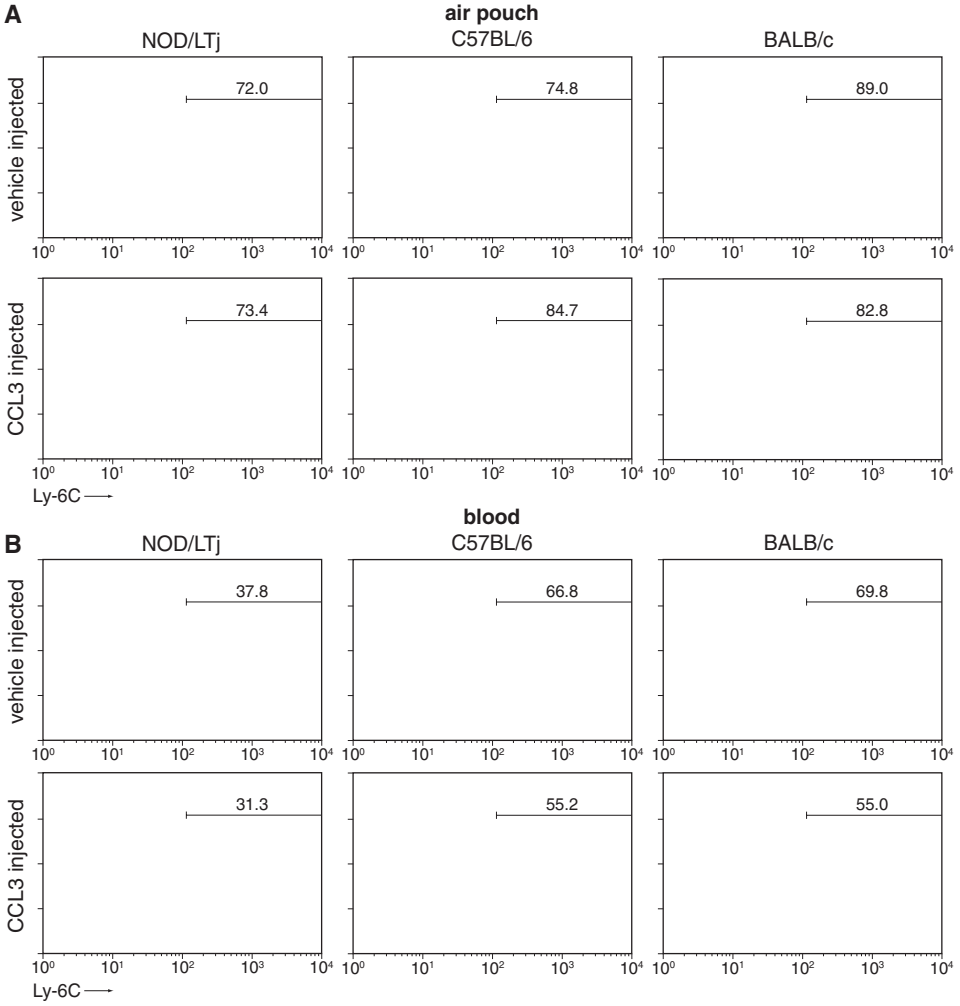

Figure 5.
The monocytes that are present in the air pouch either after CCL3 injection or after injection of vehicle were Ly-$6C^{hi}$ (A). The Ly-$6C^{hi}$ monocytes were present at lower frequency in the blood in NOD mice and after CCL3 injection a reduction in their frequency was observed, which in control mice is more pronounced (B). Representative histograms are shown (controls n=3; CCL3 n=6).

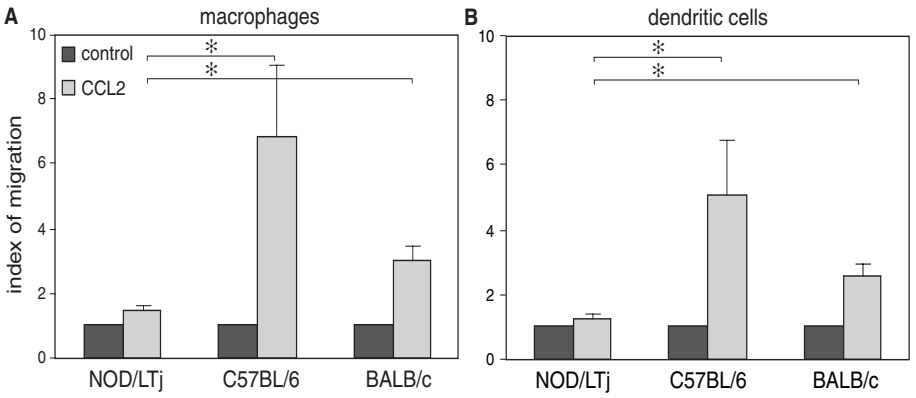

Figure 6.
The air pouch of C57BL/6 and BALB/c contained a high number of mφ (A) and DC (B) after CCL2 injection, while NOD mice did not. Mφ and DC were defined as shown in figure 4. Data are cumulative of two experiments and are represented as mean ± SEM, each bar represents at least four animals (5–7 wks), * means $p<0.05$ as determined with Student's t test.

to analyse the various types of leukocytes in the circulation or accumulated in the air pouch.

The monocytes that were present in the air pouch either before or after the injection of CCL3 were mainly of the Ly-6C^{hi} phenotype in the three mouse strains studied (figure 5A), thus demonstrating that also in the NOD mouse, the Ly-6C^{hi} monocytes preferentially accumulate at the inflammation site. In the blood of control mice a reduction of about 18% of the Ly-6C^{hi} monocytes was observed 24 hrs after CCL3 injection into the air pouch (figure 5B). NOD mice, however, only showed a mild reduction of 7% of the circulating Ly-6C^{hi} monocytes (figure 5B), which is consistent with an impaired recruitment of such monocytes into the air pouch. Interestingly, the frequency of Ly-6C^{hi} monocytes was decreased in the blood of NOD mice in comparison to control mice (figure 5B). However, when absolute numbers of these cells were calculated, the blood of NOD mice contained significantly less Ly-6C^{low} monocytes than C57BL/6 or BALB/c (not significantly) control mice (data not shown).

Low numbers of DC and mφ in the air pouch of NOD mice

To exclude the possibility that the decreased number of monocytes in inflamed air pouches in NOD mice could be the result of an enhanced differentiation of these cells into inflammatory mφ or DC, we performed a further analysis of the cells in the CCL2-injected air pouches in more detail, using flow cytometry. DC were defined as SSC^{med}CD11b$^{med/hi}$F4/80^{med}CD11c^{hi} cells and mφ as SSC^{med}CD11b^{hi}F4/80^{hi}CD11c^{low} cells (figure 4). When CCL2 was injected in control C57BL/6 and BALB/c mice an

increase in the number of both mφ and DC was observed after 24 hours (figure 6A and B). These increases were absent in NOD mice (figure 6A and B).

A partial role for IL-10 in the reduced inflammatory cell recruitment in NOD air pouches

The production of cytokines at a site of inflammation plays an important role in the attraction of inflammatory cells into that site of inflammation. Therefore, we determined the production of cytokines in the air pouch by performing ELISA on the exudate fluids that were retrieved from the air pouches. Figure 7A shows that the NOD mouse failed to produce IL-1β in the air pouch after CCL2 injection in contrast

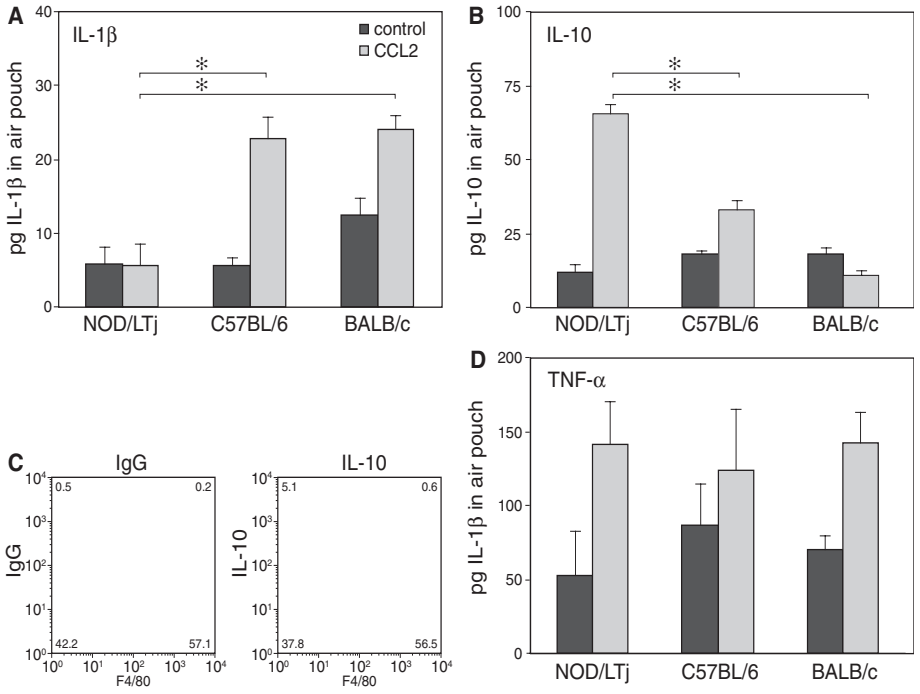

Figure 7.
The air pouch of control mice showed 24 hours after CCL2 injection an increased production of IL-1β, however not in NOD (A). NOD mice produced higher amounts of IL-10 in comparison to C57BL/6 and BALB/c mice (B). A representative flow cytometric analysis of intracellular staining for IL-10 (gated for $CD11b^+$ cells) shows that monocytes ($CD11b^{hi}F4/80^{med}CD11c^{low}$) were producing IL-10, while mφ ($CD11b^+F4/80^{hi}CD11c^{low}$) did not (C). Figures shown are representative of 5 NOD mice 24 hrs after injection with CCL2. The production of TNF-α in the air pouch of NOD mice was similar to control mice (D). Data are represented as mean ± SEM (n=5/group; 5-7 wks). * means $p<0.05$ relatively to NOD as determined with Student's t test.

to C57BL/6 and BALB/c mice. NOD mice exhibited a significantly higher production of IL-10 in the air pouch in comparison to the control mice (figure 7B). To investigate the primary source of the IL-10 in the air pouch, we performed intra-cellular flow cytometry staining of the cells isolated from the exudates of CCL2-injected NOD mice. A part of the F4/80$^{low/med}$ cells contained intracellular IL-10 and additional analyses demonstrated that these cells were CD11c^{neg}Ly-6C^{hi} monocytes (figure 7C and data not shown). The TNF-α production was not affected in the air pouches of the NOD mice (figure 7D). In all mouse strains the levels of IL-6 and IL-12 in the air pouch exudates were too low to reliably measure these cytokines (data not shown). Upon injection of CCL3 into the air pouches, similar results for IL-1β, IL-10 and TNF-α were obtained as for CCL2 (data not shown).

Since IL-10 affects the production of IL-1β (13) we argued that the high concentration of IL-10 in the air pouch might be responsible for the decreased IL-1β production and the diminished influx of inflammatory cells in the air pouch of NOD mice. To approach this experimentally, we injected recombinant mouse IL-10 together with CCL2 in the air pouch of control mice. Indeed we observed a decrease in the recruitment of inflammatory cells (figure 8A), but this did not seem to work via a reduction in IL-1β expression (figure 8B). In contrast, neutralization of IL-10 by injection of a neutralizing antibody to IL-10 together with CCL2, caused

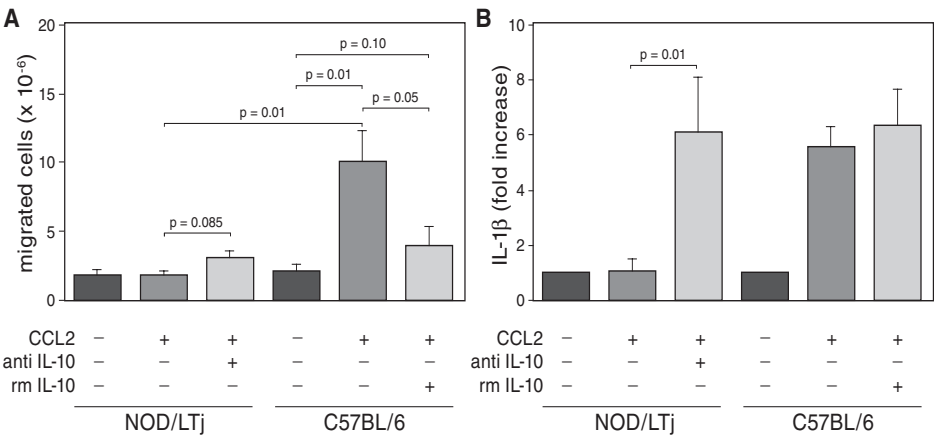

Figure 8.
Neutralization of IL-10 did not restore the cell recruitment towards CCL2 to the level of C57BL/6 mice (A). Addition of recombinant IL-10 to the air pouch of C57BL/6 mice decreased the recruitment towards the control level (A). Blocking IL-10 raised the level of IL-1β in the air pouch of NOD to a similar level of C57BL/6 mice (B). Addition of recombinant IL-10 in the air pouch of C57BL/6 did not lower the amount of IL-1β (B). Mean ± SD (n=6) are shown. In B data is presented relatively to the amount of IL-1β that was observed in control-injected animals. Statistical significance was determined with Kruskall-Wallis test and differences between groups using the Mann-Whitney test.

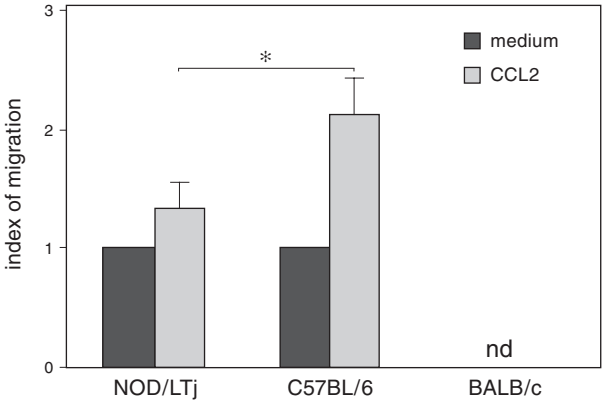

Figure 9.
Peritoneal exudate mφ of NOD mice showed a decreased migration towards CCL2 compared to C57BL/6 control mice using a Boyden chamber chemotaxis assay. Data are represented as mean ± SD (n=3), * means $p<0.05$ relatively to NOD as determined with Student's t test.

only a slight increase in the number of inflammatory cells in the air pouches of NOD mice (figure 8A), but it did enlarge the IL-1β levels significantly in the air pouch of these mice (figure 8B).

Taken together, the manipulation of IL-10 in the control C57BL/6 mice influenced the influx of inflammatory cells, but not through IL-1β. In the NOD mouse, a blockade of IL-10 contributed to the restoration of the IL-1β level, but this was not sufficient to achieve a recruitment of inflammatory cells in the air pouch compared to control C57BL/6.

