The role of monocytes and monocyte-derived dendritic cells in type 1 diabetes mellitus and autoimmune thyroid disease

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The Role of Monocytes and Monocyte-derived Dendritic Cells in Type 1 Diabetes Mellitus and Autoimmune Thyroid Disease

De rol van monocyten en van monocyten afkomstige dendritische cellen in type 1 diabetes mellitus en auto-immuun schildklierziekten

Proefschrift

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	Prof.dr. G.J. Bruining

voor mijn ouders

voor Hoy

Contents

CHAPTER 1

INTRODUCTION

Organ-specific or endocrine autoimmune diseases

Autoimmune diseases are diseases in which the immune system reacts against its own organs and organ systems. Classically, autoimmune diseases are divided into organ-specific and systemic autoimmune diseases. The majority of the organ-specific autoimmune diseases are restricted to endocrine tissues, such as the islets of Langerhans in the pancreas, the thyroid, the adrenal cortex, the parietal cells in the stomach and the steroid producing cells in the gonads.

Type 1 diabetes mellitus (DM1) is the outcome of an immune response directed to and progressively destroying the insulin producing islet cells. When less than about 70% of the initial β cell mass is left and the circulating insulin level is severely reduced, the clinical symptoms of hyperglycemia (polyuria, polydipsia, fatigue, weight loss, ketosis/ ketonuria) will develop. Important autoantigens are Glutamic Acid Decarboxylase 65 (GAD65), tyrosine phosphatase like protein (or insulinoma antigen-2 =IA2) and insulin (1-3). A T helper (Th) 1 reaction specific for these autoantigens that activates macrophages is an important mechanism for killing of the β cells.

When the immune response is directed to the thyroid gland, two major clinical syndromes of autoimmune thyroid diseases (AITD) can be distinguished: Hashimoto's autoimmune thyroiditis and Graves' disease. Hashimoto's autoimmune thyroiditis is a catabolic disorder and is characterised by autoimmune destruction of the thyroid finally leading to clinically overt hypothyroidism. Important autoantigens are thyroid peroxidase (TPO) and thyroglobulin (Tg). As in DM1, a Th1 reaction specific for these antigens and activating macrophages is an important mechanism for the thyrocyte destruction. Graves' disease is an anabolic disorder and is characterised by stimulation of the thyrocytes via antibodies directed to the TSH receptor and mimicking TSH in its function. This leads to thyrocyte growth and excessive hormone production, resulting in a diffuse goiter and hyperthyroidism, both hallmarks of Graves' disease (18).

Autoimmunity against the gastric proton pump H^+/ K^+-ATPase on gastric parietal cells results in autoimmune gastritis (AIG), hypo-/ achlorhydria, hypergastrinemia and iron deficiency anemia. When the intrinsic factor secretion is in addition hampered due to intrinsic factor antibodies pernicious anemia usually develops (4;19).

Clustering of organ specific endocrine autoimmune diseases

It is known that DM1, autoimmune thyroid disease (AITD) and autoimmune gastritis (AIG) often occur together forming the so-called autoimmune polyglandular syndrome (APS) type 3 (5). Chapter 2 represents a review on the association of type 1 diabetes mellitus, autoimmune thyroid disease and autoimmune gastritis and this chapter also includes data obtained from a study of our research group on a series of 397 Dutch DM1 children with a mean age of 9.0 ± sd 3.8 years (range from 0.8-17 years). We studied these diabetic children for TPO-Ab, gastric

parietal cell antibodies, serum TSH and serum T4 levels and more importantly, we additionally investigated thyrogastric autoimmunity and thyroid dysfunction in 260 of their siblings (125 girls and 135 boys, mean age of 11.5 ± sd 5.9 years) and in 423 of their parents (219 mothers and 204 fathers, mean age of 39.5 ± sd 5.8 years).

This review concludes that thyroid autoimmunity is evident in up to one third and gastric autoimmunity in up to a quarter of patients with DM1. Also relatives of DM1 patients, particularly mothers, have higher frequencies of these autoimmune conditions. Also, gastric autoimmunity is present in one third of AITD patients and islet autoimmunity in one out ten.

Animal models of organ-specific endocrine autoimmune diseases

For the various endocrine autoimmune diseases there exist excellent animal models. These animal models often represent APS, since frequently more than one endocrine organ is affected. Chapter 3 reviews the usefulness of these animal models for studying the pathogenesis of type 1 diabetes mellitus, autoimmune thyroid disease and autoimmune gastritis. These models are particularly useful to study the pathogenesis of the very early phases of the autoimmune reaction, since such studies are practically not possible in patients. The animal models represent disease models that develop spontaneously in specifically inbred models, such as obese strain (OS) chicken, the bio breeding diabetes prone (BB-DP) rat and the non obese diabetic (NOD) mouse, or that are artificially induced, such as neonatal thymectomised, experimental allergic models induced by immunizing with auto-antigens in adjuvantia, transgenic models, etc.

We conclude that animal models of endocrine organ-specific autoimmune disease still hold immense promise for the discovery of pathways, genes and environmental factors that determine the development of endocrine organ-specific autoimmune diseases. These models are helpful to uncover disease-associated pathways, which are complicated to define in man. However, careful interpretation of the causes of the human diseases and the involvement of various genes and environmental factors is required considering the different etiologies in the different animal models.

Dendritic cells and T cells are aberrant in the very early phases of the autoimmune reaction in these animal models.

What are dendritic cells?

Dendritic cells (DC) are the most potent antigen presenting cells (APC) of the immune system and are critically involved in the initiation of primary immune responses, the generation of T cell dependent autoantibody formation, graft rejection and autoimmune diseases (6). DC are present in the interstitium of all tissues (except the brain) and DC in the tissues migrate via the lymph to the T cell areas of the draining lymph nodes. DC in lymphatic tissues are characterised

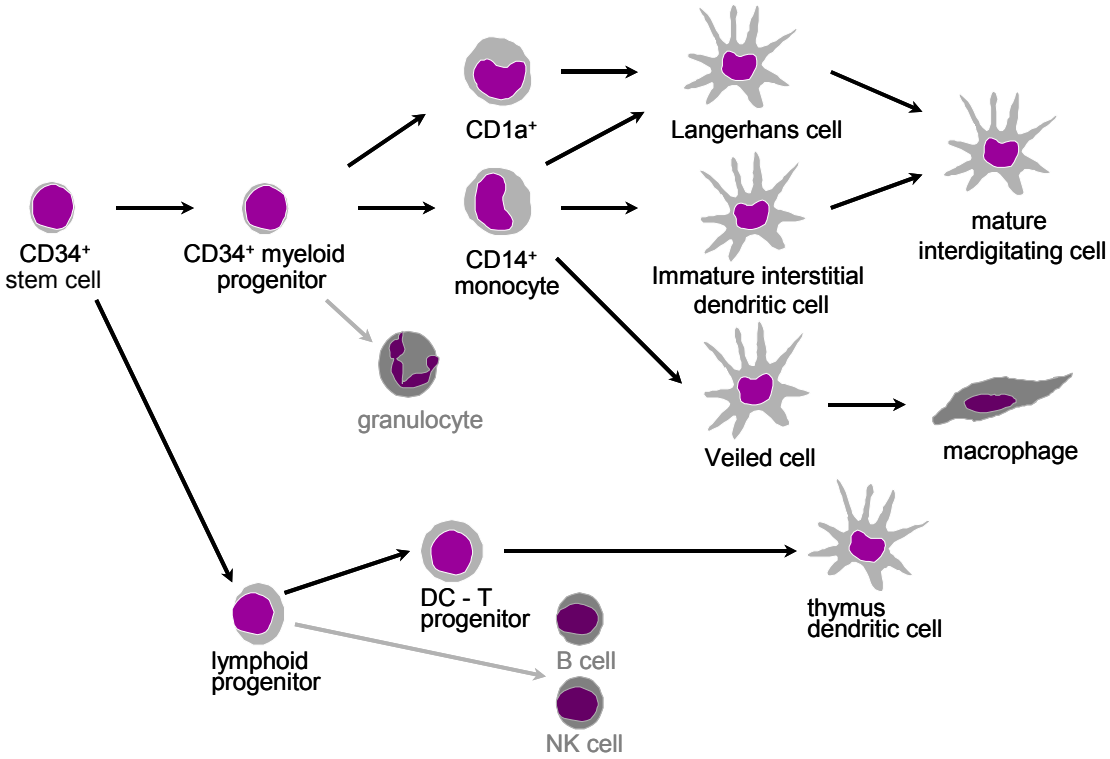
by a strong expression of MHC class II molecules and other essential costimulatory molecules (CD80, CD86, CD40, etc) essential to initiate a proliferation response of naïve T cells.

DC form an enormously heterogeneous group of APC with different lineage backgrounds (so-called lymphoid versus myeloid lineage), precursors and various stages of differentiation and maturation (figure 1). The “lymphoid” DC originate from pre T cells in the thymus and predominantly populate the thymic cortico-medullary junction where the cells are instrumental in the deletion of erroneously created autoreactive T cells. The “myeloid” DC originate from a special CD34+ precursor in the peripheral blood (giving rise to epidermal S100+ Langerhans cells) or from CD14+ circulating monocytes. The monocyte-derived DC are closely linked to other classes of APC, such as the veiled macrophages and often other types of accessory macrophages (figure 1).

Mature and immature dendritic cells. Molecular interactions with T cells.

The presently generally held paradigm (7) in Immunology is that DC present in the interstitium of non-lymphatic tissues are in an immature state, suitable for their sentinel function. The immature cells express various molecules for the uptake of foreign and damaged material (mannose receptors, Toll like receptors), and have a high endocytotic capability enabling to capture and process antigens.

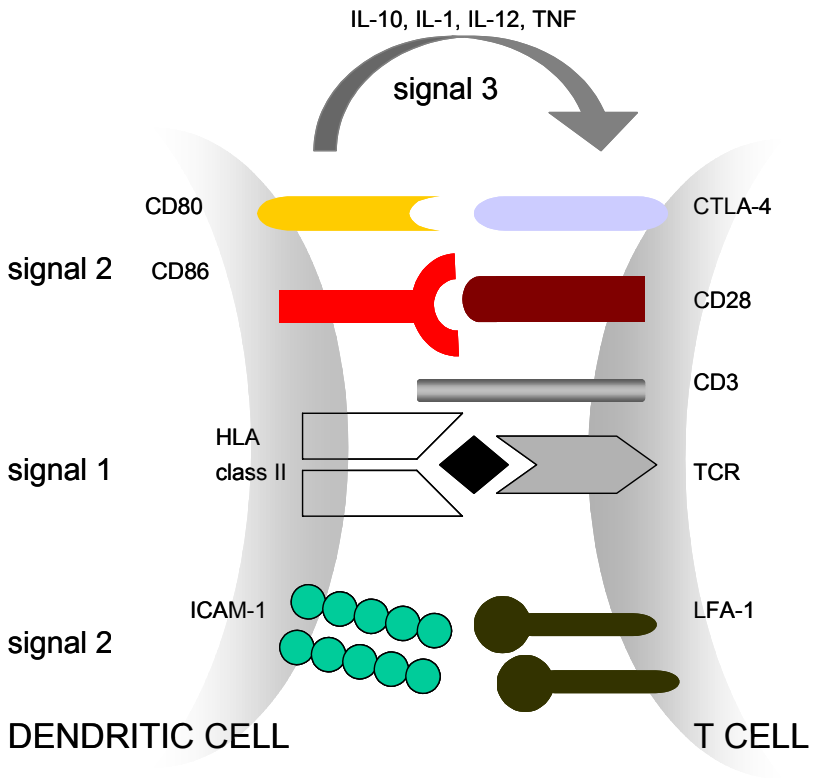
Fig. 1. A scheme of origin and maturation of dendritic cells



These immature DC have a limited potency to stimulate T cells. In response to a local inflammatory stimulus (the so-called danger signal), such as endotoxin (LPS), TNFα and bacteria, interstitial DC undergo maturation. The matured DC have lost their antigen-capturing capacity, but have acquired a strong potency to stimulate the proliferation of naïve antigen-specific T cells, by directing the antigen-loaden MHC molecules to the cell membrane and upregulating their costimulatory molecules. Thus, mature, inflammatory DC are *the* initiators of effector immune responses.

The expression of MHC class II and costimulatory molecules is critically involved in the T cell stimulation by such mature DC. MHC class II binding to the TCR is the first signal between the cells (signal 1). Additional costimulatory signals are however required (signal 2) for cross activation (Fig. 2). CD80 / CD86 binds to CD28 and in a later activation phase to CTLA-4. CTLA-4 is exclusively expressed on activated $CD4^+$ and $CD8^+$ T cells. It binds to CD80 / CD86 with a much higher affinity than CD28, down regulates T cell function and mediates apoptosis (11). The third signal in the DC-T cell cross activation is stimulation via cytokines. IL-12 stimulates in particular a Th1 skewed differentiation of the T cells, while IL-10 stimulates a Th2 skewing. The skewing signal is probably also in part dependent of the costimulatory signal, since it has been shown that a higher co-stimulation is needed for Th2 than for Th1 skewing (12;13).

Fig. 2. A simplified scheme of a DC-T cell cross-talking during antigen presentation. The HLA class II present the Ag to the TCR at low avidity. Additional binding of CD54 (ICAM-1) to CD11/CD18 (LFA-1) facilitate full ligation of the HLA-TCR complex. The T cell is now activated and upregulates costimulatory molecules (e.g. CD28, which bind to CD80 and CD86 on DC. CD80 and CD86 both bind to CTLA-4 and CD28 with a different affinity. The third signal includes the production and secretion of cytokines.

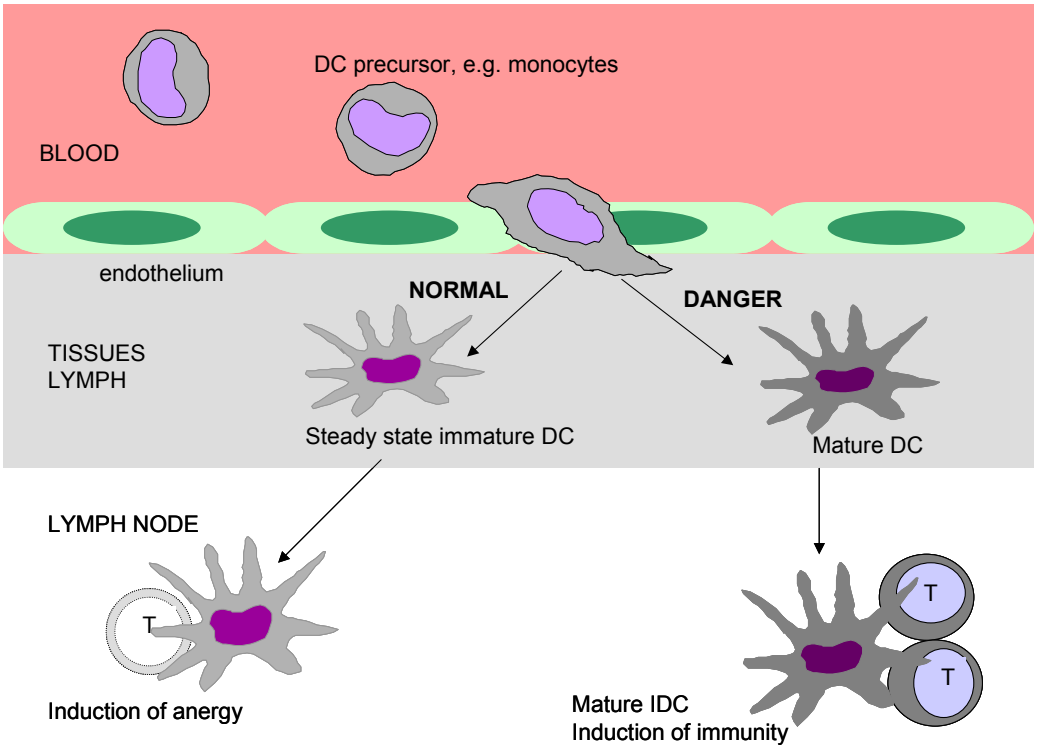


DC and T cell tolerance

The last years the idea has gained acceptance that DC are also prime inducers of tolerance (8). With regard to central tolerance, there are strong indications that thymic DC express autoantigens and can therefore act as the prime deletors of auto reactive T cells created in the thymus (20). However this deletion process is far from complete: low, but sufficient auto reactive T cells escape to the periphery. Peripheral tolerance mechanisms should keep these circulating autoreactive T cells under control.

When interstitial, immature DC are not triggered by “danger” signals and stay under “steady state” conditions, there is nevertheless a continuous travel of such immature DC (carrying auto-antigens) to the draining lymph nodes ((9), figure 3). Such DC lack sufficient co stimulatory molecules and are able to induce anergy in circulating autoreactive T cells (8). Whether these DC are capable of inducing deletion of autoreactive T cells is presently a matter of debate and research (10). On the other hand when mature DC in the lymph node give strong signals not to naïve autoreactive T cells, but to T cells that have recently expanded in multiple proliferation rounds, the latter T cells stop to proliferate and go into apoptosis (the so-called Activation Induced T cell Death, AITCD, figure 4). Hence there are various ways in which DC are indispensable for tolerance induction and ending autoreactive T cell reactions.

Fig. 3. DC precursors, such as the monocyte, continuously migrate through the epithelium via the tissues to the draining lymph nodes. Various molecules, such as adhesion molecules (integrins), interleukins and chemokines are instrumental in this traffic. In a non-danger (“steady state”) environment DC stay immature and induce after migration T cell anergy in the draining lymph node. However when DC encounter danger signals in the tissues, e.g. microbial agents or necrotic cells, they maturate and are able to induce proliferation and activation of naïve T cells in the draining lymph node.

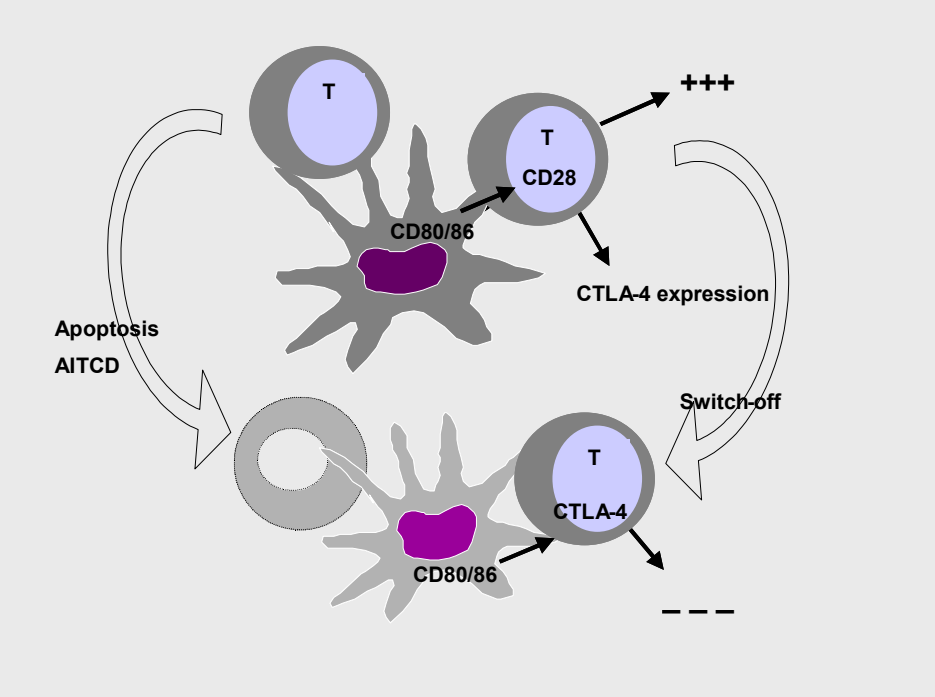


Dendritic cells in type 1 diabetes and autoimmune thyroid disease

Since DC defects are important in the animal models of endocrine autoimmune disease (see Chapter 3) and since DC are amongst the first cells that are activated in the pancreas and thyroid (14-17) of BB-DP rats and NOD mice, we decided to study monocytes and monocyte derived DC in patients with DM1 and AITD. Data on such cells in human endocrine autoimmune diseases are relatively scarce.

An important purpose of the studies described in this thesis was to gain insight to the role of these APC in the immunopathogenesis of DM1 and AITD. In chapter 4, 5 and 6 we investigated the accessory function of monocytes and monocyte derived DC in DM1, AITD and the relatives of DM1 patients. Specifically, we studied the differentiation of DC from monocytes, the adhesion molecule expression and cytokine production of monocytes and DC and the T cell stimulatory capacity of the obtained DC. We conclude from these chapters that monocytes and DC of DM1 patients have a low expression of CD54, low IL-10 production, are defective in the differentiation with reduced T cell stimulatory capacity. The first-degree relatives of DM1 patients however, showed (some opposite) features: normal expression of CD54, high IL-10 production, enhanced differentiation and enhanced stimulatory capacity of T cell proliferation. No such aberrancies were detected in AITD patients. Since we found virtually normal monocyte and DC function in AITD, we review in chapter 7 the role of DC in the pathogenesis of AITD.

Fig. 4. A scheme of the costimulatory signals in the induction of activation and in the silencing of effector T cells. Mature DC with a high expression of the costimulatory molecules CD80 and CD86 stimulate proliferation of naïve T cells via triggering of CD28 on the T cells. After such activation the T cells respond by an up-regulation of CTLA-4, which is a molecule able to down-regulate the functions of T cells. In addition, after various rounds of proliferation T cells become vulnerable to apoptosis. The same costimulatory signals given by the mature DC now induce a silencing of the activated T cells or induce apoptosis (AITCD) in these T cells.



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CHAPTER 2

THE ASSOCIATION BETWEEN TYPE 1 DIABETES MELLITUS, AUTOIMMUNE THYROIDITIS AND AUTOIMMUNE GASTRITIS.

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Summary

Type 1 diabetes mellitus (DM1), autoimmune thyroid disease (AITD) and autoimmune gastritis (AIG) often occur together forming the so-called autoimmune polyendocrine syndrome (APS) type 3. Thyroid autoimmunity is evident in up to one third and gastric autoimmunity in up to a quarter of patients with DM1. Also relatives of DM1 patients, particularly mothers, have higher frequencies of these autoimmune conditions. Also, gastric autoimmunity is present in one third of AITD patients and islet autoimmunity in one out ten.

Screening DM1 patients and their relatives (particularly females) for thyroid autoimmunity is recommended. If positive, excess iodine should be avoided and thyroxine treatment considered. Whether autoimmune thyroiditis and autoimmune gastritis patients should be screened for islet Ab is not clarified.

Introduction

There are two main categories of autoimmune diseases: "organ-specific" and "systemic" autoimmune diseases. The immune attack in the organ-specific autoimmune diseases is confined to one organ or organ system, while in the systemic autoimmune diseases the damage is throughout the body and often the consequence of immune complex formation and deposition. In the majority of organ-specific autoimmune diseases, target tissues are endocrine, hence this category of autoimmune diseases is also often referred to as “endocrine autoimmune diseases”. Important target tissues are the islets of Langerhans, the thyroid, gastric parietal cells and steroid producing cells in the adrenal and ovary.

Autoimmune endocrine gland insufficiencies regularly associate with other endocrine autoimmune and non-autoimmune diseases in patients and in their families. The associations between the various autoimmune diseases were noted not to appear at random but in particular combinations. Consequently, in 1980 Neufeld and Blizzard (1) organized and classified these clinical clusters in four main types defined as polyglandular autoimmune diseases, also termed autoimmune polyendocrine syndromes (APS).

The most frequent co-occurrence between endocrine autoimmune diseases is that between DM1, AITD and AIG. This syndrome is sometimes referred to as “APS type 3”. In the original classification of Neufeld and Blizzard (1) APS type 3 was defined as the association between one of the clinical entities of the AITD (Hashimoto’s thyroiditis (HT), idiopathic myxedema, symptomless autoimmune thyroiditis, Graves’ disease (GD), endocrine ophthalmopathy) and one or more of other autoimmune diseases, i.e. DM1 (type 3a), atrophic gastritis/pernicious anemia (type 3b) or vitiligo, alopecia, myasthenia gravis (type 3c). Whereas the diagnosis APS type 1 and 2 requires the presence of autoimmune Addison’s disease, in APS type 3 this disease (and hypoparathyroidism) needs to be absent (Table I). APS type 3 is a polygenic trait, whereas APS type 1 is a autosomal recessive monogenetic disease, which is associated with mutations in the autoimmune regulator (AIRE) gene. This gene regulates autoimmunity by promoting ectopic expression of peripheral tissue restricted antigens in the thymus and thereby plays an important role in tolerance induction for self antigens (81).

Different and multiple clinical combinations were found in APS type 3 and it has become evident that the classification of APS type 3 may be more complicated than initially reported. New classification criteria for APS type 3 have recently been proposed (2;3). This review firstly aims at reviewing the literature regarding the co-occurrence of thyroid autoimmunity, autoimmune insulitis (type 1 diabetes mellitus, DM1) and autoimmune gastritis (APS types 3a and 3b), which form the most frequent combinations. It secondly focuses on what is presently known on the (putative) pathogenic mechanisms behind the more than co-incidental co-

occurrence of these endocrine autoimmune diseases. On the basis of these mechanisms recommendations for diagnostic and preventive measures are proposed.

AITD is more frequent in DM1 patients as compared to the frequency reported in non-selected control populations. The co-occurrence of AITD in DM1 patients is characterized by a persistence of islet reactive antibody (Ab) (4;5).

The reported frequency of thyroid peroxidase antibody (TPO-Ab) positivity in DM1 varies widely (from 6 to 40%) and is given in Table II. This variation is in part due to the various (generations of) thyroid antibody assays used in the last 50 years and their respective cut-off levels, but it is probably also due to differences in ethnicity, age and gender of the patients.

In DM1 patients positivity for TPO-Ab is strongly associated with sub-clinical hypothyroidism and the majority of studies report a close correlation between the presence of TPO-Ab and an increased serum thyroid stimulating hormone (sTSH) level as a sign of sub-clinical hypothyroidism (6-8). However, there is also a report that denies this association (9). Sub-clinical hypothyroidism is present in 1.4-10% of DM1 patients. Sub-clinical hyperthyroidism is present in 0-6% of DM1 patients (table II.).

With regard to the *development* of an actual clinically overt thyroid dysfunction several studies exist in DM1 patients. Diabetic patients who are positive for thyroid Ab and have a sub-clinical thyroid dysfunction are more prone to develop clinically overt thyroid disease (10-12). Two follow up studies of TPO-Ab positive DM1 patients showed high percentages of thyroid failure in such patients: MacLaren et al reported that 38% of TPO-Ab positive DM1 children and young adolescents developed overt hypothyroidism and 7% hyperthyroidism within 5 years (12). The other study described that half of the patients with TPO-Ab developed thyroid dysfunction in a median period of 3.5 years and that this development was associated with the highest TPO-Ab titers (13).

Table I. The autoimmune polyglandular syndromes (APS) type 1, 2, 3a and 3b (modified from Muir et al 1999 and Betterle et al 2002).

<table>
<tr><th>APS type 1</th><th>APS type 2</th><th>APS type 3a</th><th>APS type 3b</th></tr>
<tr><td rowspan="2">At least two of the following:

autoimmune Addison disease

hypoparathyroidism

candidiasis</td><td>autoimmune Addison’s disease with</td><td colspan="2">autoimmune thyroid disease (Hashimoto’s disease) with</td></tr>
<tr><td>autoimmune thyroid disease
and/ or
type 1 diabetes</td><td>type 1 diabetes</td><td>chronic atrophic gastritis
or
pernicious anemia</td></tr>
<tr><td colspan="3">exclusion of autoimmune Addison’s disease, hypoparathyroidism and candidiasis</td></tr>
</table>

The prevalence of overt thyroid dysfunction in DM1 patients ranges from 2.6% in young DM1 patients to 8% in adult patients (4;9;14-21), hypo- and hyperthyroidism in these patients have generally been reported to occur at the same rate, i.e. in 0.5-4% of DM1 patients (4;14;15;17;19;20;22;23). One study reported an excessively high prevalence of GD in 9.3% of DM1 patients (24).

We recently studied a series of 397 children with ages ranging from 0.8-17 years with DM1 and observed 6% TPO-Ab positivity, 10% sub-clinical and 0.7% overt hypothyroidism (Table II). Hyperthyroidism was not detected in our study.

The prevalence and incidence of AITD latent autoimmune diabetes of the adults

In a special subgroup of diabetes, the Latent Autoimmune Diabetes of the Adults (or LADA), an increased frequency of other organ specific autoimmune diseases has been found. LADA represents a slow progressive form of autoimmune diabetes and is a complex and heterogeneous disorder with different phenotypes. Since the presence of Glutamic Acid Decarboxylase 65 (GAD)-Abs is an important characteristic, LADA is considered to be an intermediate form between DM1 and DM2. Compared to diabetic patients without GAD-Abs, patients with these Abs exhibit a lower body mass index, higher insulin requirement, lower basal C-peptide and less metabolic complications (25-28). Moreover, the insulin requirement and the presence of other autoimmune diseases (especially AITD) is related to the presence, epitope specificity and titer of GAD-Abs, and also the co-occurrence of GAD-Abs with ICA and/ or tyrosine phosphatase like protein antibodies (IA2-Abs). LADA patients have been reported to have an higher frequency of thyroid autoimmunity. Twenty-five to 40% of GAD-Abs and/ or ICA positive patients showed TPO-Abs as compared to 5-18% in GAD-Abs and/ or ICA negative patients (25;27;28).