NOD exudate mφ show an impaired in vitro CCL2 induced migratory capability

The impaired recruitment of inflammatory cells in NOD might not only be due to specific alterations in the air pouch environment (such as the cytokine environment), but also to intrinsic migratory aberrancies of the inflammatory cells. Therefore, we performed in vitro chemotaxis using a Boyden chamber. Since these experiments would require more blood monocytes than could be obtained practically, we used recently emigrated monocytes, i.e. peritoneal exudate mφ that had been elicited with TG. We observed that NOD mφ showed a decreased migration in vitro towards CCL2 in comparison to C57BL/6 mice (figure 9).

DISCUSSION

This study shows that NOD mice exhibit a strongly reduced recruitment of monocytes, but also of granulocytes, into the peritoneal cavity and into artificially made skin air pouches after injection of various chemokines (C5a, CCL3 and CCL2) and after injection of the non-specific inflammatory compound thioglycollate (TG). Our air pouch model confirmed, that the recruited monocytes are of the inflammatory, recently bone marrow-emigrated Ly-6C^{hi} subset. During, such recruitment Ly-6C^{hi} monocytes concomitantly decrease in the circulation, shifting the balance from Ly-6C^{hi} in the direction of Ly-6C^{low} cells. This inflammation-induced shift in blood monocyte subsets was hardly observed in NOD mice after eliciting inflammation. This observation additionally supports the view that the NOD mouse is severely hampered in the recruitment of monocytes to the mentioned sites of inflammation. It could be speculated that NOD mice have a reduced potential to recruit monocytes due to the accumulation of monocytes and monocyte-derived DC and mφ in the autoimmune insulitis, sialoadenitis or other autoimmune inflammations characteristic of the NOD mouse. However, our experiments show that 3-week-old mice, that do not yet show such autoimmune inflammation, do show the inability to recruit monocytes into the peritoneal cavity. Moreover, granulocytes failed to be recruited too, and granulocytes are hardly seen in the NOD autoimmune inflammations.

In relation to the impaired recruitment, we observed that NOD mice failed to increase the production of IL-1β in the artificially induced air pouch. This low level of IL-1β likely contributes to the decreased recruitment of inflammatory cells, since IL-1β induces the expression of adhesion molecules on endothelial cells and thus plays a role in the influx of leukocytes from the blood (8, 9). We also observed an increase in the production of IL-10 in NOD mice. This cytokine has been implicated in a reduced trafficking of leukocytes (20, 21). The increased IL-10 production observed in the CCL2-inflamed air pouch of NOD mice may even be considered a primary abnormality since IL-10 is able to suppress the local production of pro-inflammatory cytokines, such as that of IL-1β (13). Indeed, neutralization of IL-10 at the site of inflammation by a neutralizing antibody increased the level of IL-1β in the air pouch of NOD mice. However, it only partially restored the full influx of cells to the level of control mice. Taken together our findings indicate a definite, but only limited role of the enhanced local IL-10 production in the reduced influx of inflammatory cells in NOD mice and imply that other factors must also be involved. The cells responsible for the altered IL-10 and IL-1β production in the NOD air pouch have not been studied, but - though few in number - the mφ and monocytes present in the air pouch are likely candidates, since mφ and monocytes are in general prime sources of these cytokines.

Which could be factors other than cytokines playing a role in the severely hampered recruitment of inflammatory cells, including that of monocytes, mφ and

DC in the artificially induced inflammations in the NOD mouse? NOD mice exhibit a C5 deficiency, a constitutive lower number of circulating inflammatory monocytes, a defective differentiation of DC and mφ, an altered expression of adhesion molecules on endothelium in inflammation and a diminished chemotactic responsiveness of mφ and DC and these are all factors that could be responsible.

NOD mice have a complete C5 deficiency (22), which could play a role in the here-described hampered recruitment response to C5a. However the accumulation of inflammatory cells after injection of other chemo-attractants, such as CCL2 and CCL3 was also defective.

In the steady state, non-inflammatory condition a constitutive decreased number of inflammatory monocytes is observed in the blood of NOD mice as compared to that of control mouse strains (this report), which might certainly be a factor of importance for the reduced recruitment of the Ly-6C^{hi} monocytes into the inflammations. However, the lower quantity of Ly-6C^{hi} blood monocytes is not the decisive factor, since of those that are present, a low percentage did leave the circulation and entered the air pouch inflammation. Thus, the NOD mice are able to recruit Ly-6C^{hi} monocytes into inflammation, but in strongly decreased numbers, suggesting that not so much the number of circulating Ly-6C^{hi} monocytes, but rather the migratory potential of the monocytes is of importance in the decreased monocyte recruitment.

Mouse monocytes have the potential to differentiate rapidly into DC (23), raising the possibility that the decreased monocyte recruitment in comparison to control mice could be due to an increased differentiation of monocytes into descendent DC. However, we were not able to find increases in the number of DC or mφ in the air pouch after CCL2 injection in NOD mice. Moreover there is ample evidence that the development of precursors to descendent DC and mφ is hampered in the NOD mouse (24-26).

Aberrancies of the NOD endothelial cells, such as a low expression of adhesion molecules, may also have determined a poor emigration of the cells from the blood stream. However there is ample evidence in the literature that adhesion molecules are over-expressed on endothelial cells of the NOD mouse in inflammations, be it that all studies concentrated on the infiltrated islets (27), the thyroiditis (28) or the sialoadenitis (27). We have not determined the expression levels of adhesion molecules in the artificially induced inflammations used here.

Last but not least aberrancies in the inflammatory cells themselves may have played a role in the decreased recruitment of cells in the artificially induced inflammations. A lower responsiveness to the used chemokines or an altered capability to adhere to endothelial cells may have been causes for “lazy leukocytes” in the NOD mouse. We recently found a lower chemotactic responsiveness of bone marrow-derived DC towards the chemokines CCL2 and CCL19 (manuscript in preparation) and here we show that also NOD exudate mφ display a reduced migratory response to CCL2 in vitro. A diminished chemotactic response of

monocytes of type 1 diabetes patients to casein and C5a in a Boyden chamber (29) and to zymosan-activated medium using an underagarose assay (30) was reported previously. In addition, we found in type 1 diabetes patients a decreased responsiveness of monocytes to fMLP and CCL2, but not to CCL3, CCL4 and CXCL12 using the various migration assays (manuscript in preparation). Also a diminished fMLP-induced cytoskeletal rearrangement of monocytes has been observed in autoimmune thyroiditis patients (31, 32) and in type 1 diabetes (unpublished observations). With regard to a putative altered adhesiveness of inflammatory cells to endothelium we have actually found a raised adhesiveness of monocytes of type 1 diabetes patients to endothelium and the extra cellular matrix protein fibronectin (unpublished observations and ref (33)).

It is also important to note the contrast that exists between the here-reported poor recruitment of NOD monocytes, mφ and DC into artificially induced acute inflammations and the well-established “spontaneous” accumulation of monocytes, mφ and DC in the pancreas and other sites of chronic autoimmune inflammation of the NOD mouse. It is generally assumed that the mφ and DC in these chronically inflamed tissues of NOD mice are derived from infiltrating monocytes and/or precursors circulating in the blood. However it cannot be ruled out that at least part of the accumulation of DC and mφ stems from the proliferation of local precursors. Such local precursors have been found in the liver (34), the thymus (35) and the peritoneal cavity (36).

There are also other not-mutually exclusive possibilities to explain the difference. An accumulation of monocytes, mφ and DC at a site of inflammation is not only the outcome of an influx of precursors from the blood (and/or a local proliferation of precursors), but also of the efflux of such cells. Furthermore it should be noticed that we investigated the recruitment of NOD monocytes, mφ and DC into sites of acute inflammation in response to single chemoattractants, whereas the chronic autoimmune inflammations in the NOD mouse are the result of a range of inflammatory factors.

In conclusion this is the first report that shows that NOD mice have an impaired ability to recruit infiltrating leukocytes into sites of artificially induced inflammation (peritoneal cavity and air pouch). To explain this phenomenon we favour a view that not only an increased IL-10/IL-1β ratio, but also a deficient migratory capacity of the monocytes of the NOD mouse plays a role. This defect is probably present in cells of the myeloid lineage, since granulocyte recruitment in NOD mice was also strongly reduced. Whether the here-described impaired migration also applies to cells accumulating in the well-known autoimmune inflammations in the NOD mice is the focus of our current investigations.

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CHAPTER 4

ADHESION AND MIGRATION OF DENDRITIC CELLS IN THE NON-OBESE DIABETIC MOUSE

CHAPTER 4.1

EVIDENCE FOR AN ENHANCED ADHESION OF DENDRITIC CELLS TO FIBRONECTIN AND A ROLE OF THE LYMPHOID TISSUE-RELATED CHEMOKINES CCL19 AND CCL21 IN THE VERY EARLY ACCUMULATION OF DENDRITIC CELLS AROUND THE PRE-DIABETIC ISLETS OF THE NOD MOUSE

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Submitted

ABSTRACT

The non-obese diabetic (NOD) mouse is a widely used animal model for the study of human diabetes. The lymphocytic (peri-)insulitis is preceded by an early accumulation of dendritic cells (DC) close to and around the islets of Langerhans. This DC accumulation is thought to derive from an influx of inflammatory monocytes attracted by pro-inflammatory chemokines. Besides chemokines, extra-cellular matrix (ECM) proteins play an important role in the accumulation of immune cells in tissues. We studied the expression of the chemokines CCL2, CCL5, CXCL10, CCL19 and CCL21 by ELISA in pancreas lysates of NOD mice over time as well as by immunohistochemistry. In addition, we studied the adhesive capacity of bone marrow-derived DC (BMDC) of NOD and control mice to the ECM component fibronectin. DC in the NOD pancreas accumulated at sites with an intense expression of fibronectin (the isthmus of the islets). In vitro, NOD immature and mature BMDC showed an increased adhesion to fibronectin and a corresponding increased expression of VLA-5. At the time of early DC accumulation (before 10 weeks of age) the lymphoid-tissue related chemokines CCL19 and CCL21 were found increased. Our findings support a view that the early accumulation of DC around the NOD islets is not the consequence of an enhanced attraction of precursors and immature DC by pro-inflammatory chemokines. It rather might be the consequence of an aberrantly enhanced adhesion of NOD DC to fibronectin and of an aberrant retention of mature DC by lymphoid-tissue related chemokines.

INTRODUCTION

The non-obese diabetic (NOD) mouse is widely used as a spontaneous animal model to study the development and progress of autoimmune type-1 diabetes. Prior to the massive infiltration of lymphocytes around and into the islets of Langerhans in the pancreas of NOD mice (7-12 wks of age) professional antigen presenting cells such as the dendritic cells (DC) accumulate at the islet periphery particularly at the ductal-vascular poles (4-7 wks of age) (1, 2). The mechanisms regulating the accumulation of such cells remain to be clarified; yet it is generally assumed that pro-inflammatory chemokines are important mediators in the attraction of leukocytes to the islets (3-5).

Chemokines form a super-family of small (8-10 kDa), inducible cytokines that play a crucial role in infection and immunity (6). Upon inflammation the expression of chemokines like CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES) and CXCL10 (IP-10) is induced and these chemokines attract leukocytes from the bloodstream to the inflammatory site (7). Receptors for these chemokines are abundantly expressed on circulating monocytes, an important precursor population of the DC (8). In inflammation, the locally produced pro-inflammatory cytokines induce maturation and a switch in chemokine receptor expression of the DC by down regulating CCR1, CCR2 and CCR5 (receptors for the pro-inflammatory chemokines CCL2, CCL3, CCL4 and CCL5) and up regulating the expression of CCR7, the receptor for CCL19 (MIP-3β, ELC) and CCL21 (6Ckine, SLC)(9, 10). CCL19 and CCL21 are constitutively and abundantly expressed in lymphoid tissue. The migration of DC from inflamed tissues to the lymph node is thought to be mediated via above-mentioned switch in chemokine receptors on the DC, since the cells loose their ability to dwell at the inflammatory site, but gain in capacity to home to lymphoid tissue (“weigh the anchor, hoist the sail”) (10, 11).

We determined the expression of CCL2, CCL5, CXCL10, CCL19 and CCL21 in NOD pancreases from 5 through 20 weeks of age by quantitative ELISA on pancreas lysates and by immuno-histochemistry. The findings urged us to also test the migratory response of immature and mature bone marrow-derived DC (BMDC) of NOD and control mice to CCL19.

Besides chemokines, extra-cellular matrix (ECM) proteins play an important role in the accumulation of immune cells in tissues. To effectively migrate, immune cells interact with the surrounding extracellular matrix (ECM) proteins. This contact is mediated via integrins. Integrins are trans-membrane glycoproteins that are composed of a non-covalently associated α and β chain (12). Via the extra-cellular domain integrins bind components of the ECM and intracellular they are linked to the cells’ cytoskeleton, thus linking the cells’ cytoskeleton to the surrounding ECM. A major constituent of the ECM is fibronectin (FN), to which cells bind using the very late antigen-4 (VLA-4; CD29/CD49d) or VLA-5 (CD29/CD49e). In a flanking study we showed that pancreas macrophages (mφ) accumulate at sites of intense

FN labelling (13). We therefore also studied the expression of FN in 5-week-old NOD mice pancreases and determined the adhesive capacity of NOD DC to FN coated onto plates and the surface expression of VLA-4 and VLA-5 on NOD DC.

MATERIALS AND METHODS

Animals

NOD/LTj mice were bred in our own facilities under specified pathogen-free conditions and female mice of 5-7 weeks of age were used for the experiments. Female C57BL/6 and BALB/c mice were obtained from Harlan (Horst, The Netherlands) and female NOR/LTj mice were purchased from the Jackson Laboratory (Bar Harbor ME, USA). All mice were fed standard pellets and received water ad libitum. The cumulative incidence of diabetes at 30 weeks of age in our NOD colony was 90% in females and 60% in males. All animal procedures were carried out with the approval of the Erasmus University Animal Welfare Committee.

Chemokine determination

Pancreas lysates were prepared by homogenization of half of the pancreas in ice-cold phophate-buffered saline (PBS) supplemented with protease inhibitor cocktail (1 tablet in 10 ml; Life Technologies, Paisley, UK). The other half of the pancreas was used for immunohistochemistry. The lysates were sonificated twice for 30 seconds and centrifuged at 10 000g at 4°C for 10 min. The supernatant was collected and stored at -20°C. The protein concentration in the pancreas lysates was measured using the Bradford method (Bio-rad laboratories GmbH, München, Germany). Samples were assayed for murine CCL2, CCL5, CCL19, CCL21 and CXCL10. All ELISAs were ordered from R&D Systems (Minneapolis MN, USA) and performed according manufacturers protocol.

Immunohistochemistry

Pancreases were embedded in Tissue-Tek (Sakura, Zoeterwoude, The Netherlands) and snap-frozen in liquid nitrogen. Cryostat sections of 6 µm thickness were fixed for 5 min in 4% pararosanilin (Sigma, St. Louis, MO) and rinsed in PBS (pH 7.8) with 0.1%Tween20 (Fluka, Buchs, Switzerland). Slides were incubated with primary antibodies specific for fibronectin (kindly provided by dr. S.B. Geutskens), CD11c (N418), CD3 (KT3), B220 (B220), insulin (Dako, Glostrup, Denmark), CCL5 (Santa Cruz Biotechnology Inc, Santa Cruz CA, USA), CXCL10 (Santa Cruz), biotinylated CCL21 (R&D Systems) and the IgG control (PH2-4a, own laboratory). Subsequently, slides were washed with PBS-Tween-20 and incubated with appropriate peroxidase-coupled second step: goat-anti-hamster (Jackson ImmunoResearch Laboratories, West Grove PA, USA), rabbit-anti-

guinea, rabbit anti-rat or peroxidase-coupled streptavidin (Dako) in the presence of 2% normal mouse serum. The 3-amino-9-ethylcarbazole substrate (AEC; Sigma) in 50 mM sodium acetate/0.02% hydroxyperoxide or nickel-3,3'-diaminobenzidine tetrahydrochloride (Ni-DAB; Sigma) in 1% hydroxyperoxide was used to detect peroxidase activity and haematoxylin (Merck, Darmstadt, Germany) or nuclear fast red (Fluka) as counterstaining, respectively. Insulitis was evaluated by the analysis of at least 50 islets according to the following scale: 0, no infiltrating cells; 1, few infiltrating cells para- or peri-insular; 2, large numbers of infiltrating cells around the islet and 3, large numbers of infiltrating cells in the islet.

For immunofluorescence staining, slides were incubated with avidin and biotin (Vector Laboratories Inc, Burlingame CA, USA) to block endogenous biotin. For double staining, slide were incubated with biotinylated CCL21 (R&D Systems), followed by FITC-coupled extravidin (kindly provided by dr. S.B. Geutskens) or Texas Red-coupled streptavidin (Caltag, San Francisco CA, USA) in the presence of 2% normal mouse serum. As second antibody, slides were stained for: fibroblasts (ER-TR7, own laboratory), von Willebrand factor (kindly provided by dr. S.B. Geutskens), macrophages (F4/80 and BM8, BMA Biomedicals, Augst, Switzerland; ER-MP23, own laboratory), dendritic cells (FITC-coupled HL3; BD Pharmingen, Alphen aan den Rijn, The Netherlands), monocytes (Ly-6C; ER-MP20, own laboratory), B cells (B220) or T cells (KT3), followed by Texas Red-coupled goat anti rat (Southern Biotechnology Associates Inc, Birmingham AL, USA) in the presence of 2% normal mouse serum. The slides were mounted in DAPI-containing vectashield (Vector Laboratories Inc) and examined by conventional fluorescence microscopy using a Zeiss Axioplan 2 imaging fluorescence microscope (Zeiss, Göttingen, Germany).

Generation of bone marrow-derived DC (BMDC)

Mice were euthanized with CO_2 and bone marrow cells were isolated form the femora. DC were generated from bone marrow cells in RPMI medium 1640 (Cambrex, Verviers, Belgium) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin (Cambrex) in the presence of GM-CSF (20 ng/ml; BioSource, Camarillo CA, USA) for 7 days. DC were enriched for immature cells by immuno-magnetic depletion of mature cells. Cells were labeled with undiluted supernatant of rat anti-CD86 hybridoma (GL-1, cultured in own laboratory), followed by incubation with rabbit anti-rat IgG beads (Miltenyi, Bergisch Gladbach, Germany) and applied over the AutoMACS (Miltenyi). To generate mature DC, enriched immature cells were stimulated overnight with 100 ng/ml lipopolysaccharide (LPS, Sigma, St. Louis MO, USA).

Purification of spleen DC

Splenic DC were isolated using CD11c magnetic bead isolation kit (Miltenyi) and the AutoMACS following manufacturers protocol. A purity of over 95% $CD11c^+$ cells was obtained.

Adhesion

Adhesion of BMDC and splenic DC was performed as described previously (14). Briefly, cells were allowed to adhere for 60 min to 96-wells plates coated with fibronectin (10 µg/ml; Sigma), collagen I (10 µg/ml; Sigma) collagen IV (10 µg/ml; Sigma) or VCAM-1 (5 µg/ml; R&D systems). Cells were fixed with 20% formaldehyde (Merck) and stained with 1% methylene blue (BDH Chemicals, Poole, UK) in 0.01 M boric acid. The staining intensity was measured at 650 nm using an ELISA reader (Thermo labsystems, Amersfoort, The Netherlands). Adhesion to endothelial cells was studied as previously described (15). Pancreatic endothelial cells (MS1; ATCC, Manassas VA, USA) were grown to confluence in a flat-bottomed 96 wells plate in DMEM (Cambrex), supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. Prior to the experiment, the endothelial cells were washed with RPMI 1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin, which was the same medium as used for the culturing of BMDC and the rest of the experiment. DC were labeled with $Na_2{}^{51}CrO_4$ (Amersham BioSciences, Uppsala, Sweden) and co-incubated with the endothelial monolayer for 60 min at 37 °C. Non-adherent cells were washed away and adherent cells were lysed with 0.1 ml of 1% SDS, 0.05% NaOH. Radioactivity of non-adherent and adherent cells was measured and results are expressed as percentage of adherent cells.

Transmigration

The endothelial cell line MS1 (ATCC) was used for the transendothelial DC migration. Migration assays were performed as described previously (15). Briefly, a confluent monolayer of endothelial cells was grown on fibronectin (10 µg/ml; Sigma) coated transwells (24-wells; Costar; Acton MA, USA) of 6.5 mm diameter, with 5-µm pore filters. Mature and immature DC were labeled with $Na_2{}^{51}CrO_4$ (Amersham BioSciences) and incubated for 60 minutes at 37°C. After thoroughly washing, 100 µl cells ($1x10^5$ cells) were added to the endothelial monolayer. In the lower compartment of the transwell, the chemokines CCL2 (MCP-1, 100 ng/ml; PeproTech, Rocky Hill NJ, USA) or CCL19 (MIP-3β, 100 ng/ml; PeproTech) were added. After an incubation of 90 minutes at 37°C the upper compartment was washed and together with the filter and the lower compartment collected and measured for radioactivity. Migration was calculated as the radioactivity measured from the down-side of the filter and the lower compartment divided by the total radioactivity. Migration is shown as percent of migration observed towards medium.

Chemotaxis

The in vitro migration towards CCL19 (100 ng/ml, PeproTech) of DC was evaluated using a Boyden chemotaxis chamber (Neuroprobe, Gaithersburg MD, USA) and polycarbonate membranes (5 µm pore size; Whatman, Clifton NJ, USA)

as previously described (16). DC (1.5×10^6/ml) migration was determined after 90 min and expressed as a migration index (chemokine-migrated cells divided by the medium-migrated cells). Each experiment was performed in triplicate and cells were counted in five high power fields (1000X magnification).

Flow cytometry

Fluorescence flow cytometric analysis was performed using a FACSCalibur apparatus (Becton Dickinson, Amsterdam, The Netherlands). The antibodies used were: CD11b-FITC, CD11c-FITC, biotinylated CD11c, CD18-PE, biotinylated CD29, CD49d-PE, biotinylated CD49e, CD80-PE, CD86-FITC (all from BD Pharmingen), biotinylated MHC class II (NOD and NOR, 10.2.16, own laboratory; C57BL/6 and BALB/c ER-TR3, BMA Biomedicals) and streptavidin-APC (BD Pharmingen) was used as conjugate. Viability of the cells was determined using 7-aminoactinomycin D (7-AAD; Molecular Probes, Leiden, The Netherlands). Flow cytometry was performed on BMDC that were stimulated with LPS (100 ng/ml, Sigma), CCL19 (100 ng/ml, PeproTech), CCL21 (100 ng/ml, PeproTech) or unstimulated.

Statistical analysis

Data are shown as means ± SEM, and comparisons between groups were carried out by unpaired, two-tailed Student's t test. A p-value of less than or equal to 0.05 was considered as statistically significant.

RESULTS

Expression of pro-inflammatory chemokines

We investigated the expression of chemokines in the pancreas of NOD mice by quantitative ELISA on pancreas lysates and by immunohistochemistry. Figure 1A-C shows that CCL2 (MCP-1), CCL5 (RANTES) and CXCL10 (IP-10) were hardly expressed in the pancreas lysates of the two control mouse strains and that none of these inflammatory chemokines showed an increased expression during the early phases of DC accumulation in 5-7 week-old NOD mice. The expression of CCL5 (figure 1B) increased with age, being clearly increased above the level of control mice at later age, i.e. from 10 wks onwards (during the lymphocyte accumulation). The level of CCL5 in the pancreas stayed at a constant, high level throughout the further period of observation (figure 1B). The expression of CXCL10 increased with age up to the age of 10 weeks, being increased in NOD over control mice from 7-10 wks of age onwards (figure 1C). After 10wks the expression of CXCL10 declined and approached the level of the control animals at 20 wks of age (figure 1C). The expression of CCL5 (figure 1D) and to a lesser degree that of CXCL10 correlated to the degree of lymphocyte insulitis (figure 1E), while that of CCL2 expression did not (data not shown).

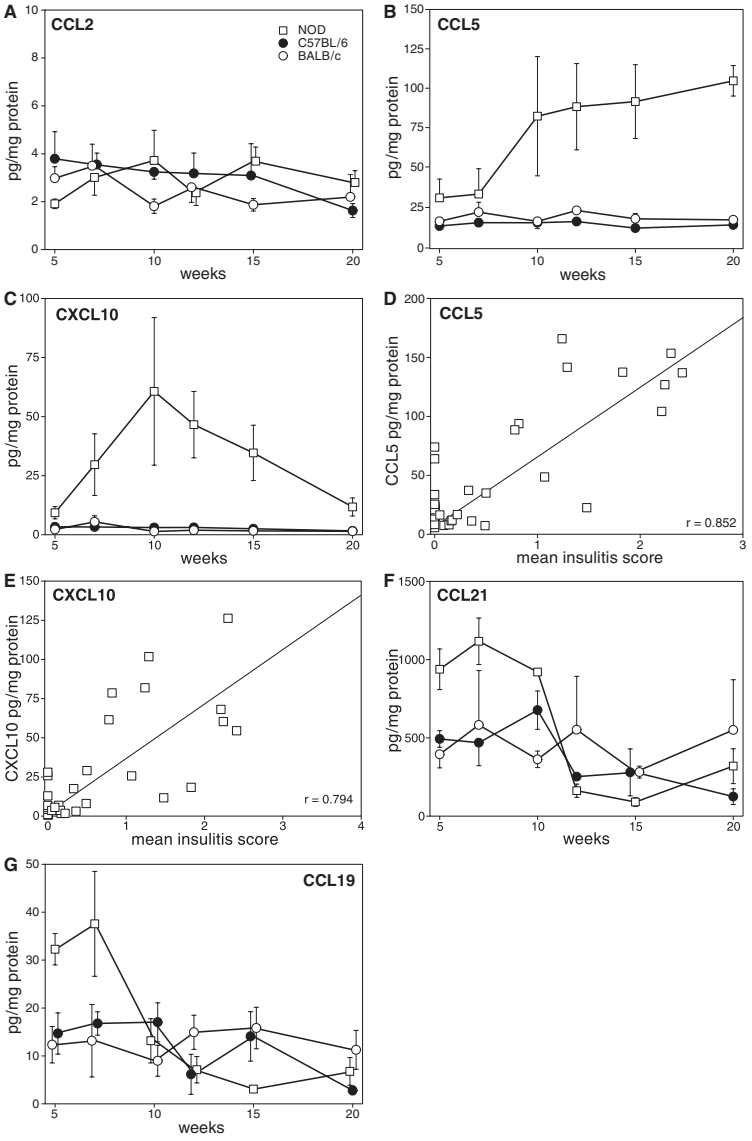

Figure 1. Distinct chemokine expression in the pancreas of NOD mice during diabetes development.

The level of CCL2 (A), CCL5 (B), CXCL10 (C), CCL19 (F) and CCL21 (G) at different ages as determined by ELISA is shown. Data are presented as mean ± SEM and averages of five animals. The expression of CCL5 (D) and CXCL10 (E) showed a positive correlation with the mean insulitis score that was determined by immunohistochemistry.

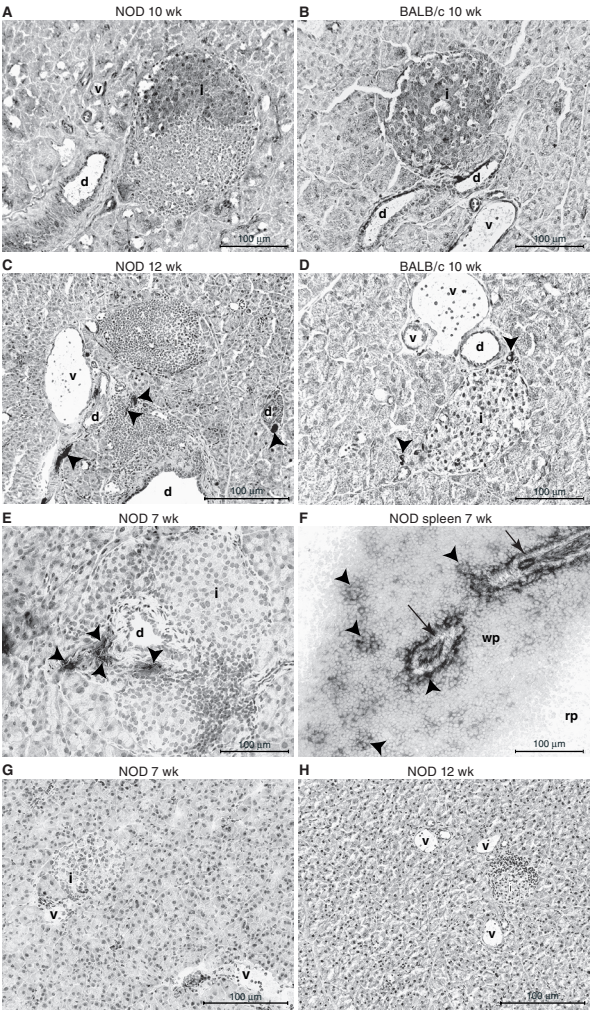

Figure 2. Chemokine expression in the pancreas of NOD mice.
NOD mice (A, C, E and D) showed increased chemokine expression compared to control animals (B and D). CCL5 (A and B) expression was located mainly in the islets itself, with NOD mice (A) showing increased expression compared to control BALB/c (B). CXCL10 (C and D) was expressed by the para-ductular nerves as indicated by the arrowheads. NOD mice (C) showed stronger expression of CXCL10 compared to control BALB/c mice (D). The expression of CCL21 was confined to the para-ductular area in the pancreas NOD mice as indicated by the arrowheads (E) and the PALS in the spleen as indicated by the arrows (F). IgG control staining of a 7 wk old and a 12 wk old NOD mouse are shown in G and H, respectively. Arrowheads indicate single cells expressing the indicated chemokines, i = islet, d = duct, v = blood vessel, wp = white pulp and rp = red pulp.