The prevalence and incidence of DM1 in established cases of AITD

Reports on the prevalence and incidence of islet autoimmunity and DM1 in established cases of AITD are scarce. This is probably due to the fact that AITD mostly starts in women at ages over 25 years, while DM1 starts more frequently at younger age in boys and girls alike. Nevertheless there are a few reports that show a higher frequency of islet related Ab in AITD patients, i.e. a prevalence of 6-8% has been reported for islet cell antibodies (ICA) and GAD-Abs in Japanese patients with GD or HT (29;30). Another study described that 2% of patients with AITD and without co-occurrent DM1 have GAD-Abs and/ or IA2-Abs, and this number increases to 87% in patients with AITD and co-occurrent DM1 (31). Insulin auto-antibodies (IA-Abs) have been found in about 4% in AITD patients.

Table II. Thyroid autoimmunity in DM1 and their relatives

		Destructive autoimmune thyroiditis		Graves' disease	
Thyroid-Abs^{+}	normal TSH and T4	TSH ↑ and normal T4	TSH ↑ and T4 ↓	TSH ↓ and normal T4	TSH ↓ and T4 ↑
DM1					
children	5.8-38% [9;13;16;32-34), *]	1.4-3% [9;20]	0.7-1.9% [9;20), *]	0% [20), *]	0-0.7% [20), *]
adults	13-40% [12;35-39]	2.9-10% [4;6;14]	0.8-4% [4;6;14;17]	0.9-6% [4;6]	1.6-4% [4;6;14;17]
FDR	7.8-13% [7;14;40]	0.7-11% [7;14;15), *]	0.5-0.9% [15), *]	1% [15), *]	0-0.4% [15), *]
siblings	0-7% [15;34;36), *]	9.2% *	0% *	1% *	1% *
parents	17-20% [15;34;36), *]	8% *	1.4% *	1.7% *	0.2% *
offspring	4% [15]				
Controls	0-9.4% [14;30;34-36;40-42]	5.7%*	0% [14]	3.4% *	

Thyroid-Abs^{+}= TPO-Abs^{+} and/ or Tg-Abs^{+}

Note: controls used are from the same studies as the patients and relatives.

* = unpublished data; In this study a series of 397 DM1 children with mean age of 9.0 ± sd 3.8 years (range from 0.8-17 years) were studied for TPO-Ab, GPAb, serum TSH and serum T4 levels. We additionally investigated thyroid autoimmunity and thyroid dysfunction in sibs (n= 260, 125 girls and 135 boys, mean age of 11.5 ± sd 5.9 years) and parents (n= 423, 219 mothers and 204 fathers, mean age of 39.5 ± sd 5.8 years) of the DM1 children separately. Control groups consisted of healthy schoolchildren of the region of Rotterdam (n=81, 54 girls and 27 boys, mean age of 15.3 ±sd 2.1 years, range 10-20 yrs) and healthy persons (n= 251, over the age of 55 years), hospitalized psychiatric patients of the region Utrecht (n= 2468, range 20-50 yrs), a series of pregnant women (n= 291, range 20-40 yrs). Family history for autoimmune diseases was negative for the schoolchildren. Informed consent was obtained for all participants and the ethical committee of the Erasmus MC Rotterdam has approved the protocol.

Although these frequencies are relatively low, they are still many times higher than in healthy control populations. IA-Abs have even been reported in up to 44% in GD patients (29;43;44). However, the assays used were later shown less reliable, thus a new assay is in place to validate these findings (45).

With regard to the *development* of overt DM1, Bosi et al showed that patients with AITD and ICA positivity developed DM1 in 16% of cases within 12 years. The progression to DM1 was dependent on the ICA titer/ persistency, the co-existence of IA-Abs and a family history of DM1 (46;47). With regard to the presence of overt DM1 in AITD patients, this has only been studied by a Japanese group. The authors reported that 7% of AITD patients with ICA have an overt DM1 (30).

The characteristics of co-occurrent DM1 and AITD

Patients with co-occurrent DM1 and AITD are characterized by higher levels of ICA, IA2-Ab and/ or GAD-Ab and an increased persistent islet autoreactivity (5; 14, 30). Interestingly GAD-Ab and TPO-Ab have been reported to closely correlate in such patients (5;6;29;37;42). To explain the close correlation between GAD-Ab and TPO-Ab (and hence the coexistence of DM1 and AITD), it has been proposed that the autoimmune reaction towards GAD in the islets cross-reacts with GAD in the thyroid, where this neuro-enzyme is indeed present. However above described correlation between GAD-Ab and TPO-Ab does not occur in Chinese DM1 patients. This might however be due to the fact that a lower percentage of Chinese DM1 patients are positive for GAD-Ab (32;35). Furthermore, (non) diabetic ICA positive individuals with an endocrine autoimmunity have higher GAD-Ab levels than without an endocrine autoimmunity. Apparently, the GAD-Ab levels are enhanced in coexistent endocrine autoimmunity (48).

A few studies showed that patients with DM1 and co-occurrent AITD are older at the age of DM1 onset compared to DM1 patients without AITD (12;30), although a negative finding in this respect has also been described (19). The effect of the presence of a thyroid dysfunction on the clinical presentation of diabetes has only been investigated to a limited extent and data are somewhat conflicting. A few studies were unable to find differences in the clinical presentation and the complications of diabetes between TPO-Ab negative and positive patients or between DM1 in isolation or co-occurring with AITD (4;13;24). In pregnant diabetic patients, the presence of TPO-Ab was related to a poorer glucose control (23). Even when pregnant diabetic women with thyroid dysfunction were suppleted with thyroxine, the glucose control was still poorer compared to pregnant diabetic women without thyroid autoimmunity. Nevertheless, treatment of diabetic patients for their thyroid dysfunction leads to decreased cholesterol levels and a better glycemic control (17).

The prevalence of AITD in relatives of DM1 patients

TPO-Ab are also more frequent in relatives of DM1 patients as compared to non-selected control populations and Table II gives the frequencies reported in the literature (ranging from 0-20%).

In our own study on children with DM1 and their relatives (see Table II) we investigated thyroid dysfunction in sibs and parents of the DM1 children separately. Siblings have a slightly increased TPO-Ab frequency of 5.4% compared to 4.2% in collected healthy sex and age matched controls (n.s.), whereas parents have a doubled frequency of TPO-Abs compared to controls (19% in parents vs 9% in controls, p=0.00001). In general, we found that siblings and parents have a somewhat higher frequency of sub-clinical hypothyroidism as compared to matched controls (9.2% vs 5.3%, n.s. and 8% vs 5.7%, p=0.08 respectively). Significant differences appeared when we took the gender of the parents into consideration: we especially found mothers of the DM1 children to have sub-clinical thyroid dysfunction compared to sex matched controls (12.3% vs 7%, p=0.04). We also evaluated TPO-Ab positive and TPO-Ab negative parents and sibs separately. In TPO-Ab positive sibs the mean sTSH was not raised as compared to TPO-Ab negative sibs. However, we found a significantly higher mean sTSH level in TPO-Ab positive parents as compared to TPO-Ab negative parents (6 vs 1.7 IU/ml, p=0.0007), indicating a higher risk for developing thyroid dysfunction in TPO-Ab positive parents. For a diagnosis on an individual basis the presence or absence of sub-clinical hypothyroidism (i.e. an individually raised sTSH) is of greater importance. Regarding this parameter we found a higher frequency of sub-clinical hypothyroidism in TPO-Ab positive parents as compared to TPO-Ab negative parents (35% vs 1.7%, p=0.00001). Again, such difference could not be found in sibs (7.1% in TPO-Ab positive sibs vs 9.3% in TPO-Ab negative sibs). It must be noted that De Block et al (2001) were unable to find such higher prevalence of sub-clinical thyroid disease in their TPO-Ab positive relatives, but these investigators did not study parents and sibs separately and the frequency of TPO-Ab positive subjects was small in comparison to our study (15).

With regard to the frequency of overt thyroid dysfunction, this frequency is definitely higher in relatives of DM1 patients than in non-selected controls, although it is lower as compared to the frequencies reported in the DM1 patients themselves. Figures range from 2 to 3 % (14;15). Relatives are in particular at high risk of developing AITD when the diabetic proband has AITD (7;16;24;41;49;50) and the relatives of such patients show a higher frequency of thyroid Ab (7). In this group again the parents are more prone to have the disease.

In our study on the relatives of DM1 children we were able to confirm the close correlation between GAD-Ab and TPO-Ab as has been reported for DM1 patients: In parents GAD-Ab status was positively correlated to TPO-Ab positivity (p=0.006) and the TPO-Ab level (p=0.05).

The prevalence of DM1 in relatives of AITD patients

To our knowledge studies on the presence of GAD-Ab and other diabetes related Ab have not been performed in relatives of AITD patients. Also the prevalence of type 1 diabetes in relatives of AITD patients is unknown.

Determinants for a co-occurrence of AITD and DM1 in patients and their relatives

There is a plethora of literature showing that the frequency and titre of the TPO-Ab in DM1 patients and their relatives are – apart from the assay used (see before) - determined by age, gender, genetic background and racial factors (4;6;8;16;19). Older individuals and females predispose to thyroid Ab positivity and thyroid dysfunction and Afro-Americans have lower frequencies of TPO-Ab than Caucasian Americans (12;41). There are however also some negative reports in this respect. Lindberg did not observe an age dependency, but his population existed of only young patients lacking adult individuals. Other studies were unable to find a female preponderance probably due to young age of the subjects or the small numbers of individuals tested (9;21;33).

Genetic association of co-occurrent AITD and type 1 DM in patients and their relatives

It is without doubt that HLA-DR3 and DR4 are clear susceptibility genes for DM1 and other organ specific AID, including AITD. Especially DR3 has been associated with DM1 and AITD separately. The presence of GAD-Abs, which is one of the most important characteristics of LADA, has been associated with DR3/ DR4 (28) or DR3 (25) and DR4 (28;51) separately in LADA patients.

Conflicting data exist however on the HLA susceptibility genes for DM1 with a co-occurrent AITD, i.e. APS type 3a. The majority of studies are unable to find any association with specific HLA susceptibility genes for such co-occurrence (6;12;24;52;53). However, some authors did find DR4 or DQ8 as risk haplotypes for APS type 3a compared to DM1 alone (54), while others reported on DR3 or DQ2 as risk haplotypes not only in the patients, but also in their relatives (8;36).

In our study we observed in the DM1 children an association between the presence of TPO-Ab (APS type 3a) and DR5 positivity (chi square test $p=0.02$), while DM1 in general was strongly associated with DR3 and DR4. In the (non-diabetic) parents TPO-Ab associated with DR4 positivity ($p=0.02$). An association of thyroid microsomal antibodies and HLA-DR5 has been reported in diabetic families before, however the non-diabetic control population also showed this association (55). Thus, this latter study supports an HLA-DR5 association with AITD rather than with APS3a. In addition, this association was found to be limited to males (55).

Only a few studies investigated genetic association other than the HLA in patients with AITD with coexisting DM1. CTLA-4 polymorphisms have been studied in such patients, since

evidence exists that these polymorphisms are associated with both AITD and DM1 (56-58). In a Japanese study, the G variant of CTLA-4 gene was associated with an onset of diabetes at young age (<30 years of age) in DM1 patients with coexistent AITD (50). Also in a Caucasian study, a more pronounced association was found for patients with the co-occurrency of AITD and DM1 as compared to patients with these diseases separately. However the differences between these groups was not significant and the role of CTLA-4 a co-occurrence of AITD and DM1 is probably weak (59). For further in depth references on CTLA-4 the reader is referred to a review by Kristiansen and colleagues (60).

APS type 3b (or Thyrogastric syndrome), the Association of AITD with Autoimmune Gastritis (AIG)

Patients and relatives

The association between AITD and autoimmune atrophic gastritis, often referred as thyrogastric disease, has been recognized as early as the 1960s (80). This combination has later been defined as APS type 3b (1).

Autoimmune gastritis (AIG) is characterized by a diffuse inflammation of the gastric body, the presence of Gastric Parietal Cell Antibodies (GPAb) and increased gastrin level in serum (61). GPAb are antibodies directed against gastric H^+/K^+ adenosine triphosphatase (ATPase) and have been associated, particularly in the presence of Intrinsic Factor Antibodies (IF-Ab), with pernicious anemia (PA) (62;63). PA is the consequence of atrophic gastritis and its subsequent deficiency of vitamin B12. Patients with PA are not only characterized by a high prevalence of GPAb (75-100%) and IF-Ab (60-100%), but also by thyroid Ab, i.e. 30-50% (64-66; 80). About 25% of PA patients has an overt AITD (66).

Vice versa, GPAb and PA have been found in high prevalence in patients with AITD (13-27% and 12-26% respectively) (64;67;80). Evidence for the association between autoimmune thyroiditis and the clinical sequelae of autoimmune gastritis has for example been given in the study performed by Centanni et al (67), who found in one third of their TPO-Ab positive patients an elevated serum gastrin level and a histologically confirmed atrophic body gastritis. Of these latter patients the majority had PA (82%). GPAb were less reliable for the diagnosis atrophic body gastritis as compared to the gastrin levels.

One of the possible explanations for the co-existence of AITD and AIG has been suggested to be an immunological cross-reaction of the two major auto-antigens in these diseases: a homologous 11 residue peptide was found in TPO and gastric H^+/K^+ ATPase (68). Elevated serum gastrin levels have also been found to occur more frequently in Graves' disease and some studies even found the gastrin levels to be correlated to the serum T3 levels (69-71).

In relatives of patients with PA or AITD, the prevalence of GPAb and/ or thyroid Ab has been demonstrated to be higher than in the general population. Up to 50% of the first degree relatives of PA patients have thyroid Ab, which is three times higher than in the general population (80). Also prevalences of 20% have been reported for latent or overt PA in relatives of patients with PA, whereas the prevalence in the general population is less than 0.2% (64).

Genetic association of AITD and AIG/ PA

It is thought that the HLA genes are not the major loci responsible for AIG and PA (3). Reported HLA associations are weak (64, 80), such as a weak association with HLA-A3 and -A7. Some stronger associations were however reported for the DR antigens DR2/DR4 and DR4/DR5 (72). There are conflicting data on an increased prevalence of DR3/DR4 in APS 3b (53;72).

Extended APS type 3a, the Association of DM1 and AIG

Patients and relatives

All studies agree that DM1 patients have a high prevalence (5-34%) of GPAb (6;12;14;34;40;73;74). The presence of GPAb in DM1 patients is correlated with the presence of atrophic gastritis with increased serum gastrin levels and an increased prevalence of pernicious anemia (73;75). Moreover, a strong association between GAD-Ab and GPAb has been reported in DM1 patients. The same study also found a weak association between TPO-Ab and GPAb (76).

The evidence in literature on a higher prevalence of GPAb in relatives of DM1 patients as compared to the prevalence in the general population is weak. Three studies found increased, although not significant, frequencies of GPAb in relatives as compared to non-selected controls (14;34;36). This was only the case for the parents of the patients, not for the siblings, and indeed when the frequencies of the Ab between parents and siblings were compared, parents were more likely to be positive for GPAb (15). In our earlier mentioned study we could not find any increased prevalence of GPAb in DM1 relatives and our observation is supported by another study (40). As is known for thyroid Ab, the occurrence of GPAb in DM1 patients and relatives is age dependent. Higher frequencies of GPAb were found with increasing age in patients as well as in relatives and controls (12;15;40;76).

In addition, the frequency of GPAb also increased with advancing age of onset (76). One study did not find an age dependency of GPAb, but this study was performed in DM1 children only (with a mean age of 12 years) (75).

Table III. Gastric autoimmunity in DM1, AITD and their relatives

	Gastric parietal cell-Ab	Pernicious anemia
DM1 patients		
children/adolescents	5-34% [19;36;74]	0.5-0.6% [8;19]
adults	5-28% [14;15;39;40;64;77]	0.8-5.6% [12;14;17;40]
FDR of DM1 patients	2-25% [14;15;34;36;40]	1-1.1% [14;40]
siblings	0-14% [15;34;36]	
parents	4-28% [15;34;36]	
AITD patients	13-27% [64;67]	12-26% [64;67]
FDR of AITD patients	?	?
Controls	0-17% [14;34;36;40;64;65;74;77] , *	0-0.7% [39;40]

DM1= type 1 diabetes mellitus; FDR= first degree relatives; AITD= autoimmune thyroid disease.
*= unpublished data of our study.

With regard to gender, in the recent study of our group on DM1 children we found the frequency of GPAb in female patients significantly higher than in their male counterparts (15.9% vs. 1.6%, p=0.01). This was in agreement with other studies (12;75). However, there was no female preponderance of GPAb in the sibs or in their parents.

HLA associations

The positivity for GPAb in DM1 patients has been associated with DR5, but not with the "diabetic genes" DR3 or DR4. Such associations could not be established in DM1 relatives (15). In our study we were unable to find any associations of GPAb with any of the susceptible haplotypes, but it must be noted that the frequency of GPAb was in fact too low to give valid data in this respect.

Recommendations for screening

Since the HLA typing studies have not yet given conclusive results and since reported relative risks are low, there is in our view no place for the determination of these genetic risk factors in patients with DM1 or AITD in isolation to predict the development of APS type 3. But with regard to the determination of a panel of relevant autoantibodies the story is different. Many authors recommend screening for thyroid autoimmunity in DM1 patients shortly after diagnosis and in particular in patients with a positive family history of AITD (11;13;18;21). Indeed it is also our view that DM1 patients and their relatives should be tested for TPO-Ab and sTSH.

Table IV. Frequencies of Islet Antibodies and DM1 in AITD patients

	ICA	GADA	IA2-Ab	DM1
AITD patients	7.6% [30]	3.8-6.6% [29;31]	3.8% [29]	6.6% [29]
HT	7.9% [30]	7.9% [29]	3.9-10% [29;43;44]	4.4% [78]
GD	2.4-7.4% [30;78]	6.1-13% [29;78]	3.8-44% [29;43;44]	4.4% [31]
controls	0.7% [30]	0.9-1.1% [29;31]	0-3% [29;44]	0.5% [78]

AITD= autoimmune thyroid disease; HT= Hashimoto's thyroiditis; GD= Graves´disease; ICA= islet cell antibody; GADA= glutamate decarboxylase antibody; IA2-Ab= tyrosine phophatase like protein-antibody; DM1= type 1 diabetes mellitus.

As described in this review, especially female DM1 patients and patients with high titres of TPO-Ab have an increased risk to develop AITD.

If the patient or its relative is TPO-Ab positive (without any further signs of a low thyroid reserve) a yearly follow up of the thyroid function is recommended. Treatment of this condition might be considered if the patient or its relative is a female with a child-wish, not only because this condition constitutes a higher risk for spontaneous abortion, but also because a TPO-Ab positive women is at high risk to develop post-partum thyroiditis after child delivery (24). Let alone that there are also reports that TPO-Ab positive pregnant women with a low thyroid reserve might give birth to offspring with a lower IQ (24). Furthermore one should be careful with dietary iodine (e.g. kelp tablets) and iodine-containing desinfectants or drugs (e.g. amiodarone) in such TPO-Ab positive individuals, since an exposition to high iodine is able to induce the development of hypothyroidism in these susceptible individuals.

If the patient or its relative is positive for TPO-Ab and has in addition subclinical hypothyroidism (a raised sTSH), replacement treatment should be initiated particularly in older individuals, since this condition is not without risks for the development of cardiovascular disease (79).

With regard to screening autoimmune thyroiditis patients, the determination of GPAb is useful, since there is a high prevalence of gastric autoimmunity in these patients and since the risk for the development of PA is increased in such individuals. Also DM1 patients should be tested for GPAb. If GPAb are present, a yearly follow-up for the development of PA (serum gastrin levels) should be carried out.

Whether autoimmune thyroiditis and autoimmune gastritis patients should be screened for islet Ab, is still not clarified. In AITD patients, a higher prevalence of these Ab has been described and the risk of the development of DM1 is indeed increased. A suggested strategy is

the combined islet Ab determination, especially the combination of GADA and IA2-Ab (31). However, this has to be investigated in a large prospective study, before a reliable advise for screening can be given. For this moment, the approach is a regular testing for urine/ blood glucose levels in these individuals.

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CHAPTER 3

ANIMAL MODELS OF ENDOCRINE ORGAN-SPECIFIC AUTOIMMUNE DISEASES: DO THEY REALLY HELP US TO UNDERSTAND HUMAN AUTOIMMUNITY?

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Abstract

Organ-specific or endocrine autoimmune diseases are complex, polygenic afflictions the penetrance of which is heavily dependent on various environmental influences. Important target tissues are the thyroid, the islets of Langerhans, gastric parietal cells and steroid producing cells in the adrenal and ovary. The etiology of these diseases remains to be clarified. The pathogenesis is strongly associated with autoimmune phenomena. None of current treatment approaches provide a cure but represents replacement therapy.

An important objective in the treatment of endocrine organ-specific autoimmune diseases is the detection of individuals at risk for the development of such diseases and the development of interventions to prevent an outbreak of the diseases. This requires an exquisite knowledge of the early etio-pathogenic stages of these diseases. This review concentrates on the usefulness of animal models for the understanding of precisely these very early stages.

It must be emphasized that studying animal models cannot answer all the problems presented by endocrine organ-specific autoimmune diseases as seen in the clinic. It must be expected –considering the different etiologies in the different animal models (see below)- that the causes of the diseases in the human and the involvement of various genes and environmental factors may also vary. Yet in the study of particularly the pre-autoimmune phases of the diseases there is hardly an alternative than to study the animal models. Only limited series of experiments can be carried out in human subjects at risk to develop such diseases. Moreover a general semblance (blueprint) of the etio-pathogenesis found in the animal models can lead the way for human studies.

Efforts to understand the pathophysiology of the early stages of endocrine organ-specific autoimmune diseases have mainly involved animal models that “spontaneously” develop such diseases. Of these the Bio-breeding Diabetes-Prone (BB-DP) rat and the Non-obese Diabetes (NOD) mouse are the most well studied, yet many studies have also been carried out in the Obese Strain (OS)-chicken. Apart from these spontaneous models there are animal models that are induced by environmental perturbations (viruses, toxic substances), by thymectomy procedures or by genetic manipulations, e.g. the RIP-LCMV model and the BDC2.5 TCR mouse model.

A general blueprint has emerged from the studies into the early stages of the pathogenesis of endocrine organ-specific autoimmune diseases in these animal models: animals at risk to develop endocrine organ-specific autoimmune diseases show various pre-autoimmune aberrancies in their target glands, T cells, macrophages (MΦ) and dendritic cells (DC). The presumably aberrant target cells, T cells, DC and MΦ need to interact abnormally before autoimmune disease can fully develop. In this abnormal interaction additional aberrancies in other regulatory systems may play a role in a further exacerbation of the self-directed immune response, such as defects in the HPA axis system. The various aberrancies are partly genetically determined

by a variety of separate genes, particularly MHC-related genes, but they may also be environmentally induced (e.g. via viruses, high iodine diet, and other experimental manipulations).

Recently, evidence has been gathered for pre-autoimmune aberrancies similar to the animal models in the DC/ MΦ compartment and the HPA-axis in humans at risk to develop endocrine organ-specific autoimmune diseases. However analogous pre-autoimmune abnormalities in human target glands or in T cell function have not yet been found with certainty.

We believe that animal models of endocrine organ-specific autoimmune disease still hold immense promise for the discovery of pathways, genes and environmental factors that determine the development of endocrine organ-specific autoimmune diseases. Animals affected by such diseases provide an unique opportunity to uncover disease-associated pathways, which are too complicated to define in man.

Introduction

One of the important functions of the immune system is the discrimination between "self" and "nonself", or perhaps better between “danger” and “non-danger”. Currently, such discrimination is thought to be made through a series of complicated and multi-step interactions between various cells and components of the immune system. Immune cells sometimes erroneously establish an immune reaction towards “self” during conditions of apparent “non-danger”. If such immune reactions are so aberrantly and vigorously self-directed, they may inflict pathological damage on tissues. So-called "autoimmune diseases" are the consequence.

Autoimmune diseases can be divided into two main categories: "organ-specific" and "systemic" autoimmune diseases. In the organ-specific autoimmune diseases, the immune attack is confined to one organ or organ system, while in the systemic autoimmune diseases the damage is widespread and often the consequence of immune complex destruction. In the majority of organ-specific autoimmune diseases, target tissues are of endocrine character, hence this category of autoimmune diseases is also often referred to as “endocrine autoimmune diseases”. Important target tissues are the thyroid, the islets of Langerhans, gastric parietal cells and steroid producing cells in the adrenal and ovary.

There are ethical and technical restrictions to studying the etiology and the pathogenesis of autoimmune diseases in man. Reliance on animal models is in part a recognition of the primacy of patient safety - *primum non nocere* - first do no harm (1). In man the endocrine organ-specific autoimmune diseases often have sub- or non-clinical pro-dromal phases, which are difficult to study since signs and symptoms are virtually absent. In animal models these studies can be done. Unlike humans, animals with endocrine organ-specific autoimmune diseases can be bred to study

and manipulate inheritance. They can be biopsied and autopsied. Their genome can be altered. Therapies to prevent or reverse the disease can readily be tested.

Over the past 50 years a plethora of animal models of various endocrine/ organ-specific autoimmune diseases have been developed (table I). These animal models have greatly contributed to the knowledge concerning the etiology and the pathogenesis of endocrine organ-specific autoimmune diseases and their possible prevention and treatment. A word of caution is, however, necessary when trying to extrapolate data obtained in these animal models to the human situation. The animal models clearly show a caricature of the more complex human disorder. The animal disease is often studied in specifically inbred animals to generate homogenous and extreme forms of the autoimmune diseases. In this way the disease will not only differ from the human disorder but also between various animal models. Hence, general conclusions drawn from studies in one of the animal models should always be verified in other animal models and in patients. Recent studies have culminated in the awareness that endocrine organ-specific autoimmune diseases must be regarded as polygenic diseases, in which the penetrance of a combination of genes is strongly influenced by environmental factors (Fig. 1). Firstly, multiple genes determine part of the aberrant immune response towards self. The most important genes are those in the MHC region (21,72,103). However other genes are also involved, including those with a role in the regulation of the immune response in general, e.g. the CTLA-4 gene (60), genes determining aberrancies in the target gland eliciting the abnormal self response (16,118), genes playing a role in the sensitivity of the target gland to the autoimmune attack (115) and genes controlling T cell development and differentiation (44, 74). However, genetic polymorphisms or mutations are clearly not always explaining the etiology. Mono-zygotic twin studies, for example, have shown a concordance rate ranging from an 80% for thyroid auto antibody positivity (13), via a 30-40% for type 1 diabetes (30) to a meagre 20% for Graves' disease (12). This demonstrates the important role of environmental eliciting factors in the development of these diseases.

An important objective in the area of endocrine organ-specific autoimmune diseases is the detection of individuals at risk for the development of such diseases and their early treatment to prevent disease. This requires an exquisite knowledge of the early stages in the etio-pathogenesis of these diseases. This review will concentrate on the usefulness of the animal models for the study of very early stages of disease. Here the animals that develop a “spontaneous” disease are of main importance. For the role of the various immune cells and immune mechanisms in the late effector phases of the autoimmune reactions and the importance of the animal models in this respect the reader is referred to excellent recent reviews (7,21,116,117).

Some frequently used animal models of endocrine organ-specific animal models

Efforts to understand the pathophysiology of endocrine organ-specific autoimmune diseases have involved animal models of the diseases that develop "spontaneously", or are induced by either environmental perturbations, or by genetic manipulations (transgenics and knockouts) (Table I).

Spontaneous animal models

Of the spontaneous animal models the Bio-Breeding Diabetes-Prone (BB-DP) rat and the Non Obese Diabetes (NOD) mouse are the most well studied.

The BB-DP rat. The BB-DP rat is primarily a model for autoimmune diabetes (40). In fact, this animal model is a model for autoimmune polyglandular syndrome (APS) type 3a. The spontaneous diabetic BB rat was discovered in a commercial breeding colony in Canada in the 1970's. Inbred diabetes-prone BB (BB-DP) rats develop spontaneously a T cell dependent, ketosis-prone diabetes, that is clinically very similar to type 1 diabetes in humans. The animals were subjected to inbreeding of different lines with variable frequency of diabetes. During the course of this work it was discovered that the rats had profound T cell lymphopenia. The lymphopenia is a recessive trait and the animals are lymphopenic from birth due to a mutation in one of the Immune associated nucleotide (Ian) genes on rat chromosome 4 (74). The diabetes develops in most DP lines at around the age of 8-12 weeks. Histologically infiltrative insulitis develops for each islet rapidly (in a few days), but each islet is not affected at the same time. Insulitis is not characterized by a large peri-insular accumulation of lymphoid cells, as in the NOD mouse (see below). Females and males are equally affected.