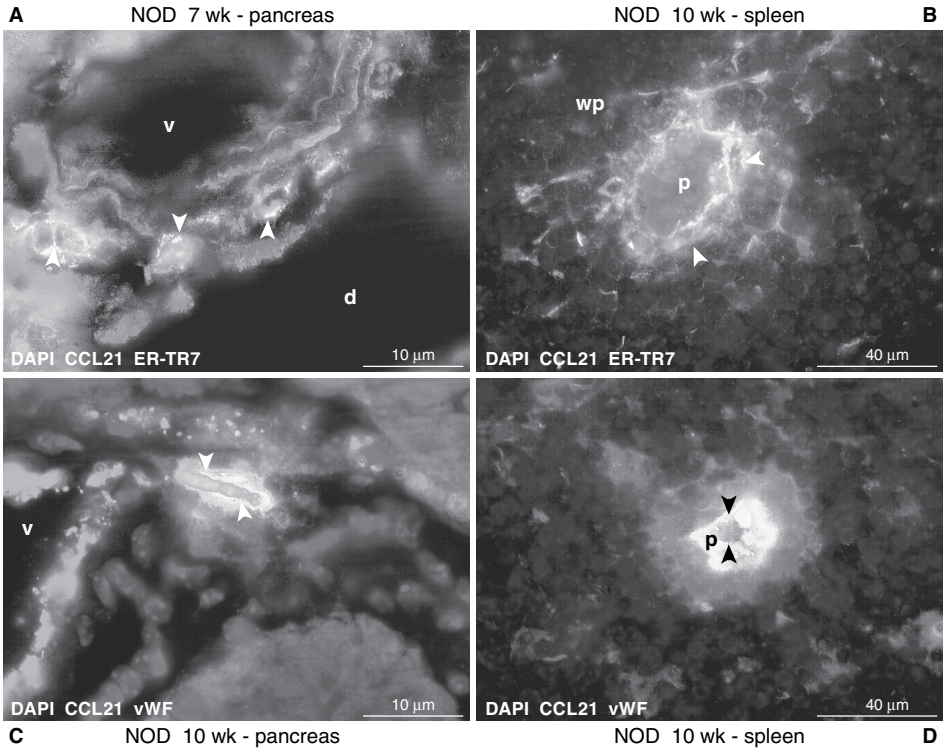

Figure 3. CCL21 expression in the pancreas and spleen of NOD mice.
The expression of CCL21 in the NOD pancreas was localized in the vicinity of ER-TR7^{+} (A) and von Willebrand factor-positive cells (B), although no double staining was observed. In the spleen, CCL21 did show double staining with ER-TR7^{+} (C) and von Willebrand factor-positive cells (D). CCL21 expression is shown in green, ER-TR7 and von Willebrand factor in red and DAPI in blue. The arrowheads indicate the CCL21 expression, d = duct, v = blood vessel, wp = white pulp and p = PALS.

Immunohistochemical analysis of the pancreases of NOD mice (5-15 wks) and control C57BL/6 and BALB/c mice confirmed the age-dependent expression of the pro-inflammatory chemokines and showed the location where the increased expression of the chemokines was found. CCL5 was mainly expressed in islet and duct cells of the NOD mice (figure 2A-B). CXCL10 was expressed in para-ductular nerves, confirming previous results (17), and to a lesser degree in the islets, possibly by recruited lymphocytes (figure 2C-D). We did not stain for CCL2 since it came hardly to expression in the lysates of the pancreases. The pictures also show that lymphocyte infiltrates – though correlating with the expression grade of these cytokines – were not found in clear topological connection with the chemokine expressing structures.

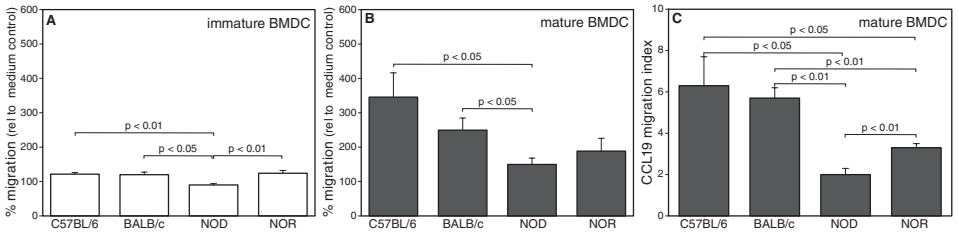

Figure 4. Migration of BMDC is impaired in NOD mice.
Migration towards CCL19 was reduced in NOD immature BMDC using the Transwell system (A). Mature BMDC of NOD and, to a lesser extent, NOR mice showed decreased migration towards CCL19, compared to C57BL/6 and BALB/c when tested using the Transwell system (B). Also using the Boyden chamber, migration was impaired in NOD, but also NOR mice (C). Migration is presented relative to migration observed with medium only. Data are shown as mean ± SEM and averages of at least four animals (5-7 wks) are shown. Statistical significant differences were determined with Student's t test.

Expression of the constitutive non-inflammatory chemokines CCL19 and CCL21

Unexpectedly, we observed that at an early age (5-7 wks) NOD mice showed an increased level of intra-pancreatic expression of the constitutive, lymphoid-tissue specific, non-inflammatory chemokines CCL19 (MIP-3β or ELC, figure 1F) and CCL21 (6Ckine or SLC, figure 1G) when compared to the pancreases of the two control strains. The expression level of these two chemokines rapidly declined with age and was at the same low level as that of the control mice from 10 wks onwards.

Since the expression of these chemokines correlated to the early DC accumulation we also aimed at investigating the location of CCL19 and CCL21 expressing structures in immunohistochemistry. We were not able to stain the tissue for CCL19, probably due to a failure of the used antibody in immunohistochemistry (this is also the experience of others; R. Mebius, personal communication). CCL21 was detectable in immunohistochemistry and found mainly in single cells in the para-ductular area (figure 2E) and the PALS in the spleen (figure 2F). To determine the cell type that was responsible for the expression of CCL21 in the pancreas, we performed double immunofluorescence microscopy. We were unable to determine the cell type responsible for the expression of CCL21. CCL21 did not label double with monocytes (Ly-6C$^+$), mφ (BM8$^+$, ER-MP23$^+$ or F4/80$^+$), DC (CD11c$^+$ or NLDC45$^+$), T cells (KT3$^+$) or B cells (B220$^+$) (data not shown). We observed expression of CCL21 in close vicinity to fibroblasts (ER-TR7$^+$) and endothelial cells (von Willebrand factor-positive; vWF) as shown in figure 3A and C. In the control staining of the spleen we did observe double labelling of CCL21 with ER-TR7$^+$ and vWF$^+$ cells (figure 3B and D), confirming observations of Ato et al of expression by fibroblasts and endothelial cells (18).

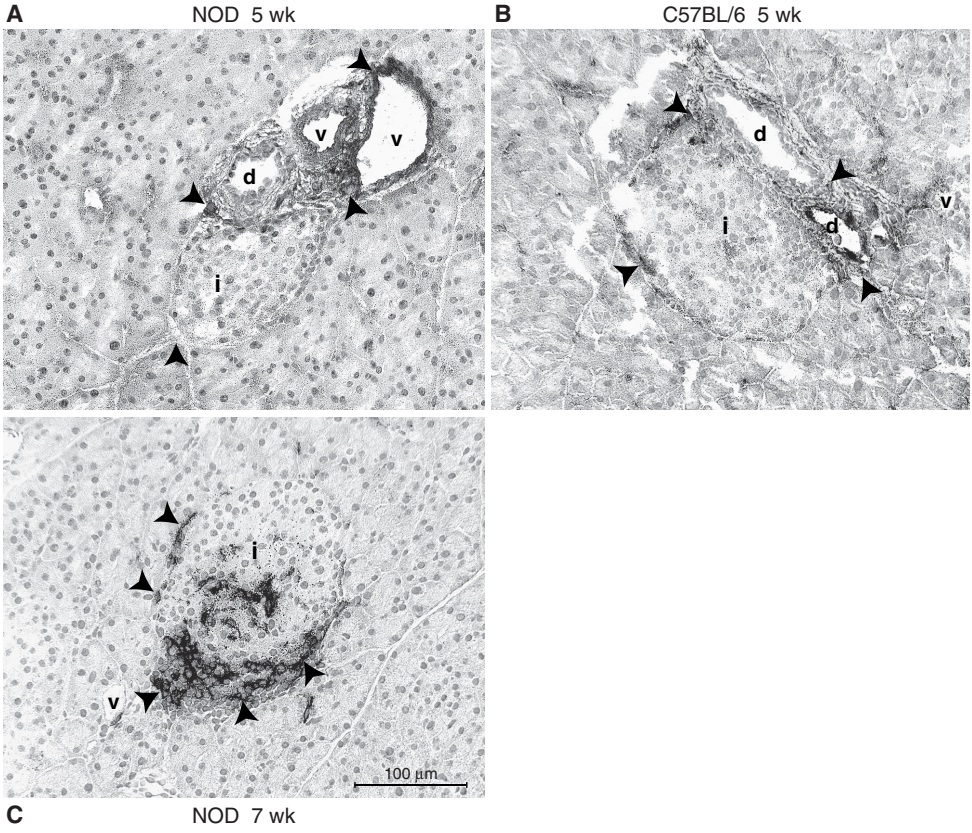

Figure 5. FN expression.
Both NOD mice (A) and control C57BL/6 mice (B) showed a similar expression of FN that is located at the perivascular area, the ductular pole and the periphery of the islet as indicated by the arrowheads. The DC that accumulation around the islets in young (5-7 wks) NOD mice showed a similar localization as the FN labelling (C). i = islet, d = duct and v = blood vessel.

To functionally test the response of NOD DC to lymphoid tissue related chemokines we determined the in vitro migratory capacity of NOD DC to CCL19. We used immature and mature bone marrow-derived DC (BMDC) in these experiments, since pancreatic DC are difficult to obtain in sufficient quantities (for maturation stages of the DC see supplementary figure 1). Figure 4 shows the migration of the DC as relative migration to the spontaneous migration towards culture fluid alone, the latter set to a hundred percent. No differences between the different mouse strains were found in the spontaneous migration towards culture fluid alone of either immature or mature cells (data not shown).

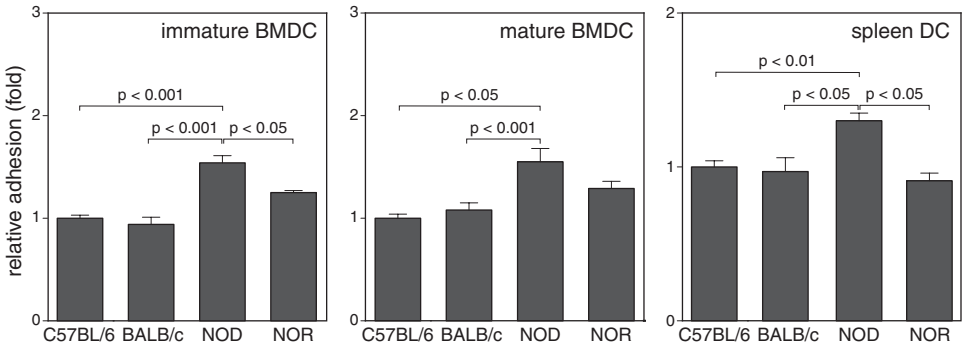

Figure 6. Adhesion of DC to extra-cellular matrix components is increased in NOD mice.
NOD mice showed increased adhesion to fibronectin of immature BMDC, mature BMDC and splenic DC. Adhesion is presented relative to adhesion of C57BL/6. Data are shown as mean ± SEM and averages of at least four animals (5-7 wks) are shown. Statistical significant differences were determined with Student's t test.

As expected the chemotactic migration towards CCL19 of immature BMDC was of very low magnitude. It was in fact absent for NOD immature DC (figure 4A). Upon maturation the DC increased their responsiveness to CCL19 (19). Indeed, migration towards CCL19 of mature BMDC of C57BL/6 and BALB/c mice increased more than two-fold compared to their immature counterparts (figure 4B). In NOD mice, the response of mature BMDC to CCL19 was also increased compared to NOD immature BMDC, but significantly decreased in comparison to C57BL/6 and BALB/c DC (figure 4B). It is worthy to note that in flow cytometry we did not find the surface expression of CCR7 (the receptor for CCL19) to be different between NOD and control mice DC (M.E. Wildenberg, personal communication). In NOR mice as a genetic background control for the NOD, the migration of mature BMDC was decreased, but not statistically significant compared to either C57BL/6 or BALB/c, nor was it statistically significant increased compared to NOD (figure 4B). When we tested the migratory potential of mature BMDC using the classical Boyden chamber, we observed again that NOD DC were impaired in their migration towards CCL19 (figure 4C). DC of NOR mice also showed an impaired migration, however, not as severe as DC of NOD mice (figure 4C).

ECM and adhesion studies

In addition to chemokine receptors, cell migration is dependent on the contact of the cell with the surrounding extra-cellular matrix (ECM) via integrins. Figure 5 shows the FN staining of pancreases of NOD (figure 5A) and control mice (figure 5B) at 5 weeks of age. No significant difference was observed in the FN expression between the mouse strains. FN staining was particularly strong at the islet-ductal pole, peri-vascular area and to a lesser degree at the islet periphery. Typically the

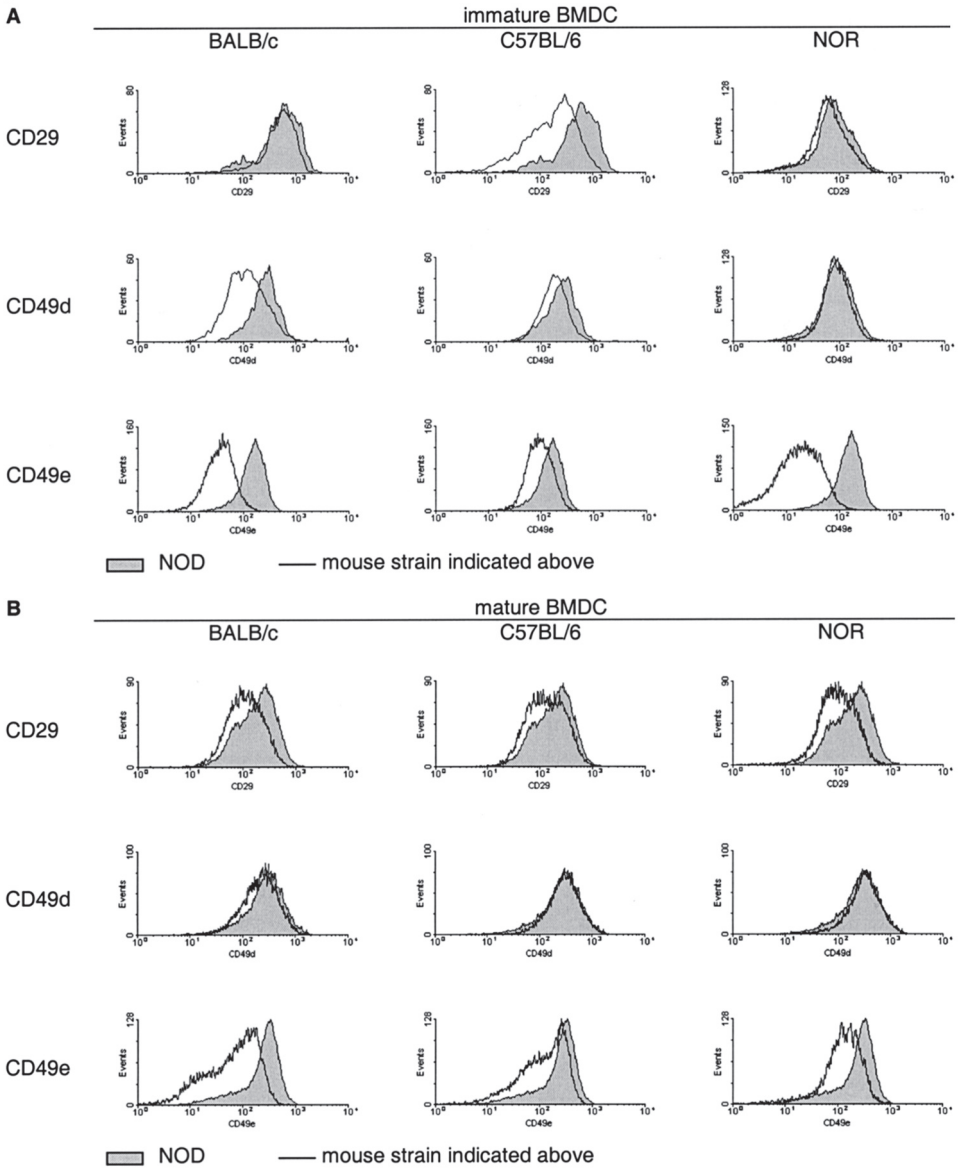

Figure 7. Surface expression of adhesion molecules on BMDC is increased in NOD.
NOD immature BMDC (A) and mature BMDC (B) showed an increased expression of CD29 and CD49e, but not CD49d in comparison to control mice. Representative histograms of nine BALB/c, eleven C57BL/6, five NOR and eleven NOD mice are shown, all mice were between 5 and 7 wks of age.

early DC accumulation is found at the FN-rich vaso-ductular pole of the islets as illustrated in figure 5C. The distribution of FN is different from that of laminin, which was described to be strong at basement membranes of exocrine acini, ducts and blood vessels (13, 20).