There also exist sublines of the BB-DP rats, that are not lymphopenic and do not develop diabetes. These lines are referred to as Diabetes Resistant or BB-DR. The peripheral lymphopenia of the BB-DP rat is primarily due to a lack of $RT6^+$ T cells. RT6 is a marker for regulatory T cells. Transfers of $RT6^+$ T cells from BB-DR rats to BB-DP rats prevent disease (39). Although diabetes-resistant BB-DR rats are sufficient in $RT6^+$ T cells, they are still prone to diabetes: infection with Kilham Rat Virus (KRV) is a known inducer of autoimmune diabetes in these rats (26). The virus does not infect islet cells, but the macrophages of the animal and perturbs the immune system of the BB-DR rats resulting in changes in the balance from T helper (h) 2 to Th1 mechanisms (25). Also treatment with poly I:C induces diabetes in these rats. A standardized approach is to treat the BB-DR rats with monoclonal anti-RT6 antibodies and poly I:C, a treatment that effectively accelerates the onset of insulitis and diabetes. Reciprocal cross-intercross breeding to establish a congenic BB-DR rat with lymphopenia showed that the Ian5 gene mutation only was sufficient to induce spontaneous diabetes in all rats provided that the rats were kept specific pathogen free (6).

Table I. A selection of animal models of important endocrine/ organ-specific autoimmune diseases.

	Animal	Manipulation	Genes	Environment	Disease	Remarks
Spontaneous	BB-DP rat	none	lyp (idd1)	normal	autoimmune diabetes	lymphopenic
			RTu (idd2)	high iodine diet	focal thyroid infiltrates	
	BB-DR rat	none	lacks idd1	KRV virus	autoimmune diabetes	no lymphopenia
	NOD mouse	none	H-2^{g7} (idd1)	normal	autoimmune diabetes	
			Idd3, idd5		autoimmune sialoadenitis	
			toxic iodine dose	focal thyroiditis		manipulable by cytokines
	NOD H-2^{h4}	none	high iodine diet	focal thyroiditis		
	OS chicken	none	at least 5 genes	normal	severe thyroiditis	HPA-axis disturbances
Experimentally Induced	rabbits/ rats	Tg in FCA	MHC genes	normal	thyroiditis	transient
	rats/ monkeys	MBP in FCA	MHC genes	BBB disruptor	encephalomyelitis	transient
	mice	H/K ATPase	MHC genes	normal	gastritis	transient
	Balb/C mice	TSH-R cDNA	H-2^{d}	normal	Th2 thyroiditis	Graves' model
	Balb/C mice	TSH-R cDNA + transfer	H-2^{d}	normal	eye signs	Graves' model
	NOD mice	TSH-R cDNA	H-2^{g7}	normal	Th1 thyroiditis	hypothyroidism
	AKR/N mice	transfected fibroblasts	H-2^{k}	normal	hyperthyroidism	Graves' model
	mice	thymectomy day 1-5	non-MHC	normal	gastritis, oophoritis	lack $CD4^{+}CD25^{+}$ T cells
						Balb/C, A/J mice resistant
						C57bl, DBA mice suscept.
Transgenic/ KO	mice	cytokines in β cells		normal	insulitis forms	
		H/K ATPase in GPC		normal	gastritis	
		NP in β cells		LCMV	autoimmune insulitis	
	NOD mice	BDC2.5 TCR in T cells		normal	peri-islet lymphoid cells	

BB= biobreeding; DP= diabetes prone; DR= diabetes resistant; NOD= non obese diabetic; OS= obese strain; KO= knock out; Tg= thyroglobulin; FCA= Freund's complete adjuvant; MBP= myelin basic protein; TSH-R= thyroid stimulating hormone receptor; GPC= gastric parietal cell; NP= nucleoprotein; KRV= Kilham rat virus; HPA= hypothalamus pituitary adrenal; LCMV= lymphocytic choriomeningitis virus

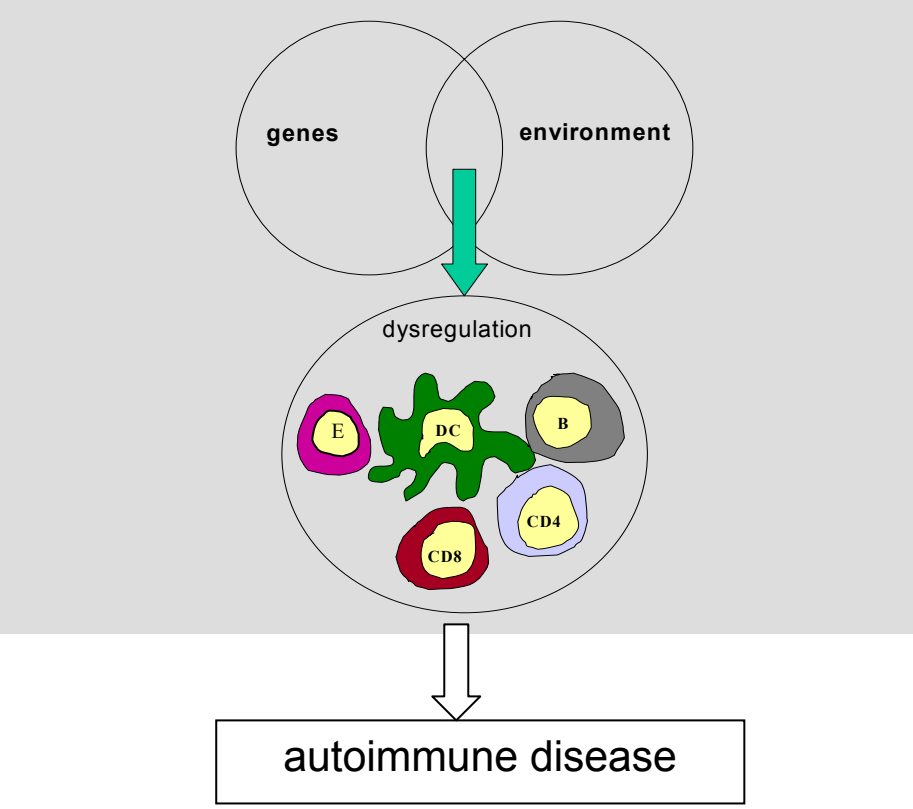


Fig. 1. A cartoon illustrating that the combined effect of several genes and environmental factors must act together to bring about a disturbed mutual interaction between various subtypes of immune cells and of these cells with target cells to elicit an endocrine organ-specific autoimmune disease.

BB-DP rats also suffer from a form of focal lymphocytic infiltrations that under normal conditions do not lead to hypothyroidism (99).

Yet thyroid failure becomes apparent after hemi-thyroidectomy of the animals. Aggravation of focal infiltrations can also be observed when the animals are fed a high iodine diet (3,77). The thyroiditis is genetically linked to the MHC class II RT1u rather than to the Ian5 gene mutation (80).

The NOD mouse. (4) The NOD mouse is predominantly studied for its diabetes and sialo-adenitis. The animal is also deaf. The NOD mouse has been extensively reported on since the 1980's. NOD mice develop on an early age (from 5 weeks of age onwards) an initially non-destructive peri-insular infiltration of dendritic cells, accessory MΦ, T cells and B cells that persists for several weeks before it develops into a destructive form of insulitis (from 12 weeks of age onwards). Mild diabetes follows. Animals can survive without insulin administration and keto-acidosis seldom occurs, unlike the BB-DP rat and humans. Typically female mice are more severely affected.

There are many genetic loci (over 15) on different chromosomes that associate with diabetes and/ or insulitis and/ or sialo-adenitis in the NOD mouse. The most important diabetic loci

(idd1 loci) are linked to the MHC complex; NOD mice express an unique I-A locus, i.e. I-A g7 (histidine as residue number 56 and serine as residue 57, homologous to "diabetogenic" HLA-DQ ß non-aspartic acid 57 containing alleles in the human), but lack expression of I-Ea (homologous to DR α in humans) (21,72). Idd1 is not related to sialo-adenitis development, here Idd5 and Idd3 are thought to play a prime role, but data are inconsistent (8,10).

In the majority of the NOD strains there only occasionally is an association of diabetes with thyroid infiltrations (unlike in the BB-rat). In general the incidence of thyroiditis is very low in the NOD mouse, but it varies from colony to colony (69). Certain dietary iodine regimens, however, have a triggering effect on thyroiditis development. In humans with a pre-existing iodine-deficient goiter, a single administration of a high dose of iodide is known to result in some individuals in an attack of autoimmune thyroiditis. In normal mice such identical high dose of iodide has a necrotic effect on hyperplastic iodine-deficient glands, but such dietary manipulation does not lead to thyroid autoimmunity. In contrast in NOD mice it does lead to autoimmune thyroiditis following the initial phase of thyrocyte necrosis (69). This shows the importance of a local factor (high antigen release, necrosis) in combination with a dysregulated immune system (NOD mouse background) in the development of at least this endocrine organ-specific autoimmune disease (69,89). Like the BB rat, this model is in fact also a model for APS type 3a.

There exists a subline of NOD mice, which has under normal dietary conditions a prevalence of around 5% of thyroiditis, but when kept on a continuously high iodine diet "spontaneously" develops autoimmune thyroiditis in virtually all animals (84). This subline is characterized by an alternative MHC haplotype, viz. the I-Ak allele in stead of the I-Ag7 on the NOD background, and the mice are called NOD-H-2^{h4} mice.

Obese Strain (OS) chicken. One of the oldest models of endocrine organ-specific autoimmune disease is the Obese Strain (OS) chicken, which suffers from lymphocytic thyroiditis with a rapid onset of hypothyroidism (33). For the last 40 years chicken of the OS strain have been used to study the disease, which resembles severe Hashimoto's disease of humans in many clinical, histopathological, serological and endocrinological aspects. Mononuclear cell infiltration of the thyroid gland commences in the second week after hatching and leads to an almost complete destruction of the thyroid architecture by 1-2 months of age. OS chicken do not develop insulitis. Limitations of the model are the scarcity of immunological reagents for chicken and the absence of avian-cloned thyroid-specific genes.

The first genetic theory of endocrine organ-specific autoimmunity as a polygenic trait was proposed by Cole (1966) and based on breeding studies with this bird (107). The three-locus model of immune response MHC and non-MHC genes and genes coding for a hypothetical primary thyroid defect emerged from genetic analysis of OS families and from F2 crosses between OS and CS chicken. Crossing experiments with another CB inbred line unrelated to OS revealed

the existence of about 5 genes regulating the full development of the disease. Approximately three genes encode the susceptibility of the target organ to the attack by the immune system (one of them recessive) and the remaining one or two genes encode the hyperreactivity of the immune system (115).

Iodine levels in food are an important environmental factor in the development of the thyroiditis in the OS chicken, and the severity of the disease can be manipulated by iodine (100). Application of anti-oxidants delay the onset of the disease, illustrating the importance of oxidative reactions in the toxicity of iodine (100).

The role of the stress system in the development of the disease in the chicken is illustrated by an altered immuno-endocrine communication via the HPA-axis in this strain of birds (114). The OS chicken show a hypo-responsiveness to glucocorticoids and in particular to inhibitory factors released by this stress hormone in immune cells (114). Moreover low levels of the central opioid peptide ß-endorphin have been shown in the hypothalamus of the OS chicken before onset of the disease, i.e. already at the embryonic stage. A further decrease in this brain peptide was observed in correspondence with the first signs of thyroid mononuclear infiltration (90). Similar HPA-axis regulating disturbances have been shown in another animal model of organ-specific autoimmune disease, i.e. the Lewis rat that is sensitive to experimental allergic encephalomyelitis (EAE) elicited by immunizations with myelin antigens (22).

Experimentally-induced animal models

Excessive exposure to autoantigen. Classical models for the induction of organ-specific autoimmune disease are models that make use of immunizations with autoantigen in an adjuvant, e.g. injections of thyroglobulin (Tg), H^+/K^+ ATPase (1), or myelin basic protein (MBP) (47) in Freunds' complete adjuvans (FCA) leading to experimental allergic thyroiditis (EAT), experimental allergic gastritis (EAG) or EAE respectively. It is of interest that similar immunizations with glutamic acid decarboxylase (GAD65) or insulin, the two major antigens in type 1 diabetes have failed to induce insulitis and diabetes. In general diseases are transient in these models, depending on the animal used for sensitization. Obviously these models can not be used to study the very early phases of "spontaneous" autoantigen presentation in "wild type-occurring" endocrine organ-specific autoimmune diseases (the focus of this review). The models have however been proven useful in the study of effector mechanisms playing a role in the autoimmune diseases as well as some preventive and therapeutic interventions.

A recent promising development in this area of experimental allergic diseases is the sensitization of mice with TSH-receptor (TSH-R) peptides, recombinant TSH-R preparations or with cDNA for the full-length human TSH-R cloned in an eukaryotic expression vector (genetic immunization) to create an animal model for Graves' disease (68). In these experiments it appeared easy to induce TSH-R antibodies (Abs) in all mice strains used with all the mentioned

regimens. However the majority of the regimens were without any effect on the histology or function of the thyroid in most of the cases: Whereas H-2b and H-2k animals did not develop thyroiditis or thyroid function abnormalities, H-2d (Balb/C) mice did and some of these mice had hypo-thyroxinemia. Also NOD mice (H-2g7) developed thyroiditis and TSH-R Abs, and particularly in the NOD mouse model the thyroiditis was destructive and of Th1 character leading to clear hypothyroidism.

Hyper-thyroxinemia and orbital pathology (both clinical hallmarks of Graves' disease) were more difficult to induce using the above described protocols. However two protocols showed some success:

1. immunizations of H-2k (AKR/N) mice intraperitoneally with MHC Class I identical fibroblasts double transfected with the TSH-R and MHC Class II led in 20% of cases to hyperthyroxinemia and
2. immunizations of out bred Balb/C mice with TSH-R cDNA vectors led in 10% of cases to hyperthyroxinemia and TSH-R Abs that were able to stimulate c-AMP in cultured fibroblasts, the so-called Thyroid Stimulating Antibodies (TSAbs).

Even more interesting is the observation that transferring T cells from the latter mice to naïve mice (after an in vitro restimulation of the T cells with a recombinant TSH-R preparation) led to a Th1 thyroiditis with no signs of eye muscle infiltration when NOD mice were used. It led to a Th2 type thyroiditis with mild signs of eye muscle infiltration when Balb/C mice were used. Clearly these models are promising and need further exploration.

Thymectomy models. The most well-known and studied model is that induced by thymectomy of Balb/C mice at day 3 (101). This procedure results in a variety of organ-specific autoimmune diseases, including thyroiditis, gastritis, and oophoritis, but not insulitis. Hence this model is a model for APS type 3b. The inflammations are characterized by the presence of T cell infiltrates in the affected organs and the development of organ-specific antibodies in the serum. There is a strict temporal relationship between the development of the autoimmune syndrome and the day of thymectomy, which has to occur between the second and the fifth day after birth (91). Treatment of neonatal Balb/C mice with immuno-suppressive or cytostatic drugs such as cyclosporine and cyclophosphamide has similar effects.