Since DC were found to accumulate at sites in the NOD pancreas which are also characterized by FN labelling we determined the adhesion of BMDC to FN coated onto plates. NOD BMDC, both immature and mature, showed an increased adhesive capacity to FN (figure 6). To exclude the possibility that the increased adhesion of NOD DC was the result of the specific conditions of bone marrow culture, we also studied the adhesion of DC isolated from spleen using immuno-magnetic bead separation. As also shown in figure 6, we confirmed an increased adhesive capacity to FN of NOD DC in comparison to control strains including the NOD-related NOR mice.

Since the adhesion to FN of NOD BMDC was increased, we also studied the expression levels of the FN-binding receptors VLA-4 (CD29/CD49d) and VLA-5 (CD29/CD49e) on the cells. We found in particular the expression of CD29 and CD49e increased on BMDC of NOD mice, both on immature (figure 7A and table 1) and mature cells (figure 7B and table 1). Since splenic DC of NOD mice also showed increased FN adhesion we also determined the expression of VLA-4 and VLA-5 on these cells. We did not detect significant differences in the expression of VLA-4 or VLA-5 on splenic DC of NOD mice compared to control strains (data not shown).

Although DC of NOD mice are less sensitive to maturation as determined by the expression of co-stimulatory molecules (supplementary figure 1), we did observe an increase in both VLA-4/VLA-5 expression and adhesive capacity of BMDC after LPS maturation (data not shown), which was shown to be related to DC maturation (21).

Table 1. Expression of adhesion molecules relative to the expression observed in NOD mice.

immature BMDC			
	BALB/c (n=9)	C57BL/6 (n=11)	NOR (n=6)
CD29	0.59 ± 0.11$^{\$}$	0.73 ± 0.11$^{\#}$	0.71 ± 0.20$^{\#}$
CD49d	0.82 ± 0.29	1.29 ± 0.30	1.17 ± 0.14
CD49e	0.57 ± 0.10$^{\$}$	0.60 ± 0.08$^{\$}$	0.58 ± 0.09$^{\$}$
mature BMDC			
	BALB/c (n=9)	C57BL/6 (n=11)	NOR (n=6)
CD29	0.68 ± 0.08$^{\$}$	0.66 ± 0.04$^{\$}$	0.57 ± 0.09$^{\$}$
CD49d	1.04 ± 0.12	1.17 ± 0.06$^{\#}$	0.97 ± 0.14
CD49e	0.44 ± 0.10$^{\$}$	0.50 ± 0.07$^{\$}$	0.64 ± 0.10$^{\$}$

Data are shown mean ± SEM and are combined results of five independent experiments. $^{\#}$ $p<0.05$, $^{\$}$ $p < 0.01$ to NOD (n=11) as determined with Student's t test

DISCUSSION

Dendritic cells (DC) start to accumulate at the edges and the isthmus of the islets of Langerhans in the NOD mouse model of diabetes from 4-7 weeks of age onwards and prior to the massive accumulation of lymphocytes at the same sites (1, 2). This report shows that NOD pancreas connective tissue expresses at these sites in particular the ECM protein FN and that NOD immature and mature DC are characterized by an increased adhesion to FN and an increased VLA-5 (CD29/CD49e) expression. VLA-5 is the prototypic FN receptor, binds to the FN domain containing the RGD sequence (12, 22) and adhesion of human monocyte-derived DC to FN is mediated by VLA-5 (23). Our observation thus supports a view that an aberrant increased expression of VLA-5 on NOD DC results in an abnormally high accumulation of such cells at sites of high FN expression, such as the islet edges and the para-ductular pole. An interesting parallel can be drawn with the monocytes of type-1 diabetic patients, which also show an increased adhesion to FN (though in the absence of a higher VLA-5 expression, but in the presence of a raised CD11b and CD18 expression) (14). This increased adhesion of type-1 diabetic monocytes to FN is induced by myeloid-related proteins (MRP)8/14 and 14, which are elevated in the serum of type-1 diabetic patients and produced by recently migrated monocytes (14).

Contrary to our expectations we did not find support for a prime role of the pro-inflammatory chemokines CCL2, CCL5 and CXCL10 in the early DC accumulation in the NOD pancreas. At the time of early DC accumulation (between 5 – 10 weeks of age) these chemokines were only expressed at very low level in the pancreas of NOD mice. We did detect increased levels of the non-inflammatory, lymphoid-tissue related chemokines CCL19 and CCL21 in the pancreas of NOD mice at the time of early DC accumulation and these chemokines were particularly expressed in the para-ductular connective tissue (we were unable to identify the exact cell type responsible for the expression of the lymphoid-tissue related chemokines). CCL19 and CCL21 are normally abundantly expressed in lymphoid tissues and this report confirms the expression of CCL21 by fibroblasts and endothelial cells close to the PALS of the spleen (18). Further support for a potential role of the non-inflammatory cytokines in the early accumulation of the DC in the NOD pancreas is given by experiments showing that transgenic over expression of CCL19 and CCL21 in islets of C57BL/6 mice results in cellular infiltrates containing DC (24). Interestingly, a recent report shows that a special subset of monocytes, i.e. the Ly-6C Gr-1^{int} monocyte subset, expresses CCR7 (the ligand for CCL19 and CCL21) and gives rise to lymphatic-migrating DC (25). This makes this subset of monocytes possible candidates for the early infiltration into the NOD pancreas in response to the increased levels of CCL19 and CCL21. Moreover, the early expression of lymphoid-tissue related chemokines might disrupt the natural gradient of these chemokines that is involved in homeostatic trafficking of DC to lymph nodes. Such

disrupted gradient could delay the emigration of DC and contribute to the observed accumulation.

It could also be that the expression of CCL19 and CCL21 is involved in or the consequence of the formation of secondary lymphoid tissue in the pancreas of NOD mice, although expression of CCL19 and CCL21 at later ages would also be expected. These lymphoid structures have been described in BDC2.5 TCR transgenic NOD mice and occasionally also in wild-type NOD mice (26). In the RIP-LCMV model for autoimmune diabetes, lymphoid tissue structures have also been found and an important role in their formation has been proposed for DC (27). Furthermore, transgenic mice with an over-expression of CCL19 or CCL21 in the islets, developed lymphoid structures in the pancreas containing lymphocytes, stromal cells and DC (24, 28). Moreover, we have reported on the presence of lymphoid tissue in the normal foetal and newborn human pancreas (29) and such lymphoid tissue was particularly prominent in a case of a diabetic infant (30).

However a clear role for CCL19 and CCL21 in the early accumulation of DC at the islet periphery in NOD mice can also be disputed. CCL19 and CCL21 also attract T and B lymphocytes (31), and these cells were virtually absent from the pancreas of 5-7 week–old NOD mice. Moreover we found the expression of CCL19 and CCL21 to decline after 10 weeks of age to levels similar to control strains. At this time DC do further increase in number and T and B cells start to accumulate close to the islets. It must, however, be noted that others have reported - in contrast to our data - an up regulation of CCL21 in NOD pancreases with aging (32), but these studies solely used morphological techniques. A further objection against a prime role of the lymphoid-related chemokines in the early accumulation of DC in the NOD pancreas is given in this report where we show that the in vitro chemotactic response of mature DC of NOD and NOR mice towards CCL19 was in fact very poor. The migratory potential of NOR mice was slightly higher than that of NOD mice. These data suggest that such impaired migration may be at least partly related to the NOD background. NOR mice have with the exception of some C57BL/KsJ-derived alleles on chromosomes 2, 4, 11 and 12, the genetic background of NOD mice including H-2 on chromosome 17 (33). Interestingly, NOR mice also develop accumulation of DC and mφ at early age, which is followed by an influx of T and B cells, although to a lesser degree as NOD mice (supplementary figure 2A-C). However, in NOR mice the insulitis does not progress towards overt diabetes (33). Obviously the role of these lymphoid-tissue related chemokines deserves further study.

It could be argued that the in vitro defective migration towards CCL19 and increased adhesion to FN is the consequence of the defective maturation of NOD BMDC. Indeed we confirm a defective DC maturation in NOD mice (34-36) as shown by the decreased expression of co-stimulatory markers (supplementary figure 1). However, DC maturation is also accompanied by an increased responsiveness to CCL19 and CCL21 (9, 10) and we observed that NOD mature

DC did show increased migration towards CCL19 compared to immature DC, albeit to a lesser degree than control mice. Furthermore, DC maturation was shown to be accompanied by an increase in the expression of VLA-4 molecules in humans (21). We observed that mature NOD DC show an increased expression of these adhesion molecules as well as increased adhesion to FN in comparison to immature DC. Taken together, our findings indicate that the defect in DC maturation in NOD mice seems selective. Although the expression of co-stimulatory molecules and lymphocyte stimulation is not appropriately upregulated upon maturation, the increases in adhesive and migratory capacity seem not to be affected. Therefore, we assume that the increased adhesion and decreased migration of NOD DC cannot be entirely ascribed to a simple defect in maturation.

With regard to the pro-inflammatory chemokine expression pattern in the NOD pancreas, our studies are in general congruent with the majority of similar other studies (3, 4). Others and we found levels of such chemokines in general increased at the time of clear lymphocyte para- and peri-insulitis. Here we show that CCL5 and CXCL10 expression closely correlated to the extent of lymphocyte infiltration and that CCL5 was primarily localized in islet cells, while CXCL10 also came to expression in neurons. Although we were not able to see evident topological connections between the chemokine expressing ducts/neurons and the actual lymphoid cell accumulations in this study, macrophages accumulating around neurons in the NOD pancreas have been described as well as an autoimmune reaction towards neuronal cells in autoimmune diabetes (17).

In conclusion, our findings support a view that the early accumulation of DC at the ductal-vascular pole of NOD islets prior to the lymphocyte accumulation is rather the consequence of an aberrantly enhanced adhesion of NOD DC to ECM proteins and perhaps also to a peculiar attraction or retention of mature DC to the site by lymphoid-tissue related chemokines than the consequence of an aberrantly enhanced attraction of precursor DC by pro-inflammatory chemokines from f.i. the blood stream. Congruent with such view is our recent report of an in general poor in vitro chemotactic responsiveness of NOD DC to pro-inflammatory chemokines and an in general poor in vivo migration of NOD myeloid cells to sites of inflammation (16).

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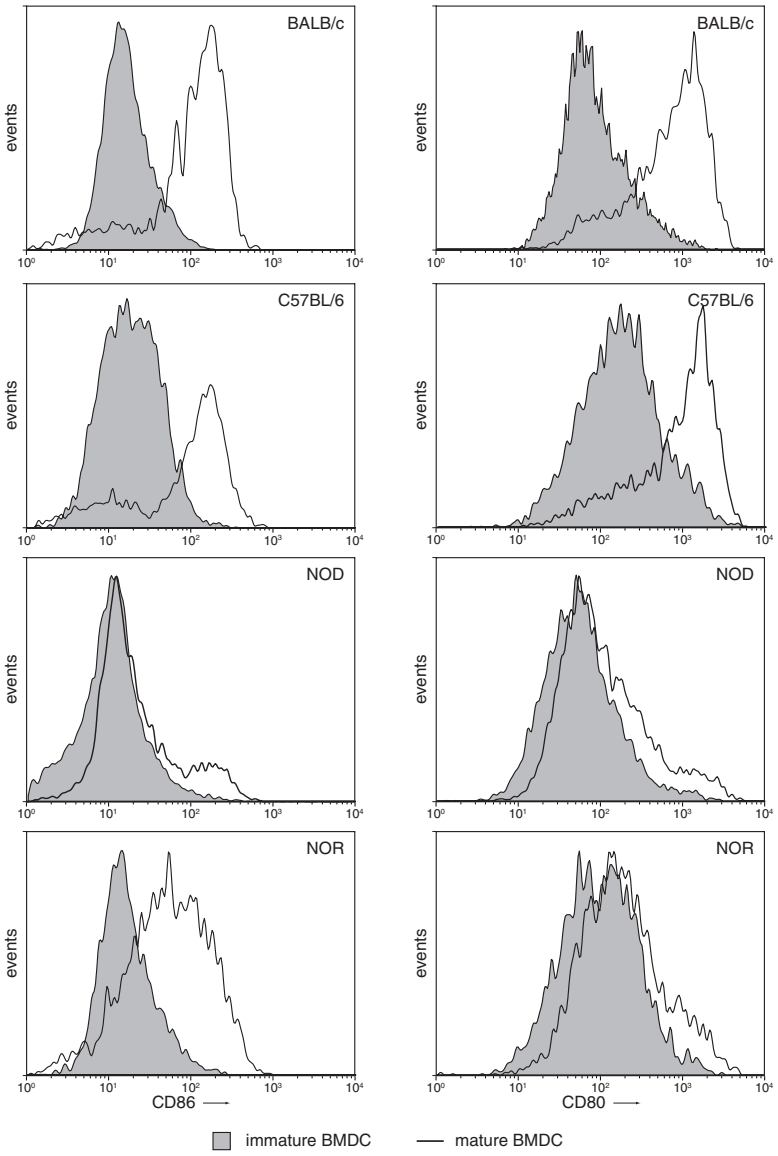
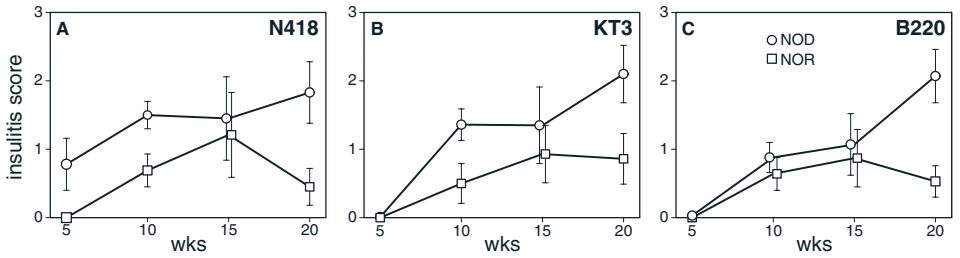


Supplementary figure 1. Surface expression of co-stimulatory molecules on BMDC.
Representative histograms of immature and mature BMDC of at least six BALB/c, C57BL/6, NOD and NOR mice are shown. Both NOD and NOR mice show less mature BMDC after maturation as determined by the expression of CD80 and CD86.