Classically the model has been used to study oöphoritis. The histopathological events of the oöphoritis in the thymectomized mice occur in an orderly manner. Initially the oöphoritis is evident as a patchy or diffuse infiltration of lymphocytes; later, developing follicles are clearly affected and monocytes, macrophages, neutrophils, and plasma cells are found between and within ovarian follicles. The onset of puberty markedly potentiates the oöphoritis, indicating that a local factor (probably a change in antigen profile due to the gonadotropin stimulation) is important in the development of the autoimmune disease (46).

The model has also recently been used to study human autoimmune gastritis (1). The features of the mouse autoimmune gastritis are remarkably similar to the human disease and include autoantibodies and T cell reactivity to H/K ATP-ase, a mononuclear cell infiltrate in the gastric mucosa with loss of parietal and zymogenic cells from the gastric mucosa, a-chlorhydria and increased serum gastrin levels. Also megaloblastic anemia develops in the mouse model.

With regard to the genetics of this model certain strains of mice are susceptible, such as the BALB/c and A/J mice, whereas other strains (C57bl/6J, DBA/2 mice) are resistant. Since susceptibility and resistance are not associated with the MHC haplotype (H2) of the mice, these antigen-presenting molecules are apparently of minor importance in this model. Using the susceptible and resistant mice strains and backcrosses of these strains in combination with a microsatellite approach, a locus has been found on chromosome 16, controlling the abrogation of the tolerance to ovarian autoantigens due to neonatal thymectomy day 3 (113). This so-called Aod1 locus was associated with the presence of oöphoritis in the mice. Interestingly, the markers on chromosome 16 failed to exhibit a significant linkage to the concomitant ovarian atrophy in this mouse oöphoritis model. Rather, this atrophy exhibited an association with markers on mouse chromosome 3. Two regions on the distal arm of chromosome 4 (Gasa1 and Gasa2) might be involved in the gastric autoimmunity of the mice (1).

The recent interest in $CD4^+CD25^+$ T cells as a specific subpopulation of thymus-derived regulatory T cells has a historical association with the day 3 mouse thymectomy model (97,105). Day 3 neonatal thymectomy-induced autoimmune disease is due to a lack of $CD4^+CD25^+$ T cell migration into the periphery, since these regulatory cells typically migrate out of the thymus in this early period and since injection of purified $CD4^+CD25^+$ T cells into neonatally thymectomized mice prevents the development of autoimmunity. $CD4^+CD25^+$ T cells develop in the thymus via a distinct pathway of thymic selection requiring the expression of endogenous TCR α chains on the cells for selection since $CD4^+CD25^+$ T cells are absent in TCR transgenic mice on a RAG-deficient background. A feature of $CD4^+CD25^+$ T cells is that the cells themselves are “anergic” to mitogenic stimuli, but are in addition capable to suppress the proliferation of $CD4^+CD25^-$ T cells when cultured together. Such suppression can be abrogated by the addition of interleukin (IL)-2 or stimulation with anti-CD28 antibodies. The mechanisms of suppression by $CD4^+CD25^+$ T cells are not clarified yet, but are presently subject of intensive research (20,73).

Chemically-induced models. Iodine is an important exogenous inducing and modulating factor of thyroid autoimmunity. In general, iodine deficiency attenuates, while iodine excess accelerates autoimmune thyroiditis in autoimmune prone individuals (89). In non-autoimmune prone individuals, the effects of iodine are, however, different. Here iodine-deficiency precipitates a mild (physiological) form of thyroid autoimmune reactivity, while iodine excess stimulates thyroid development (89). Iodine probably exerts these effects via inducing alterations

in the metabolism of thyrocytes and even via toxic thyrocyte necrosis. Iodine also has direct effects on the development and function of various immune cells (T cells, B cells, macrophages and dendritic cells) and the antigenicity of thyroglobulin (17,100).

A number of other chemicals, especially alloxan and streptozotocin, have been used to produce diabetes. These chemicals interfere at high dose with the metabolism of β cells, sometimes leading to necrosis. On the other hand (multiple) low doses of these chemicals induce an autoimmune form of diabetes in genetically susceptible animals dissimilar from the form occurring in the spontaneous models of autoimmune insulitis (21). The low-dose streptozotocin treatment needs however to be carefully titrated dependent on the strain of mice that are used. Several studies either with CD3 antibodies or using T cell knock out mice have indicated that the ß cell killing and sustained diabetes is T cell mediated.

Virus-induced models. Viruses have already been suggested for more than 70 years as implicating factors in the development of type 1 diabetes and forms of thyroiditis. To date more than 5 different viruses, e.g. Coxsackie B virus, rubella virus and mumps virus, have been reported to be associated with the development of type 1 diabetes in humans (53). An involvement of foamy viruses has been discussed in the etiology of de Quervain thyroiditis and Graves' disease (75).

In animal models of endocrine organ-specific autoimmune diseases there is clear evidence for an involvement of viruses. Viruses can act on a specific genetic background in preventing the disease, e.g. LCMV in NOD mice. In contrast viruses can also act as an eliciting factor on other genetic backgrounds, and mechanisms to induce disease differ from virus to virus and from animal species to animal species. The best known are virus models of (autoimmune) insulitis, i.e. insulitis due to Encephalomyocarditis (EMC)-D virus and Kilham Rat Virus (KRV) in mice and rats respectively (53). Coxsackievirus B4 has also recently been used in mice to induce insulitis (36). Viruses have also been described to be able to elicit autoimmune thyroiditis, i.e. when Balb/C mice are infected with reovirus type 1 (not with type 3) the mice develop thyroiditis and serum antibodies to thyroglobulin and second colloid antigen (78).

Regarding the mechanisms playing a role in the animal models several separate mechanisms have been discovered. Firstly, viruses can directly infect and destroy the endocrine target cells; secondly, viruses can infect cells of the immune system and perturb the delicate balances preserving tolerance to the endocrine cells; and thirdly, combinations of the two are possible (53).

When genetically susceptible mice (i.e. SJL/J mice) are infected with a high titre of EMC-D virus β cells are destroyed largely due to the replication of the virus in the β cells. When DBA/2 mice are infected with a low dose of EMC-D virus the virus infects not only the β cells, but also the macrophages that are attracted by the inflamed islets. Macrophage-derived

inflammatory factors, such as IL-1β, TNFα and iNOS thereafter play thereafter a critical part in the destruction of the β cells, since interference with these products ameliorates disease.

When BB-DR rats are infected with KRV the existing tolerance for islet antigens in this model (due to the presence of regulatory $RT6^+$ T cells) is broken and autoimmune diabetes develops. The virus does not infect the β cells but rather the lymphocytes and macrophages to cause a possible depletion of regulatory T cells necessary for prevention of insulitis and diabetes. However a clear picture on the mechanism in this latter model does not yet exist, although a shift in Th2 to Th1 balance is implicated in the breaking of tolerance in these rats.

Clearly further studies are needed to clarify the complex ways in which viruses are able to induce endocrine organ-specific autoimmune diseases.

Transgenic and knock-out animal models

The spectacular progress made in transgenic and knock-out technology has provided powerful new tools for the investigation of the fundamental aspects of endocrine organ-specific autoimmune diseases. There has been great interest in creating transgenic and knock-out models especially in the field of NOD mouse diabetes and autoimmune gastritis. It must be noted however that the transgenic and knock-out models are often quite artificial, far away from the etio-pathogenesis of wild-type endocrine organ-specific diseases, and thus only suitable to study some aspects of these diseases. In short the following models can be distinguished:

1. Transgenic normal or autoimmune-prone mice expressing adhesion molecules, cytokines, costimulatory molecules or receptors for these immune molecules on and in their glandular cells, for instance because the genes encoding these molecules are expressed under the influence of a promotor important in the function of the cells, e.g. the insulin-promotor for expression in β cells. It has been found that mice expressing MHC class II or TNFα on their β cells develop forms of inflammatory diabetes, but so do mice expressing IL-10 in their β cells (116). Apart from these diabetic mouse models mice expressing GM-CSF in their gastric parietal cells have been constructed and these mice develop a form of autoimmune gastritis (1).
2. Transgenic mice expressing a viral protein, e.g. the NP protein of LCMV in specifically their β cells, the so-called RIP-LCMV mice (110). Infection of these mice with LCMV leads to a form of autoimmune diabetes, because virus-cytotoxic T cells generated in the viral infection also vigorously attack the β cells. Subsequent epitope spreading causes an immune reaction to insulin and GAD, leading to a more typical form of autoimmune diabetes.
3. Transgenic mice expressing a diabetogenic T cell receptor (TCR) on subpopulations of their T cells, e.g. the BDC2.5 TCR Tg mice expressing the TCR of the diabetogenic T cell clone BDC2.5 on their $CD4^+$ T cells (57). Interestingly when these diabetogenic TCRs are

expressed on a largely non-diabetogenic background (NOD mice not yet fully inbred in the process) insulitis and diabetes do occur, however when expressed on an full NOD-background large peri-insular lymphoid accumulations can be seen, but these “infiltrates” do not progress to diabetes (87). This has been explained by the co-occurrence of diabetes-protective genes in the full NOD mouse background.

Studies into the very early stages of the pathogenesis of endocrine organ-specific autoimmune diseases in animal models reveal a general “blueprint”.

The studies in virtually all of the above described animal models have shown that the pathogenesis of an autoimmune failure of a gland is generally a multistep process, requiring several genetic and environmental abnormalities (or variants) to converge before full-blown disease develops. Hence, endocrine organ-specific autoimmune diseases are the outcome of an unfortunate combination of various genetic traits and environmental circumstances that by them selves do not need to be harmful, and may even be advantageous.

Studies of the initial etio-pathogenic phases of endocrine organ-specific autoimmune diseases is difficult in man, there is - apart from the limited study on still unaffected family members- obviously only one reasonable alternative, i.e. the study of the very early phases of the diseases in the spontaneous animal models. Interestingly two major early aberrancies can be detected in these models (fig 2):

1. An early local aberrancy in the gland resulting in an early local accumulation of macrophages (Mϕ) and dendritic cells (DCs) before there is any noteworthy local lymphocytic infiltration.
2. An early systemic aberrancy in the immune system apparently leading to an easy breakdown of tolerance.

These two types of aberrancies need to act together to start the autoimmune process.

Role of Mϕ and MHC class II-positive DC

Increased numbers of Mϕ and MHC class II-positive DC have not only been found in the very early stages of the endocrine organ-specific autoimmune diseases in all the spontaneous animal models (BB-DP rat, NOD mouse and OS chicken) (35,42), but also in the thymectomy mouse model (1), the virus- and chemically-induced models (76,119), and the RIP-LCMV mouse model (110). Also in the human Mϕ and MHC class II-positive DC are present inside and outside lymphocytic accumulations in the glands of patients with Graves' disease, Hashimoto goiter (56),

autoimmune insulitis (51) and sialo-adenitis (106), be it that only later stages of the diseases have been studied.

DC are antigen-presenting cells (APC) par excellence, and essential for stimulation of naive T cells (35,98). Mϕ have various functions, ranging from the production of factors for wound healing and remodelling of bone (63), via the phagocytosis and degradation of unwanted material to the regulation of immune responses.

The (local) presence of Mϕ and DC has been shown to be indispensable for the development of endocrine organ-specific autoimmune diseases, since prevention of their accumulation in the pancreas of NOD mice (49,62), in the pancreas of BB-DP rats (43) or the brain of Lewis rats (47) results in a prevention of insulitis/diabetes and EAE development. Also splenic lymphocytes from macrophage-depleted NOD mice fail to transfer diabetes to recipients (119).

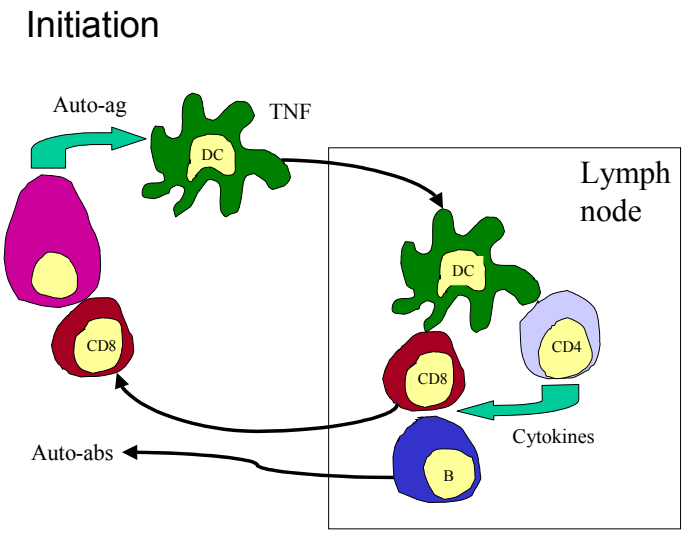
The destiny of the majority of Mϕ and DC accumulated in tissues is to enter the lymphatics (35,98) and to travel to the draining lymph nodes while transporting antigens to these nodes and – under steady state, i.e. non-inflammatory, conditions - inducing tolerance induction. In the spontaneous animal models, however, a sensitization reaction is induced in the local draining lymph nodes after the early accumulation of the Mϕ and MHC class II-positive DC in the glands (55,111). This apparently occurs in the absence of any obvious “inflammatory condition” of the gland. An expansion of autoreactive $CD8^+$ and $CD4^+$ T cells takes place in the reacting draining lymph nodes as well as the production of auto-antibodies of IgG class. Later, however, such immune reactivity is taken over by a lymphoid tissue that locally develops in the glands themselves, e.g. a local thyroid lymphoid tissue in the thyroid gland of the BB-DP rat (55,111) and the earlier mentioned peri-insular lymphoid tissue in the NOD mouse (87). These tissues are often erroneously called “focal thyroiditis” and “peri-insulitis” respectively, since they are not destructive inflammations, but have a high degree of histological architecture, with clearly distinguishable T-cell areas, B-cell follicles and germinal centres, and areas and cords of plasma cells in the periphery of the lymphoid tissue. The plasma cells in this tissue produce specific antibodies, such as anti-thyroglobulin (anti-Tg) antibodies in the BB-rat thyroid (55,111). Such local lymphoid tissue can also be found in human glands affected by autoimmune disease and are also there generally non-destructive and show a peaceful coexistence with adjacent endocrine/exocrine cells (55). In fact, the adjacent endocrine/exocrine cells often show signs of metabolic and proliferative stimulation. In the early peri-insular lymphoid tissue of the spontaneous NOD mouse model, the BDC2.5 TCR Tg mice and the RIP-LCMV mouse a predominance of Th2 type cytokines has been shown, again underlining the anabolic nature of this locally developed lymphoid tissue (87,117).