Supplementary figure 2. Insulitis in NOD and NOR mice.
The infiltration of DC (A; N418), T cells (B; KT3) and B cells (C; B220) in the pancreas of NOD (n=7) and NOR (n=4) was determined by semi-quantative immunohistochemistry. Data are represented as mean ± SEM.

CHAPTER 5 DISCUSSION

CHAPTER 5.1 GENERAL DISCUSSION

Monocyte subsets in type 1 diabetes

It has been proposed that there are, in the circulation of both humans and mice, two monocyte subpopulations. In humans the division is based on the expression of CD14 and CD16 (1). In the mouse the division is based on the expression of Ly-6C (2), but also of CD62L and of the chemokine receptors CCR2 and CX_3CR1 (3).

When monocytes leave the bone marrow, they enter the circulation as immature cells and express high levels of Ly-6C in the mouse. Immature mouse Ly-6C^{hi} monocytes correspond to the previously described CX_3CR1^{neg} CCR2^{pos} CD62L^{pos} monocytes (3) and are thought to be analogous to the human CD14^{hi} CD16^{neg} monocytes (Table 1). Immature circulating blood monocytes can rapidly be recruited to sites of inflammation ((2) and chapter 3.2). The monocytes that are not rapidly recruited, mature in the circulation to Ly-6C^{low} CX_3CR1^{pos} CCR2^{neg} CD62L^{neg} monocytes, corresponding to CD14^{pos} CD16^{pos} cells in the human (1-3), as shown in Table 1. The mature subpopulation is thought to consist of the monocytes that enter the periphery to give rise to resident macrophages (mφ) or dendritic cells (DC) (1-4).

Table 1. Characteristics of human and mouse monocyte subsets.

	human	mouse	references
immature monocytes	CD14^{hi} CD16^{neg}	Ly-6C^{hi} CX_3CR1^{neg}	(1-3)
APC function	↓	↓	(5, 6)
response to inflammation	↑	↑	(1-4)
adhesion to fibronectin	=	↑	chapters 2.1 and 3.1
mature monocytes	CD14^{pos} CD16^{pos}	Ly-6C^{low} CX_3CR1^{pos}	(1-3)
frequency	~ 10%	~ 40%	(1) and chapter 3.1
APC function	↑	↑	(5, 6)
response to inflammation	↓	↓	(1-4)
adhesion to fibronectin	=	↓	chapters 2.1 and 3.1

In several chronic inflammatory conditions, such as rheumatoid arthritis (7), HIV-infected individuals (8), sepsis patients (9) and other chronic inflammatory diseases (1) an expansion of the mature CD14^{pos} CD16^{pos} monocyte population has been observed. I was unable to observe such increase in mature CD14^{pos} CD16^{pos} monocytes in type 1 diabetes (T1D) patients (chapter 2.1). However, in the NOD mouse I did find the frequency of mature Ly-6C^{low} monocytes increased in the blood (chapter 3.1). This dissimilarity between human T1D subjects and the NOD mouse questions whether the markers used to subdivide monocytes result in the same subpopulations in mice and men. There are more indications that the human CD14^{pos} CD16^{pos} monocytes are dissimilar from the mouse Ly-6C^{low} monocytes. In humans the mature CD14^{pos} CD16^{pos} population comprises of about 10% of the total monocyte pool (10, 11), while in mice the corresponding population comprises of up to 40% of the cells (chapter 3.1 and Table 1). In addition, we have shown that in mice the immature Ly-6C^{hi} monocytes display a high adhesive capacity to fibronectin that is down-regulated when these cells become mature Ly-6C^{low}

cells (chapter 3.1 and Table 1). In the human we were however unable to find differences in adhesive capacity to fibronectin between the CD14CD16 monocyte subpopulations (chapter 2.1).

Hence there is ample evidence that the mouse and human monocyte subpopulations divided on CD14/CD16 and Ly6C respectively are not overlapping subpopulations. Moreover, various subdivisions in monocyte populations are possible based on the adhesion to fibronectin and expression of other marker molecules (see below). This suggests that the circulating monocyte population is heterogeneous, that the various stages in monocyte development are flexible and adaptable and hence that a subdivision in monocytes can not be that robust as in the case of subdivision of circulating T cell populations, e.g. $CD4^+$, $CD8^+$ and NK cells.

An increased adhesion of monocytes to fibronectin

In this thesis I describe that monocytes of T1D patients showed an increased adhesion to fibronectin as compared to monocytes of healthy controls or subjects with type 2 diabetes (chapter 2.1). I was also able to detect an increased adhesion to fibronectin of NOD monocytes (chapter 3.1) and bone marrow-derived NOD DC. The increased adhesion to fibronectin seems to be a shared feature of monocytes of both human subjects with T1D and of the NOD mice. Interestingly, the adhesive capacity to fibronectin has been used previously to subdivide monocytes into two populations (10, 11). These so-called fibronectin-adhesive p-monocytes form a subpopulation of monocytes in the circulation that comprises about 20% of all monocytes in healthy individuals (12) and that is increased in type 1 diabetics.

The work in this thesis also shows that in T1D patients an increased serum level of myeloid related protein (MRP)8/14 (a protein secreted by infiltrating monocytes) could be hold, at least in part, responsible for the increased adhesion to fibronectin of the circulating monocytes (chapter 2.1). Sera of T1D patients stimulated monocytes of healthy individuals to adhere in larger numbers to fibronectin as compared to healthy sera and this capability of T1D sera was lost after depleting the sera of MRP8/14. Also T1D monocytes produced and expressed more of MRP8/14, particularly after adhesion to fibronectin (chapter 2.2). Hence a positive feed-back mechanism exists with regard to the enhanced fibronectin adhesion of T1D monocytes: an increased expression and production of MRP8/14 by T1D monocytes leads to an enhanced adhesion to fibronectin leading to an even greater expression and production of MRP8/14. Another interesting aspect with regard to MRP expression by monocytes is that yet another subdivision has been proposed, which was based on the expression of MRP8/14 using the monoclonal antibody 27E10 (12). About 20% of the circulating monocytes express this antigen in healthy volunteers (13, 14), but again – as stated above - is higher in T1D patients (chapter 2.2). Possibly, the $27E10^{pos}$ monocyte population may overlap or even be identical to the fibronectin-adhering p-monocytes.

A decreased migratory response of monocytes to pro-inflammatory chemokines

MRP8/14 has not only been implicated in the adhesion to fibronectin, but also in the transmigration of monocytes from the circulation into inflamed tissues (15). Therefore, the increased serum MRP8/14 could indicate that monocytes of T1D patients also have an increased migratory potential to inflamed tissues. To investigate this hypothesis, we studied the chemotaxis and transmigration of monocytes of T1D patients towards inflammatory chemokines. Although I observed an increased adhesion of T1D monocytes to human endothelial cells, I did not observe an increased, but even a decreased transmigration of the monocytes to inflammatory chemokines in the transwell assay. Also in the classical Boyden assay the chemotaxis of the monocytes of T1D patients in response to pro-inflammatory chemokines was decreased (chapter 2.2).

The poor migratory response of monocytes to pro-inflammatory stimuli became also evident in the NOD mouse model of T1D. NOD mice were severely impaired in their in vivo recruitment of monocytes into either a peritonitis model or into the artificially induced air pouch (chapter 3.2). Also in vitro, when I studied the migration of mφ (chapter 3.2) or DC (chapter 4.1), I observed a reduced migratory potential of the cells. Others have also described reduced migration of NOD mφ (16) and also NOD thymocytes share this decreased migratory potential (17, 18). In the air pouch model I observed an increased expression of IL-10 and a low expression of IL-1β in NOD mice, which was reversed in control mice. Neutralizing the IL-10 only partially rescued the cellular recruitment, although the expression of IL-1β was restored to the level that was observed in control mouse strains. This indicated that the high IL-10/IL-1β ratio specific for the NOD air pouch only partially played a role in the hampered in vivo migration of NOD monocytes towards pro-inflammatory stimuli. Interestingly, I identified monocytes as producers of the IL-10 in the air pouch (chapter 3.2).

An increased migratory response of monocytes to constitutive chemokines

An unexpected finding in both the NOD mouse and the human studies was an increased tissue expression and chemotactic response of monocytes towards the so-called constitutive non-inflammatory chemokines CCL19 and CCL21. Although the chemotactic response of monocytes of T1D patients to pro-inflammatory chemokines was decreased, that towards the lymphoid tissue-related chemokine CCL19 was increased (chapter 2.2). Also CCL19 and CCL21 (but not pro-inflammatory chemokines) were expressed at increased levels in the NOD pancreas at the time of early DC and mφ infiltration (chapter 4.1). It must be noted, however, that the chemotactic response of NOD bone-marrow derived DC was lower towards CCL19.

The aberrant adhesion and migration of type 1 diabetic monocytes: an intrinsic property of diabetic monocytes, mφ and DC?

I like to propose that the increased adhesion to fibronectin, the overall reduced migratory response to pro-inflammatory stimuli and the increased migratory response to constitutive chemokines reflects an intrinsic aberrant property of the monocyte lineage in T1D, since patients and NOD mice shared most of these features. All patients had longstanding disease (>10 years) and at that time virtually no β cells were left, indicating that the monocyte phenotype that I observed cannot be related to the activity of the autoimmune process in the pancreas. Moreover in the NOD mouse similar monocyte aberrancies were found prior to the lymphocytic insulitis process.

The parallel between human T1D patients and the NOD mouse concerning the altered adhesive and migratory capacity of monocytes suggests an important role for such aberrant adhesion and migration in the development of T1D. Other defects of monocytes, DC and mφ have been described in T1D, further suggesting that the increased fibronectin adhesive and decreased pro-inflammatory migratory potential of the cells originate from intrinsic monocyte aberrancies. An aberrant prostaglandin synthase 2 (PGS2) and/or nuclear factor kappa B (NF-κB) expression may be key phenomena in this aberrancy.

Monocytes of patients with T1D show an increased PGS2 expression (19) and are resistant to IL-10 induced suppression of PGS2 (20). High levels of PGS2 (21) result in an abundant expression of prostaglandins like prostaglandin E_2 (PGE_2). PGE_2 is able to induce a down-regulation of the expression (22) and possibly function (23-25) of the pro-inflammatory chemokine receptor CCR5, but is required for CCR7 expression and responsiveness though in monocyte-derived DC (26). Possibly, the constitutive active PGS2 in monocytes of T1D patients yields high levels of PGE_2 that induces the decreased migration towards the pro-inflammatory chemokines CCL2 and CCL3 and the increased responsiveness to CCL19 that I observed in monocytes of T1D patients.

Interestingly, PGE_2 has also been described to enhance the maturation of monocyte-derived DC (27, 28). However, our group was unable to find an enhanced maturation of antigen-presenting cells in T1D patients. Culturing DC or antigen presenting veiled Mφ from monocytes of T1D patients yielded fewer APC and these APC performed less well in T cell stimulatory assays (29). In the circulation of T1D patients an altered balance in myeloid/plasmacytoid DC has been observed (30), although this was not found by others that described a similar ratio of myeloid/plasmacytoid DC (31-36). In NOD mice, many studies have described an abnormal differentiation and maturation of mφ and DC (37-40). A hyperactivation of the transcription factor nuclear factor kappa B (NF-κB) is thought to be the underlying defect for this defective maturation and differentiation (41, 42). NF-κB has been implicated in the expression of adhesion molecules of DC (43) and inhibition of NF-κB by cyclosporin A impairs the migration of bone

marrow-derived DC (37-40). However, literature is not ambiguous about the role of NF-κB in the function of DC in NOD mice. Both an increased maturation of DC and NF-κB hyperactivation (44) as well as a decreased DC maturation and decreased NF-κB activation have been reported (45). Clearly, further studies are required to unravel the molecular background of the here found T1D monocyte aberrancies in adhesion and migration.

Para- and peri-islet mφ and DC accumulation: an increased influx of monocytes by pro-inflammatory chemokines?

What might be the actual contribution of the increased adhesion of T1D monocytes to fibronectin, the impaired migration of T1D monocytes to pro-inflammatory chemokines and the enhanced migration of T1D monocytes to constitutive chemokines to the development of islet autoimmunity? An impaired monocyte migration has been found in other autoimmune diseases such as rheumatoid arthritis (46) and systemic lupus erythematosus (47). In addition, patients suffering from the Wiskott Aldrich syndrome have a defective monocyte chemotaxis (48) and are prone to develop autoimmunity (49), further indicating a possible relation between a disturbed migratory potential of monocytes and the development of autoimmunity (50-52).

The in this thesis described reduced migration of monocytes, mφ and DC to pro-inflammatory chemokines is seemingly in contradiction to the previously reported para- and peri-islet accumulation of mφ and DC in the pancreas of NOD mice. The failure to respond properly to inflammatory stimuli urged me to investigate the nature of the signals that drives the mφ and DC accumulation in NOD mice, which was thought to be inflammatory. Therefore, I determined the expression of chemokines at several stages of diabetes development (chapter 4.1). I did not observe an increased expression of CCL2 at an early stage, which is an important monocyte-attractant. I did observe an increase in the expression of the pro-inflammatory chemokines CCL5 and CXCL10, but only from 10 wks of age onwards. At 10 wks of age, the mice already showed mφ and DC accumulated close to and around the islets of Langerhans (51, 53) indicating that the pro-inflammatory CCL2, CCL5 and CXCL10 can not be involved in the early attraction of mφ and DC. From 10 wks of age onwards a massive para- and peri-islet infiltration of lymphocytes is observed in the NOD pancreas (54) and CCL5 and CXCL10 probably play an important role in this lymphocytic infiltration, since there was a good positive correlation between the expression of these pro-inflammatory chemokines and the infiltration of lymphocytes (chapter 4.1). My findings furthermore suggest that the expression of pro-inflammatory chemokines that we detected from 10 weeks of age onwards rather follows the early mφ and DC accumulation and most likely the accumulating mφ and DC produce or induce these chemokines. Indeed, the early accumulating mφ and DC produce tumor necrosis factor-α (TNF-α), which promotes an inflammatory environment (55). Furthermore, the mφ and DC will be

in contact with the ECM of the pancreas and these interactions may also induce the expression of pro-inflammatory cytokines. Stimulation of human monocyte-derived DC with collagen induces the expression of TNF-α (56) and murine DC have been described to increase the expression of IL-1 and IL-6 upon collagen stimulation (57). In addition, cyclophoshamide treatment of young BDC2.5 TCR transgenic NOD mice that induces a rapid switch from insulitis to diabetes, has been shown to be accompanied by increased expression of IL-18, IL-12 and TNF-α in the pancreas (57). This early cytokine expression is thought to be primarily derived from mφ and DC. Later in time increased IL-1β, IL-6 and interferon-γ (IFN-γ) could be detected (58, 59) that probably set off the destruction of the β cells, leading to overt diabetes (60-63). The increased cytokine expression will also induce chemokine expression by β cells as has been shown for isolated islets or isolated β cells that are induced to express CCL2, CCL5 and CXCL10 expression upon IL-1β or IFN-γ stimulation that will recruit more leukocytes into the pancreas (64).

In conclusion: The early para- and peri-islet accumulation of DC and mφ can hardly be the consequence of a regular inflammatory influx of precursors, i.e. monocytes from the circulation.

Para- and peri-islet mφ and DC accumulation: a role for lymphoid-tissue chemokines?

I showed in this thesis an increased expression of CCL19 and CCL21 at an early age in NOD pancreases that declined after 10 wks (chapter 4.1). The expression of CCL21 was located in the perivascular area of the NOD islets, up till now I have not been able to define the cell type expressing CCL21 in the NOD pancreas. It is not on lymphatic endothelium.

One can easily imagine that the early increased expression of CCL19 and CCL21 is involved in the early para- and peri-islet mφ and DC accumulation. Although the receptor for these ligands, CCR7, is mainly expressed on mature DC (65), it is also present on naïve lymphocytes (6) and on monocytes (see chapter 2.2). Recently, a third mouse monocyte subpopulation has been described that shows an intermediate expression of Ly-6C and gives rise to lymphatic-migrating DC (6). These Ly-6C^{int} monocytes express CCR2 to similar levels as Ly-6C^{hi} monocytes and in addition express CCR7 and CCR8 as determined by mRNA expression (66). Although the distinction between the Ly-6C immature and Ly-6C mature monocytes can be made in the NOD mouse (chapter 3.1), a distinction between the three populations based on Ly-6C expression may be blurred due to the aberrant expression of the Ly-6C molecule in NOD mice (67). It could be imagined that in particular the Ly-6C^{int} subpopulation of NOD monocytes would infiltrate the pancreas in response to the increased expression of CCL19 and CCL21. These monocytes then would differentiate into DC and mφ resulting in the observed early para- and peri-islet accumulation of cells. Hence it would be interesting to study the migration of Ly-6C^{int} monocytes of NOD mice to CCL19 in

further studies. Here I show that T1D monocytes show an increased chemotactic response to CCL19.

There is however also another consequence thinkable of the early increased expression of the constitutive chemokines CCL19 and CCL21 in the NOD pancreas. The increased CCL19 and CCL21 might not only stimulate the influx of monocytes into the pancreas, but it might also reduce the efflux of DC to the draining lymph node. Normally mature DC are retained in the lymph node by the predominant expression of CCL19 and CCL21 in the draining lymph node. In NOD mice, mature travelling DC would already be retained in the pancreas by the increased expression of CCL19 and CCL21 in the pancreas. It must be noted, however, that I found mouse bone-marrow-derived DC to show a reduced migratory response to CCL19. Hence a reduced efflux by perivascular islet CCL19/CCL21 expression would perhaps not affect DC that vigorously.

It could also be that the pancreatic CCL19 and CCL21 are involved in or the consequence of the formation of secondary lymphoid tissue in the pancreas of NOD mice. These lymphoid structures have been described in BDC2.5 TCR transgenic NOD mice and occasionally also in wild-type NOD mice (67). In the RIP-LCMV model for autoimmune diabetes, lymphoid tissue structures have also been found and an important role in their formation has been proposed for DC (68). Also transgenic mice with an over-expression of CCL19 or CCL21 in the islets, developed lymphoid structures in the pancreas containing lymphocytes, stromal cells and DC (69, 70). Moreover, we have reported on the presence of lymphoid tissue in the normal foetal and newborn human pancreas (71) and such lymphoid tissue was particularly prominent in a case of a diabetic infant (72).

In conclusion: The early para- and peri-islet accumulation of DC and mφ might be the consequence of a “non-inflammatory” attraction of (certain subsets of) monocytes and/or precursors by aberrantly high expressed lymphoid-tissue related chemokines in the pancreas.

Para- and peri-islet mφ and DC accumulation: the consequence of an enhanced adhesion to fibronectin and endothelial cells?

Could there be another driving force behind the very early accumulation of para- and peri-islet mφ and DC in the pancreas in type 1 diabetes, apart from the effect of the constitutive chemokines? An accumulation of leukocytes is the result of both an influx and an efflux of cells. In this thesis I describe that the para- and peri-islet mφ and DC accumulate at sites of intense fibronectin labelling at the ductal-vascular pole and the periphery of the islets (chapter 4.1 and (73)). In addition, I showed that NOD and T1D monocytes and DC display an increased adhesion to endothelial cells and fibronectin. Therefore I like to propose that the increased adhesion of the diabetic monocytic cells to endothelial cells and fibronectin is also a major contributor in the para- and peri-islet accumulation of such cells in the pancreas.

In conclusion: collectively my data show that the early mφ and DC accumulation in the NOD pancreas is not likely the result of a regular inflammation-driven increased influx of monocytes and precursors from the circulation. The increased MRP8/14 induced adhesive potential of monocytes to fibronectin and endothelium (intrinsic to monocytes, mφ and DC of NOD mice, but also of monocytes of human T1D subjects) combined with an alternative attraction by lymphoid tissue-related constitutive chemokines, likely contributes to the retention and hence the early accumulation of these cells in the para- and peri-islet environment of the pancreas (figure 1).

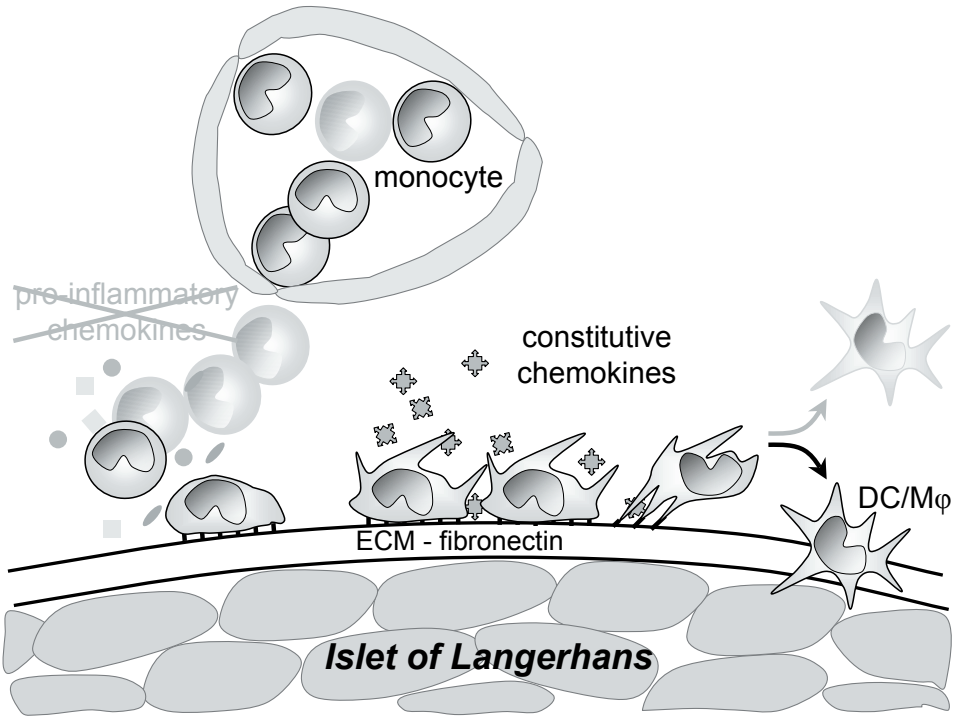


Figure 1.
Schematical representation of the accumulation of mφ and DC around the islets of Langerhans. Monocytes enter the pancreas, but not in response to pro-inflammatory chemokines as occurs during inflammation (depicted in light gray). Possibly the monocytes are attracted by constitutively expressed chemokines. The monocytes adhere to the ECM and mature to mφ or DC. As a result of the intrinsicly increased adhesion and the increased expression of constitutively expressed chemokines, the mφ and DC do not emigrate to the draining lymph node (gray arrow), but stay in the pancreas (black arrow).

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CHAPTER 5.2

FUTURE DIRECTIONS

FUTURE DIRECTIONS

The actual contribution of the enhanced adhesion and alternative attraction by constitutive chemokines of monocytes and monocyte-derived cells to the actual development of islet autoimmunity and/or loss of tolerance towards islet antigens remains to be established. Normal homeostatic trafficking of DC is thought to be important for the maintenance of peripheral tolerance (1-3). However, little is known about the kinetics of trafficking of monocytes, mφ and DC in the pre-diabetic pancreas. When APC are retained in the tissue they may acquire an immunocompetent rather than a tolerogenic phenotype. Indeed contact of DC with ECM induces maturation of the cells (4, 5). Therefore a prolonged contact with ECM may induce complete maturation of the DC in NOD mice resulting in the induction of an immune response in the draining lymph node rather than the induction or maintenance of tolerance. Isolation of mφ and DC from the pancreas of NOD mice will provide knowledge on the competence of the cells, although isolation techniques may influence the function of the cells to such extend that differences may be lost. Labelling monocytes with dyes allows kinetic studies of the time these cells and their descendants spend in the pancreas. In the future imaging techniques may become available that allow tracking of the monocytes from the blood, through the pancreas and to the draining lymph node in vivo.

I observed an increased expression of the lymphoid tissue-related chemokines CCL19 and CCL21 in the NOD pancreas at the time of mφ and DC accumulation and as stated above these chemokines might be involved in the formation of lymphoid tissue in the pancreas. This will be an interesting topic for further investigations.

In addition, in patients with T1D I observed that the MRPs that were raised in the serum play an important role in the increased adhesion of monocytes to fibronectin. Further investigations must provide insight in a possible role of the MRPs in the altered migration of the monocytes, as described in this thesis. It will also be interesting to study the role of MRPs in the development of diabetes in the NOD mouse.

In conclusion, my data indicate that monocytes of human T1D patients and of NOD mice show an increased adhesion to fibronectin and endothelial cells and a reduced migration to pro-inflammatory stimuli that likely is the result of an intrinsic defect of the monocytes. Further research is required to gain more insight in the molecular nature of this defect to allow a better diagnosis of people at risk and to provide targets for pharmaceutical intervention.

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ABBREVIATIONS

$1,25(OH)_2D_3$	1α,25-Dihydroxyvitamin D_3
APC	antigen presenting cell
BB-DP	bio-breeding diabetes prone
BB-DR	bio-breeding diabetes resistant
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CTLA-4	cytotoxic T lymphocyte associated-4
CY	cyclophosphamide
DC	dendritic cells
EAE	experimental autoimmune encephalomyelitis
ECM	extracellular matrix
DM1	type 1 diabetes
DM2	type 2 diabetes
GAD	glutamic acid decarboxylase
HLA	human leukocyte antigen
ICA	islet cell antigen
ICAM-1	intercellular adhesion molecule-1
IDDM	insulin-dependent diabetes mellitus
Ig	immunoglobulin
IL	interleukin
IFN	interferon
JAM-A	junctional adhesion molecule-A
LFA-1	leukocyte function antigen-1
lip-Cl_2MDP	liposomal dichloromethylene diphosphonate
MAdCAM-1	mucosal addressin cell adhesion molecule-1
MHC	major histocompatibility complex
mφ	macrophages
MRP	myeloid related protein
NF-κB	nuclear factor-κB
NOD	non-obese diabetic
NON	non-obese non-diabetic
NOR	non-obese resistant
PECAM-1	platelet-endothelial adhesion molecule-1
PGE_2	prostaglandin E_2
PGS2	prostaglandin synthase 2
PNAd	peripheral lymph node addressin
poly I:C	polyinosinic polycytidilic acid
PSGL-1	P-selectin glycoprotein ligand-1
scid	severe combined immunodeficiency
SLE	systemic lupus erythematosus
SPF	specified pathogen free
T1D	type 1 diabetes
TNF-α	tumor necrosis factor-α
VCAM-1	vascular cell adhesion molecule-1
VLA	very late antigen
VNTR	variable number of tandem repeats

SUMMARY

Type 1 diabetes is characterized by a T cell mediated destruction of the insulin-producing β cells in the islets of Langerhans that are situated in the pancreas. Prior to the infiltration of lymphocytes into the pancreas, an accumulation of macrophages (mφ) and dendritic cells (DC) is observed. It is generally thought that these mφ and DC originate from blood monocytes that have entered the pancreas and have differentiated into mφ or DC. On the basis of their normal physiological role, these cells presumably take up self-antigens and process these into peptides, which they present, after a so-called “steady-state” or “homeostatic” trafficking of the DC to the draining lymph nodes, to T lymphocytes in the para-cortical area. Normally this leads to tolerance induction and T regulatory cells are induced. However in the case of diabetes development, not regulatory T cells, but erroneously effector T lymphocytes become activated and islet autoimmunity is induced. These effector T cells that were primed in the lymph node, become re-activated after re-circulation upon recognition of the self-antigens in the pancreas and consequently – together with macrophages - initiate inflammation and mediate β cell destruction, which is a hallmark of type 1 diabetes.

In this thesis I have studied the processes involved in the early accumulation of mφ and DC in the pancreas prior to the infiltration of lymphocytes. The extravasation of monocytes from the circulation into the pancreas is a complex process. Amongst other factors, adhesion molecules, chemokines and myeloid related proteins (MRPs) play an important role in the adhesive and migratory responses of the monocytes that enable effective extravasation.

I studied the adhesive and migratory behaviour of human monocytes of type 1 diabetic patients and compared these functions with those of monocytes of type 2 diabetic patients and healthy control subjects. First of all, I was not able to detect any differences between patients and control subjects regarding the subdivision of circulating monocytes in mature and immature cells based on the expression of CD14 and CD16 (chapter 2.1). Secondly, monocytes of patients with type 1 diabetes displayed an intrinsically increased surface expression of the pro-inflammatory molecule MRP8/14 and an increased serum level of MRP8/14. When monocytes were allowed to adhere to the extra cellular matrix component fibronectin the cells showed an enhanced expression and production of MRP8/14. The monocytes of type 1 diabetes patients showed the strongest expression and production of MRP8/14 which was significantly increased over that of healthy control monocytes (chapter 2.2). Furthermore, such activated type 1 diabetic monocytes showed an even stronger adhesion to fibronectin, which was indeed found to be the effect of exposition of the monocytes to MRP8/14 (chapter 2.1). My findings suggest a positive feedback mechanism regarding the adhesive capacity of monocytes in type 1 diabetes: circulating monocytes express and secrete higher levels of MRP8/14 as compared to healthy control subjects, resulting in increased MRP8/14 in the

serum. The increased serum MRP8/14 induces an increased adhesive capacity to fibronectin of the monocytes, which leads to an even larger secretion of MRP8/14 compared to healthy controls.

In this thesis I also describe that the increased MRP8/14 in the serum induced an increased expression of CD11b/CD18 on the monocytes that is likely involved in the increased adhesion of the type 1 diabetic monocytes to endothelial cells that I observed (chapter 2.2).