Role of B cells

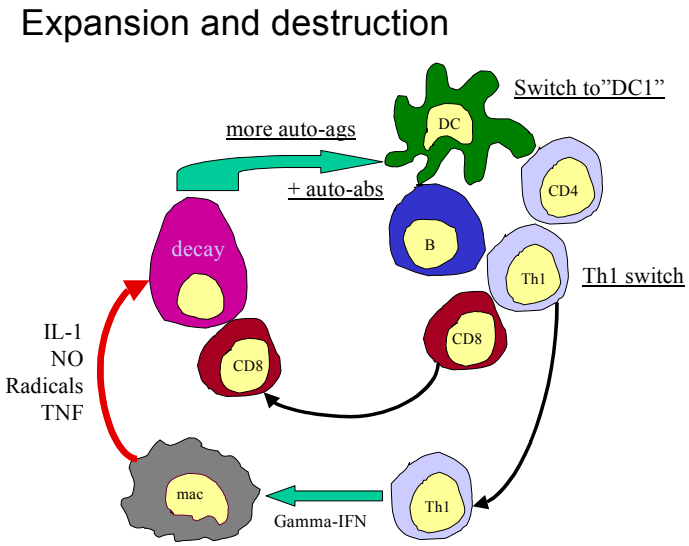
Not only Mϕ and DC are essential in the early phases of the development of endocrine organ-specific autoimmune diseases, but it has also been argued that B cells and $CD8^+$ T cells need to be present in the first weeks of life for diabetes to develop. T cells from B cell-deficient NOD mice could not be sensitised to GAD and these mice did not develop diabetes (93). Also B cell-deficient $H2^{h4}$ mice do not develop thyroiditis (9). Since this inability was not due to the absence of antibodies and could also not be restored by the transfer of B cells later in live it has been suggested that B cells might - next to DC and Mϕ - function as early APC in the development of endocrine organ-specific autoimmune diseases in the NOD mouse. However there are also reports showing that B cell-deficient mice do develop in an early stage autoreactive T cells, but that these T cells are not pathogenic (23). Moreover B cells are not required in the initial stages of the autoimmune process in the BB-DP rat, and there is a recent report of a child with a severe B cell deficiency that developed type1 diabetes (71). Hence a B cell requirement for diabetes development is certainly not a general rule and probably restricted to the NOD mouse model only, be it also in this model in a relative fashion. It is presently thought that in the NOD mouse B cells might be required for an expansion of the early autoreactive T cell response (Fig 2).

Role of $CD8^+$ T cells

There is also evidence that CD8+ T cells are required early in the process of the NOD islet autoimmune response. When NOD mice are depleted of CD8+ T cells during discrete age windows from about 2 to 5 weeks after birth insulitis development is severely hampered (112). Also NOD mice carrying a null mutation at the β_2-microglobulin locus and thereby lacking MHC class I molecules and $CD8^+$ T cells are insulitis and diabetes resistant (94). Transfer studies indicate that $CD4^+$ T cells from these protected mice, although they were isolated after repopulation of the CD8 compartment, were not capable of transferring disease (24). This indicates that at least in the NOD mouse model $CD4^+$ T cells need $CD8^+$ T cells for an early activation. A further series of experiments have made it plausible that early in the process $CD8^+$ T cells are probably needed to initiate β cell necrosis in the NOD islets, where after sufficient antigens become available to perpetuate the autoimmune response (Fig 2). It is also important to note that such requirement for $CD8^+$ T cells has not firmly been established for BB-DP rats, though there are indications that also in this model $CD8^+$ T cells are required in an early window in the disease (41).



A.



B.

Fig. 2. A scheme on the immuno-pathological events that take place during the development of endocrine organ-specific autoimmune diseases.
(A) In a first afferent phase APC – predominantly monocyte derived - accumulate in the gland. The APC influx can be induced by a-specific inflammatory stimuli, e.g. a necrosis of target cells by toxins or viruses. The accumulated APC take up relevant auto-antigens and leave the tissue to travel to the draining lymph nodes. In the draining lymph nodes the APC seek contact with T cells and B cells. Apparently, an aberrant immune response results. Instead of reinforcing tolerance the APC generate an autoimmune reaction. The text of this chapter lists the various abnormalities found in animal models underlying this aberrant regulation of the autoimmune response. Generated cytotoxic T cells attack the glandular target cells and more antigens are released. B cells produce auto-antibodies, which are in the majority of endocrine organ-specific autoimmune diseases just markers of the process (except in Graves' disease)
(B) In later phases the autoimmune reaction is perpetuated and expanded. The generated autoreactive T cells and B cells gain access to the target glands and often form focal accumulations. Such focal accumulations are mostly harmless for the target cells. When a switch takes place of the APC to Th1 stimulating cells (DC1) a switch to a more aggressive type of inflammation is induced. In such inflammations IFN-γ activates scavenger macrophages to kill off the target cells. (APC = antigen presenting cell, E = endocrine cell, B = B cell, DC = dendritic cell, Mac = macrophage, Th1 = T helper 1 cell, abs = antibodies, NO = nitric oxide.

Pre-autoimmune aberrancies in the target glands and the pre-autoimmune attraction of DC and Mϕ into the target glands.

Attraction signals for the DC and MΦ may -in the first place- be simply of environmental origin and inflammatory in nature. Early aspecific necrosis of glandular cells caused by toxins, e.g. iodine in the thyroid (5,65) and streptozotocin in the islets (21); bacterial infections, e.g. Helicobacter pylori in the stomach (1) and viral infections (e.g. EMC-D virus in the islets) with the concomitant release of self antigens and pro-inflammatory and chemotactic factors for DC and Mϕ have all been described as eliciting factors for endocrine organ-specific autoimmunity.

Secondly, DC and MΦ may initially accumulate in target glands not to exert a function in inflammation and the removal of cell debris, but to regulate the growth and function of the glandular tissue. It is important to note that DC and MΦ are normal constituents of the thyroid, the islets and other glandular structures, and that the cells have been proven to regulate the growth and function of glandular cells *in vitro* (2,45,95) predominantly via IL-1 and IL-6 signals. The recognition that such cells constitute a recruitable cellular force that, on the one hand, is capable of regulating tissue homeostasis, but on the other hand is also capable of initiating immune responses, has implications for our understanding of the induction phase of endocrine organ-specific autoimmune diseases. Minor inborn errors in metabolism and minor aberrancies in the structure, growth and function of tissues may therefore necessitate an influx of DC and MΦ to regulate tissue homeostasis. This 'non-infectious" influx may, however, be a first step on the way to endocrine organ-specific autoimmunity. There is an argument for such a view: Very early (and even fetal) abnormalities in the proliferative capability and hormone production of thyrocytes, salivary gland cells and islet cells have been reported in the OS chicken, the BB-DP rat and the NOD mouse.

Already in 1983 a decreased in vitro growth rate of fetal OS chicken thyrocytes has been described (5,14,100,115). It has been suggested that this intrinsic, abnormal low proliferative rate of OS chicken thyrocytes in the pre-autoimmune state reflected an inborn derangement of function-that is, a defect in the handling of iodine, i.e. fewer iodine atoms were found to be built into the chickens' Tg. Other pre-autoimmune thyroid abnormalities found in the OS chicken include TSH-independent hyperfunction, such as a high oxidative metabolism, a high iodine uptake and an incomplete suppression of this uptake (100). These signs of hypermetabolism were suggested to be mechanisms compensating for the iodine-handling defect and also explain the sensitivity of this birds' thyroid for the toxic effects of high dose iodine diets (100).

Similar pre-autoimmune aberrancies have been described for the BB-DP rat thyroid. Again the in vitro proliferative rate of thyrocytes was significantly lower than that of a control rat strain, while the output of thyroid hormones and of IL-6 was higher in the pre-autoimmune stage (96).

In the NOD mouse histomorphological analyses of neonatal (1 day postpartum) submandibular glands, the primary target for autoimmune sialo-adenitis, revealed a delayed morphological differentiation during organogenesis when compared to control C57BL/6 mice. Acinar cell proliferation was reduced, while the expression of apoptotic molecules was increased (16). During the following pre-weaning, but pre-autoimmune period matrix metalloproteinases (MMPs) were aberrantly expressed and the composition of the saliva altered (118). In congenic NOD strains not developing sialoadenitis, i.e. lacking Idd3 and Idd5, there were no such pre-autoimmune target abnormalities (10).

Pancreas aberrancies do also already exist at birth in the NOD mouse. NOD female neonates have, compared to C57BL/6 neonates, more hyperactive β cells, assessed by in situ preproinsulin II expression (81). This β-cell hyperactivity disappears within the first week of age and is accompanied, in NOD (and NODscid) neonates, by high percentages of small glucagon$^+$-immunoreactive (immature) islets. These data are suggestive of islet neogenesis in the very young NOD mouse in relationship with neonatal β-cell hyperactivity. At the time of these early postnatal aberrancies MΦ and DC are present in the pancreas around or close to the developing islet and ductal structures (19). Densities of DC are higher in NOD and NODscid mice as compared to controls. High numbers of BM8$^+$ scavenger MΦ are normally observed at birth in mouse pancreases and such cells are mainly seen at sites of islet neogenesis and predominantly at the duct-islet interface. After birth, such scavenger MΦ disappear progressively, but this is not the case in NOD and NODscid mice. After the first few weeks of pancreas development NOD mice show further morphologic aberrancies in islet development: enlarged, irregular islets characterize the NOD mouse pancreas up till the age of around 12 weeks, when particularly these large mega-islets become the targets of the autoimmune attack (86).

Suffice to say, that there is ample evidence for aberrancies in the morphogenesis and function of the target glands prior to the development of the specific autoimmune reaction in the OS chicken, the BB-DP rat and the NOD mice. These early phases of aberrant growth and function of the gland coincide with an aberrant influx and accumulation of MΦ and DC. Considering the broad function of these cells, it is plausible that the cells primarily play a role in correcting the aberrancies in the development of these glands. Conversely it is possible that the DC and MΦ induce the early glandular abnormalities.

Pre-autoimmune aberrancies in the immune system

The pre-autoimmune influx and glandular accumulation of MΦ and DC and their trafficking to the draining lymph nodes are as such not sufficient for a development of pathological

endocrine organ-specific autoimmunity (35). A local or systemic aberrancy in the functioning of immune cells themselves is an additional prerequisite to initiate the inappropriate, excessive and destructive immune response towards self antigens, characteristic of the endocrine organ-specific autoimmune diseases.

The exact mechanisms for maintaining tolerance in non-autoimmune-prone individuals are far from clear. There exist various mechanisms to avoid self reactivity, including mechanisms of central tolerance induction, such as the deletion of autoreactive T cells in the thymus, and mechanisms of peripheral tolerance induction, such as anergy induction (e.g. via the presentation of antigens in the context of MHC-class II molecules, but in the absence of co stimulatory molecules), the action of regulatory T cells, and Activation-Induced T Cell Death/ apoptosis (AITCD). The above listed animal models are practically all characterized by one or more defects in either one or more of these tolerance mechanisms.

Many of the models show a defect in regulatory T cells. Particularly the mouse thymectomy model is known for its specific lack of $CD4^+CD25^+$ T regulator cells (1). The BB-DP rat, however, also lacks a particular subpopulation of T cells, the so-called $RT6^+$ T cells, which clearly form a regulator (suppressor) population in the rat (39). Thymic abnormalities are also evident in the BB-DP rat, suggesting deviations at the level of central tolerance in this animal as well (38,41). The OS strain of chickens also has defects in its regulatory T cell system (115). The NOD mouse has defects in T cell apoptosis, leading to a decreased ability of the cells to undergo AITCD, a major mechanism in both central and peripheral tolerance induction (82). Moreover, NOD mice are characterized by an abnormal thymic architecture (28,88). Taken together there are subtle T cell abnormalities that need to be fully characterized to understand these spontaneous organ-specific autoimmune diseases.

Interestingly the spontaneous animal models also show aberrancies in the development of MΦ and DC from bone-marrow precursors. Studies in the BB-DP rat and NOD mouse model have established that in both the NOD mouse as well as the and BB-DP rat aberrancies exist in the maturation process of myeloid cells from their precursors (35). There is a shift in balance between MΦ and DC development from precursors in favour of the first, leading to an enhanced maturation of scavenger macrophages in the animals (Nikolic, to be published). The macrophages show in addition an enhanced migration and an enhanced production of pro-inflammatory factors (64, Nikolic, to be published). This hyper-reactivity of scavenger macrophages likely contributes to the enhanced cytotoxic potential of the cells for target glandular cells in endocrine organ-specific autoimmunity. At the same time there is a maturation defect in the DC compartment of the animals. This defect leads to a low expression of MHC class II and CD80 and CD86 molecules on the generated DC of these animals resulting in a low capacity of the APC to stimulate T cells particularly via the MHC class II-TCR and CD80-CD28 route (29,31,32,37, Groen, to be published). Precisely such triggering via the latter route is

essential to prevent the development of diabetes in the BB-DP rat and NOD mouse model (41, Groen, to be published): in vivo treatment of BB-DP rats with stimulatory anti-CD28 monoclonal Abs completely prevents the development of both insulitis and diabetes, whereas treatment with a blocking anti-CD80 monoclonal Abs accelerates diabetes development. Knocking out CD28-CD80 interaction in the NOD mouse model has also proven to result in an acceleration of the disease (92). Another piece of evidence that fully active and mature DC are required for optimal tolerance induction in the BB-DP rat is the observation that the immature DC of the BB-DP rat can not sufficiently expand the $RT6^+$ T cell population, which represents the "suppressor" cell population in the rat system (32).

In sum, the aberrant development of MΦ and DC in BB-DP rats and NOD mice may contribute to the poor tolerogenic capability of the animals and the heightened aggressiveness to glandular cells.

What do we learn from the animal models for endocrine organ-specific autoimmune diseases in the clinic.

It must be emphasized again that studying animal models cannot answer all the problems presented by endocrine organ-specific autoimmune diseases when seen in the clinic. It must be expected -considering the various etiologies in the different animal models- that the causes of the diseases in the human and the involvement of various genes and environmental factors may differ from patient to patient. Yet in the study of particularly the pre-autoimmune phases of the diseases (the focus of this chapter) there is hardly an alternative than to study the animal models. Only limited series of experiments can be carried out in individuals at risk to develop such diseases. Moreover the etio-pathogenesis found in the animal models may provide novel information of mechanisms that are relevant to human studies.