After the adhesion studies I investigated the migratory behaviour of monocytes of type 1 diabetes patients and observed a remarkably decreased response towards the pro-inflammatory chemokines CCL2 and CCL3. Both the transendothelial migration (measured in a Transwell system) and the chemotaxis (measured in the classical Boyden assay) towards these pro-inflammatory chemokines were decreased for monocytes in type 1 diabetes. In contrast, the chemotaxis of monocytes of type 1 diabetes patients to the constitutively in lymphoid-tissue expressed CCL19 was increased in comparison to healthy controls (chapter 2.2).

Since in human subjects it is not feasible to study the diabetes development in the pre-diabetes stage, I also studied the adhesive and migratory capacity of monocytes of the NOD mouse, a widely used spontaneous animal model for type 1 diabetes, before the actual development of lymphocytic insulitis. Unlike patients, NOD mice showed increased numbers of mature (in the mouse Ly-6C^{low}) monocytes in the circulation and a preferential differentiation of monocytes towards mφ (chapter 3.1). In contrast to control mice, NOD mice did not down regulate the fibronectin adhesive capacity of their monocytes upon maturation and the NOD Ly-6C^{low} monocytes displayed an increased adhesion to fibronectin as compared to the NOD Ly-6C^{low} monocytes of control mice. Hence a shared feature of monocytes of human type 1 diabetic subjects and NOD mice is an increased adhesive capacity to fibronectin (chapters 2.1 and 3.1).

With regard to the migratory potential of monocytes in NOD mice, I studied the in vivo recruitment of monocytes in response to inflammation using two models. In the peritonitis model, in which a sterile inflammation is induced in the peritoneum by injection of thioglycollate, the recruitment of monocytes was severely decreased as compared to control mice to various pro-inflammatory stimuli (chapter 3.2). Also in the air pouch model (a model in which sterile air is injected subcutaneously on the back of the mouse, creating a body cavity in which chemokines can be injected) the monocyte recruitment to the pro-inflammatory chemokines CCL2 and CCL3 was severely hampered (chapter 3.2). Like for adhesion these findings show a parallel between NOD mice and type 1 diabetes patients: in both situations monocytes show a poor reaction to pro-inflammatory chemokines.

I also studied the expression of chemokines in the pancreas of NOD mice during the development of diabetes. An increased expression of the pro-inflammatory chemokine CCL2 could never be detected as compared to control mouse strains. The expression of the pro-inflammatory chemokines CCL5 and CXCL10 was found

only to be expressed in the NOD pancreas at a higher level at a later stage of the insulitis, i.e. at the time of the infiltration of T and B lymphocytes, hence after the early accumulation of the mφ and DC (chapter 4.1). At the time of the early mφ and DC accumulation an increased expression of the lymphoid tissue-related chemokines CCL19 and CCL21 was observed. Interestingly DC of NOD mice showed in vitro a decreased migration to the lymphoid tissue-related chemokine CCL19 (chapter 4.1), but an increased adhesion to fibronectin. NOD monocytes were not tested for their chemotactic response towards this chemokine.

It is likely that the increased adhesion to endothelial cells and fibronectin and the altered response of monocytes (and DC) to pro-inflammatory and constitutively expressed chemokines are variables determining the enhanced accumulation of mφ and DC in the pancreas in the early stages of the diabetic process. My findings suggest that particularly the increased adhesion of the cells to fibronectin may lead to a retention of mφ and DC in the pancreas. There the mφ and DC may contribute to an inflammatory environment by the production of e.g. TNF-α. Such local inflammatory environment induces the expression of pro-inflammatory chemokines by the β cells and likely also by the mφ and DC. Indeed we observed an increased expression of pro-inflammatory chemokines in the NOD pancreas at stages in which already an accumulation of mφ and DC was observed. The infiltration of T and B lymphocytes correlated with the expression of these pro-inflammatory chemokines. Our data hence suggest that the inflammatory environment in the pancreas of NOD mice is rather the consequence of the accumulating mφ and DC than the cause.

Both human and mouse monocytes showed a decreased migratory response towards pro-inflammatory chemokines, suggesting that an inflammatory-driven influx of monocytes as the origin of the early mφ and DC accumulation in the pancreas is not likely. It could be that the monocytes use the lymphoid tissue-related chemokines CCL19 and CCL21 as an alternative route to enter the pancreas. It could also be that these chemokines are involved in the formation of secondary lymphoid tissue as have been previously been described in the NOD mice and the RIP-LCMV model for diabetes. The precise role of CCL19 and CCL21 in the development of diabetes is not clear, but it provides an interesting subject for further investigation.

In conclusion, in this thesis I show an interesting parallel between the adhesive and migratory behaviour of human and mouse monocytes in type 1 diabetes, providing evidence for a relation between the aberrant adhesive and migratory capacity of monocytes and DC and the development of type 1 diabetes. The mechanisms whereby an increased fibronectin adhesion and altered migration of the monocytes and DC triggers the autoimmune process need to be investigated further, providing a new and interesting challenge.

SAMENVATTING VOOR NIET-INGEWIJDEN

Type 1 diabetes, vroeger ook wel jeugddiabetes genoemd, wordt gekenmerkt door een afweerreactie gericht tegen de insuline-producerende β cellen. Bij deze afweerreactie valt het afweersysteem (immuunsysteem) de lichaamseigen β cellen in de alvleesklier aan en vernietigt deze. Deze afweerreactie waarbij het lichaam zichzelf aanvalt wordt een auto-immuunreactie genoemd. Als gevolg van deze auto-immuunreactie vermindert de productie van insuline, die belangrijk is voor de glucose huishouding binnen het lichaam. Door de verminderde insulineproductie schommelt de hoeveelheid glucose in het bloed, wat leidt tot verminderde functie van de bloedvaten en kans op complicaties.

In de auto-immuunreactie tegen de β cellen spelen een aantal type afweercellen een belangrijke rol. In dit onderzoek hebben wij ons met name op de rol van monocyten gericht. Monocyten worden gemaakt in het beenmerg, waarna ze in het bloed terechtkomen. Vanuit het bloed kunnen de monocyten de verschillende organen binnentreden. Zodra de monocyten een orgaan zijn binnengetreden, ontvangen ze allerlei signalen waardoor de monocyten zich verder ontwikkelen tot macrofagen of dendritische cellen. Macrofagen en dendritische cellen zijn gespecialiseerde afweercellen die in het lichaam bacteriën en resten van dode cellen opruimen en zorgen dat er een immuunreactie wordt gestart. Uit onderzoek met proefdieren, maar ook bij de mens, is gebleken dat in een vroege fase van diabetes ontwikkeling een ophoping van macrofagen en dendritische cellen te zien is in de alvleesklier. De β cellen liggen in een soort eilandjes gegroepeerd, de zgn. eilandjes van Langerhans. Er vindt een ophoping plaats van macrofagen en dendritische cellen rondom deze eilandjes. Pas in een latere fase verschijnen er andere afweercellen bij de eilandjes, die infiltreren vervolgens de eilandjes en uiteindelijk worden dan de β cellen vernietigd. Tenminste een deel van de macrofagen en dendritische cellen die zich in een vroege fase van diabetes ontwikkeling ophopen rond de eilandjes is afkomstig van monocyten uit het bloed. Men denkt dat de dendritische cellen in de alvleesklier lichaamseigen stoffen opnemen en bij vergissing een immuunreactie tegen het eigen lichaam starten, met als gevolg de vernietiging van de β cellen. In mijn onderzoek, dat is beschreven in dit proefschrift, heb ik mij gericht op de functie van monocyten. Men denkt dat een verhoogd binnentreden van monocyten uit het bloed de alvleesklier in, de oorzaak is van de ophoping van macrofagen en dendritische cellen, die leidt tot de auto-immuunreactie en uiteindelijk diabetes.

Het binnentreden van monocyten uit het bloed het weefsel in is een complex proces. Allereerst wordt de monocyt in het bloed afgeremd door de interactie van structuren die zich op de bloedvatwand bevinden met structuren op de monocyt. Door deze interactie remt de monocyt af en rolt deze over de wand van het bloedvat. Vervolgens bindt de monocyt met andere structuren aan moleculen op het bloedvat. Deze interacties tussen structuren op het bloedvat en op de monocyten

zijn te vergelijken met de werking van klittenband. Deze laatste binding is dusdanig stevig dat de monocyt op de plaats blijft waar hij zit en niet meer meegesleurd kan worden door de bloedstroom. Hierna wringt de monocyt zich tussen de cellen van het bloedvat door, het weefsel in. Chemokinen zijn kleine eiwitten die door het weefsel geproduceerd worden. De monocyten kunnen deze herkennen met hun chemokinereceptoren. Deze herkenning geeft de monocyt een signaal dat die zich in die richting moet bewegen. De monocyt zal dus in de richting van de hoogste concentratie chemokine kruipen.

In hoofdstuk 2 van dit proefschrift heb ik de hechting en migratie van monocyten van type 1 diabetes patiënten bestudeerd. Hechting is de interactie tussen structuren op de monocyt met structuren op het bloedvat of op het weefsel en speelt een belangrijke rol bij het binnentreden van cellen het weefsel in. Hechting is ook een voorwaarde voor migratie. Migratie moet gezien worden als een dynamisch proces van vasthechten en weer loslaten met als resultaat het voortbewegen in een bepaalde richting, dit is vergelijkbaar met de voortbeweging dmv een rupsband. Monocyten gebruiken de extracellulaire matrix, dat is de matrix die cellen van het weefsel bij elkaar houden en het weefsel vorm geeft, als ondergrond om naar de juiste plek te migreren. Monocyten van type 1 diabetes patiënten bleken beter te kunnen hechten dan monocyten van gezonde vrijwilligers (hoofdstuk 2.1), terwijl de migratie naar bepaalde chemokinen slechter is (hoofdstuk 2.2). Een belangrijke factor bij de hechting van monocyten bleek het eiwit MRP8/14 te zijn. Dit eiwit was verhoogd aanwezig in het bloed van type 1 diabetes patiënten en kon een verhoogde hechtingscapaciteit bij monocyten induceren (hoofdstuk 2.1). Ook bleek hechting van monocyten een verhoogde uitscheiding van MRP8/14 door deze monocyten te veroorzaken bij monocyten van patiënten met type 1 diabetes (hoofdstuk 2.2). Het lijkt erop dat MPR8/14 betrokken is bij een positieve feedback mechanisme om een optimale hechting van monocyten te realiseren.

Omdat het bij patiënten met type 1 diabetes niet mogelijk is de alvleesklier te bestuderen en het ook niet mogelijk is de pre-diabetes fase bij patiënten te onderzoeken, heb ik ook onderzoek gedaan met de non-obese diabetic (NOD) muis. De NOD muis is een veelgebruikt proefdiermodel voor type 1 diabetes omdat de ontwikkeling van diabetes geschiedt op vergelijkbare wijze als bij de mens. In hoofdstuk 3.1 heb ik de verschillende typen monocyten in het bloed bestudeerd. De NOD muis bleek naar verhouding meer rijpe monocyten te hebben dan controle muizen en tevens bleken deze rijpe monocyten een hoge hechtingscapaciteit te hebben, terwijl bij controle muizen de rijpe monocyten de hechtingscapaciteit hadden verloren. De onrijpe monocyten hadden bij zowel de NOD muis als bij controle muizen een hoge hechting. Ook bleek dat zowel de rijpe als onrijpe monocyten van de NOD bij voorkeur uitrijpten tot macrofagen, meer dan tot dendritische cellen.

Zowel bij type 1 diabetes patiënten als bij de NOD muis bleek een hoge hechtingscapaciteit een kenmerk te zijn van de monocyten. Bij de mens vond ik

ook een slechte migratie in type 1 diabetes. Toen ik dit bij de NOD muis onderzocht, bleek ook hier de parallel te bestaan. De NOD muis had een slechtere migratie van monocyten dan controle muizen. Dit heb ik onderzocht op 2 verschillende manieren: m.b.v. een steriele ontsteking in de buikholte en m.b.v. de zgn. air pouch (hoofdstuk 3.2). Bij een steriele ontsteking in de buikholte door injectie van de stof thioglycollaat kan men op verschillende tijden de afweercellen die de buikholte binnen zijn gegaan eruit halen en tellen. De NOD muis bleek in vergelijking met controle muizen niet goed in staat grote hoeveelheden monocyten uit het bloed aan te trekken de buikholte in. Bij het air pouch model werd op de rug van de muis op verschillende dagen steriele lucht geïnjecteerd, zodat er onderhuids een huidzak ontstond gevuld met lucht. In deze zak werd een chemokine geïnjecteerd waarna in de tijd het aantal afweercellen dat hierdoor aangetrokken werd, geteld kon worden. Ook in dit model bleek de NOD muis minder goed in staat grote aantallen monocyten te rekruteren. In de NOD muis vond ik dus ook een verminderde migratie van monocyten. Omdat monocyten belangrijke voorlopercellen zijn van dendritische cellen, heb ik ook de hechting en migratie van dendritische cellen in de NOD muis bestudeerd. Dit is beschreven in hoofdstuk 4.1. Ook de dendritische cellen van de NOD muis bleken beter te hechten en slechter te migreren dan dendritische cellen van controle muizen.

Men dacht dat in type 1 diabetes de ophoping van macrofagen en dendritische cellen in de alvleesklier het gevolg was van een verhoogd binnentreden van monocyten uit het bloed. Mijn data wijst hier niet op. Om deze hypothese verder te weerleggen, heb ik ook in de alvleesklier van NOD muizen gekeken naar de hoeveelheid chemokinen tijdens de diabetes ontwikkeling. Hierbij heb ik ontdekt dat de zgn. pro-inflammatoire chemokinen, die heel goed monocyten uit het bloed aantrekken, pas laat tijdens de diabetes ontwikkeling verhoogd aanwezig zijn. De verhoogde aanwezigheid van deze pro-inflammatoire chemokinen viel samen met het binnentreden van andere type afweercellen. Deze cellen verschijnen pas als er al een ophoping van macrofagen en dendritische cellen in de alvleesklier is. Het is daarom niet waarschijnlijk dat dergelijke pro-inflammatoire chemokinen verantwoordelijk zijn voor een verhoogd binnentreden van monocyten als oorzaak van de ophoping van macrofagen en dendritische cellen. Veel eerder denk ik dat de verhoogde aanwezigheid van deze pro-inflammatoire chemokinen het gevolg is van de ophoping van macrofagen en dendritische cellen. Het gevolg hiervan is dat andere afweercellen worden gerekruteerd en de laatste fase van diabetes ontwikkeling, de vernietiging van de β cellen, in wordt gegaan. Naar mijn mening is de ophoping van macrofagen en dendritische cellen in de alvleesklier in type 1 diabetes het gevolg van het blijven zitten van de cellen in de alvleesklier. Hierbij speelt de verhoogde hechtingscapaciteit van de monocyten en dendritische cellen waarschijnlijk een belangrijke rol. Nader onderzoek zal duidelijk moeten maken hoe deze verhoogde hechting en veranderde migratie van monocyten precies betrokken zijn bij het ontstaan van auto-immuunreacties, en dit biedt nieuwe en interessante uitdagingen voor vervolgstudies.

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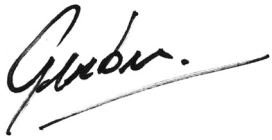

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