In summary, we believe that the animal models of endocrine organ-specific autoimmune disease still hold immense promise for the discovery of pathways, genes and environmental factors that determine the development of overt endocrine organ-specific autoimmune diseases. The BB rat is a good example of the success of this approach since the recent positional cloning of the lymphopenia gene, Ian5 identified a novel family of genes of importance to T cell development. If the findings of the animal models are verified in patients such studies will eventually lead to a better understanding of the human diseases. Until then therapies that show effect in the animal models should only cautiously be applied to humans, since knowledge is still imperfect and errors can easily be made.

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CHAPTER 4

AN ABERRANT DEVELOPMENT FROM BLOOD MONOCYTES IN TYPE 1 DIABETES MELLITUS. A ROLE FOR AN ABNORMAL LOW CD54 EXPRESSION?

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Abstract

Dendritic cells (DC), antigen presenting cells par excellence, play a pivotal role in the regulation of the islet autoimmune response in the animal models of type 1 diabetes mellitus (DM1). Interestingly the function of DC and their development from precursors have been shown defective in the animal models of DM1 as well as in patients. In this study we confirm that the generation of DC from DM1 monocytes results in populations of DC that are relatively immature. We also found that the expression of the adhesion molecules was considerably decreased on such DC.

Adhesion molecules play a role in the differentiation and maturation of leucocytes, including that of DC. We therefore went on to investigate the expression of these molecules on monocytes of DM1 patients. DM1 patients have a significant lower number of monocytes expressing CD54 (ICAM-1). Moreover, triggering of CD54 on monocytes and DC using an anti-CD54 monoclonal antibody enhanced the differentiation and maturation of the cells to fully mature DC.

We conclude that the low expression of CD54 on DM1 monocytes and immature DC likely plays a role – at least in part - in their defect to mature into fully potent APC.

Introduction

Type 1 diabetes mellitus (DM1) is a chronic T cell mediated autoimmune disease in which the ß cells of the islets of Langerhans are destroyed. Antigen presenting cells (APC), such as dendritic cells (DC) are amongst the first leukocytes accumulating around the islets of the two most important animal models of spontaneously developing DM1, i.e. the Non Obese Diabetic (NOD) mouse and the Biobreeding Diabetes Prone (BB-DP) rat (1;2). Since DC are the most potent APC of the immune system and indispensable for the initiation of primary immune responses and the generation of T cell dependent antibody formation (3), it is now generally accepted that DC are critically involved in the regulation of the islet auto-immune response in these animal models.

DC form a heterogeneous group of APC with different lineage backgrounds (lymphoid versus myeloid), with different (local) precursors and with various stages of differentiation and maturation. In the last decade the idea has gained acceptance that DC are not only cells capable of initiating immune responses, but are also inducers of tolerance (4-8). Abnormalities in the differentiation and maturation of DC from their precursors have been documented in DM1 patients, the NOD mouse and the BB-DP rat, and it is presently thought that such defect might be causal to the state of defective tolerance in DM1.

With regard to patients it proved difficult to generate veiled accessory macrophages (9) and DC (10) from circulating blood monocyte precursors. The generated veiled accessory macrophages and DC showed a lower expression of (co-) stimulating molecules and had a lower potency to stimulate T cell proliferation.

With regard to the NOD mouse, a mixture of splenic DC and macrophages were poor in accessory function (11). In accord with the abnormalities of the DC isolated from the lymph node and spleen of this animal were findings on an abnormal differentiation and maturation of DC from NOD bone marrow precursors, particularly when DC were generated in the presence of GM-CSF alone (12-18). Under such circumstances NOD bone marrow precursors yield lower numbers of differentiated DC, yet show various signs of pro-inflammation, viz. an enhanced expression of NF-κB (19) and a higher production of interleukin-12 (20). There exist however also reports for DM1 patients and NOD mice that doubt the above mentioned defects in the differentiation of DC from their precursors (21-23).

With regard to the BB-DP rat we previously showed that lymph node and spleen DC are less well differentiated and have an abnormal (“immature”) phenotype (24), i.e. the cells show a relatively low expression of MHC class II and of costimulatory molecules. These DC also showed a diminished capability to stimulate T cell proliferation, particularly of the $RT6^{+}CD8^{+}$ suppressor T cell population of the rat. We also found that a defective DC-DC interaction in so-called homotypic cell clusters played an important role in the poor differentiation and maturation state

of DC in this animal. Aggregation of DC in homotypic clusters normally occurs in the lymph, when the cells are in transit as veiled cells from the peripheral tissues to the draining lymph node. Cluster formation is dependent on the interaction between various adhesion molecules on these cells. After induction of an enhanced cluster formation (via the stimulation of cell-cell adhesion) normal rat DC have a higher expression of CD80 and CD86 and increase their T cell stimulating capabilities (25). DC of the BB-DP rat formed fewer and smaller clusters and the DC-DC clustering resulted in only a modest maturation of the cells (25). Also in DM1 patients veiled accessory macrophages generated from blood monocytes formed fewer and smaller clusters (9). Yet studies on the expression of adhesion molecules on DM1 monocytes and the role of such adhesion molecules in the differentiation and maturation of DC from precursors are lacking.

Here we report on the expression of various adhesion molecules on monocytes of patients with recent onset DM1, of DM2 patients (as hyperglycaemic /metabolic-disturbed controls), and of healthy controls. We tested monocytes of patients with a recent onset autoimmune (AI) thyroiditis as well (as controls for autoimmunity). We found that a low intercellular adhesion molecule-1 (ICAM-1 or CD54) expression on circulating monocytes was specific for DM1. We also observed that stimulation of the CD54 molecules on the surface of monocytes and DC using a stimulating CD54 antibody, stimulated the homotypic cluster formation of the cells and their differentiation and maturation. Because of the positive role of CD54 molecule in the maturation of monocyte-derived (mo-) DC and the specific lower expression of CD54 on monocytes of DM1 patients, we consider it not surprising that we could confirm previous findings of others that the generation of DC was hampered from monocytes in DM1 (10), but was normal in DM2 and AI thyroiditis.

Research design and methods

Subjects

Heparinized blood (60mls) was obtained via venapuncture from the following groups of individuals: Recently diagnosed DM1 patients (n=39), who visited the outpatient clinics of three major hospitals in the Netherlands. The patients were included according to the WHO criteria. The mean age was 13.7 ± 6.5 years, ranging from 6.5 to 33.2 years. The mean of HbA1c was 8.8 ± 2.5%. DM2 patients (n=15), who visited the outpatient clinic of the Department of Internal Medicine, Erasmus MC, Rotterdam, The Netherlands. The mean age was 55 ± 13.3 years, ranging from 36.4 to 83.3 years. The mean of HbA1c was 8 ± 1.4%. Recently diagnosed AI thyroiditis patients (n=28), who visited the outpatient clinic of the Department of Internal Medicine, Medical Centre Rijnmond Zuid, Rotterdam, the Netherlands. The mean age was 42.7 ± 12.6 years, ranging from 30.9 to 71.2 years. All patients had high levels of thyroid peroxidase

antibodies (TPO-Abs > 400IU/ml). Healthy controls with an absent family history of autoimmune diseases consisted of laboratory personnel and students. The controls were divided for some comparisons into two groups to obtain age-matched controls for the two diabetic groups (young group, n=41, mean age 28 ± 6.6 years, ranging from 19.4 to 42.5 years; older group, n=15, mean age 50.1 ± 9.5 years, ranging from 30 to 60.5 years).

Informed consent was obtained from all participants. The research protocol has been approved by the Medical Ethical Committee of the Erasmus MC, Rotterdam, the Netherlands. For the DC cultures with anti-CD54 antibody stimulation we used monocytes isolated from buffycoats purchased from the Sanquin Blood bank, Rotterdam, the Netherlands.

Serum

Serum samples were collected and stored at -800C until the analysis for soluble (s) ICAM-1 using two commercially available ELISA methods (Bio-source ELISA KH5401 and Bio-source KH5412 high sensitive, Camarillo, CA, USA).

Monocyte isolation and generation of DC

Ficoll (Pharmacia, Uppsala, Sweden; density 1.077g/ml) and Percoll (Pharmacia; density 1.063g/ml) density gradient centrifugation were used to isolate monocytes from heparinized blood and buffy-coats (Sanquin Blood bank, Rotterdam, the Netherlands). The monocytes were cultured at a concentration of $0.5x10^6$ cells/ml on 24-well culture plates under plastic-adherent conditions in RPMI 1640 with 25mM HEPES and L-glutamine (BioWhittaker Europe, Verviers, Belgium) (hereafter referred to as $RPMI^+$) containing ultraglutamine (UG) (2mM, BioWhittaker), penicillin/streptomycin (P/S) (100U/ml penicillin, 100µg/ml streptomycin, BioWhittaker) and 10% heat inactivated FCS (FCSi) (BioWhittaker) in the presence of GM-CSF 400U/10^6 cells/ ml and IL-4 500U/10^6 cells/ ml (both Pepro Tech EC, London, England). The cells were incubated at 37^0C, 5% CO_2 and 95% humidity. On day three, half the culture fluid was refreshed with GM-CSF 200U/10^6 cells/ ml, IL-4 250U/10^6 cells/ ml. After six days DC were collected by resuspending and washing the wells thoroughly with cold phosphate buffered saline (PBS) pH 7,4 (BioWhittaker), with 0,1% bovine serum albumin (BSA) (Bayer, Kankakee, IL, USA) and 3mM ethylene diamine tetraacetic acid, pH 8 (Sigma-Aldrich, Steinheim, Switzerland). T cell isolation was performed by washing the pellet in the Percoll gradient twice with PBS/ 0,1%BSA and incubating the cells with 20µl/10^7 cells anti-CD3 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 minutes on ice. A magnetic cell sorting system (auto MACS sorter, Miltenyi Biotec) was used for the selection of CD3 positive T cells.

Phenotype of monocytes and DC

The following monoclonal antibodies (mAbs) were used for flowcytometry: anti-IgG1 FITC (1:10, Becton Dickinson (BD), San Jose, CA, USA), anti-IgG1 PE (1:10, BD), anti-CD14 FITC (1:250, Beckman Coulter, Hialeah, FL, USA), DC-SIGN PE (1:10, R&D systems, Minneapolis, MN, USA), anti-HLA-DR PE (1:200, BD), anti-CD80 PE (10µl/10^5 cells, BD), anti-CD86 FITC

(10µl/10^5 cells, Pharmingen, San Diego, USA), anti-CD40 FITC (10µl/10^5 cells, Serotec, Oxford, England), anti-CD1a PE (1:100, Beckman Coulter), anti-CD18 FITC (1:10, BD), anti-CD11b PE (1:50, BD), anti-CD29 FITC (1:160, Beckman Coulter), anti-CD54 PE (1:4, BD), anti-CD49a FITC (VLA-1), anti-CD49b FITC (VLA-2), anti-CD49d (VLA-4) (10µl/10^5 cells, Serotec), anti-CD49e FITC (VLA-5) (10µl/10^5 cells, Immunotech, Marseille, France), anti-CD3 FITC (1:20, BD), anti-CD19 PE (1:25, BD) and 7AAD (1:250, Molecular probes, Eugene, Oregon, USA). Whole blood (50µl) was incubated in polypropylene tubes (BD) with mAbs for 15 minutes and washed with PBS/ 0,1% BSA. Red blood cells were lysed with lysing solution (BD) and washed twice with PBS/ 0,1% BSA. DC were incubated in polypropylene tubes with mAbs for 15 minutes, then washed twice. The cells were measured immediately following cell staining using a FACScan flowcytometer and analysed using CellQuestPro (BD, Mountain View, CA, USA). Routinely 10,000 events were collected. Debris and dead cells were gated out on basis of their light scatter properties. The gated DC population consist no CD3+ and CD14+ cells. The background staining was determined by staining of cells with IgG1-FITC and IgG1-PE alone and subtracted from the values. Data were expressed as mean ± SD of percentage of positive cells and mean ± sd of mean fluorescence intensity (MFI).

Mixed Leucocyte Reaction (MLR)

For the MLR, DC were irradiated with 20Gy and placed in flat-bottom 96-wells-plates (Nunc, Roskilde, Denmark) in $RPMI^+$ medium containing UG, P/S and A^+-serum. We added 100µl of the concentration of $3x10^5$, $1.5x10^5$, $0.75x10^5$ and $0.37x10^5$ cells/ml of DC and an equal volume of T-cells at concentration of $1.5x10^6$ cells/ml to the wells resulting in a total volume of 200µl per well. As positive control these T-cells were stimulated with phytohemagglutamin (PHA) (Sigma). Proliferation was determined after 18 hours of 0.5µCi/ well ^{3}H-thymidine addition on day 5. Cells were harvested on filter papers and radioactivity was counted in a liquid scintillation analyzer (LKB Beta plate, Wallac, Turun, Finland). The values are the mean of triplicates.

Stimulation with CD54

To investigate the role of CD54 triggering in the actual differentiation of DC from buffy-coat monocytes a mouse anti-human (anti-) CD54 mAb (1:50 and 1:100, Pelicluster, CLB, Amsterdam, The Netherlands) or recombinant human (rh-)CD54 (30ng/ml and 300ng/ml, R&D) was added throughout the entire 6 days culture period in the presence of GM-CSF and IL-4. To study the role of CD54 triggering in a further maturation of the DC, DC were harvested after 6 days of culture and re-suspended in the presence of anti-CD54 mAb, rh-CD54 or anti-CD18 mAb (1:100, Serotec) for 24 hours. Pooled normal mouse serum (1:500, C57BL/6 mice, C3H/FEJ and BALB/C) was used as a negative control. The effect of the various treatments was assessed by investigating the expression of DC-SIGN, HLA-DR and of the co-stimulatory molecules CD80, CD86 and CD40 on the various treated DC populations. For assessment of the homotypic cluster formation, we harvested and resuspended DC of two healthy donors in flat-bottom 96-

wells plates (Nunc) at concentrations of 0.5 x10^5 and 1x10^5 cells/ 200µl/ well. These DC were allowed to aggregate in presence or absence of anti-CD54 mAb for 20 hours in the incubator. Formed clusters were counted with an inverted microscope, and values were expressed as numbers of clusters per 5 microscopic fields (250x magnification). A cluster was defined as an aggregate of four or more cells.

Statistical analysis

Statistical analysis was performed using Spss 11.0. Paired Student's T-test was used to detect differences between data of untreated and CD54 treated DC experiments. For the other experiments the Mann Whitney U-test was used. P–values lower than 0.05 were considered significant.

Results

DC derived from monocytes of DM1 patients show a less differentiated phenotype.

The yields of DC, measured as the percentage of generated viable cells of the original number of monocytes put into GM-CSF/ IL-4 culture, were comparable for DM1 patients, DM2 patients, AI thyroiditis patients and healthy controls (data not shown).

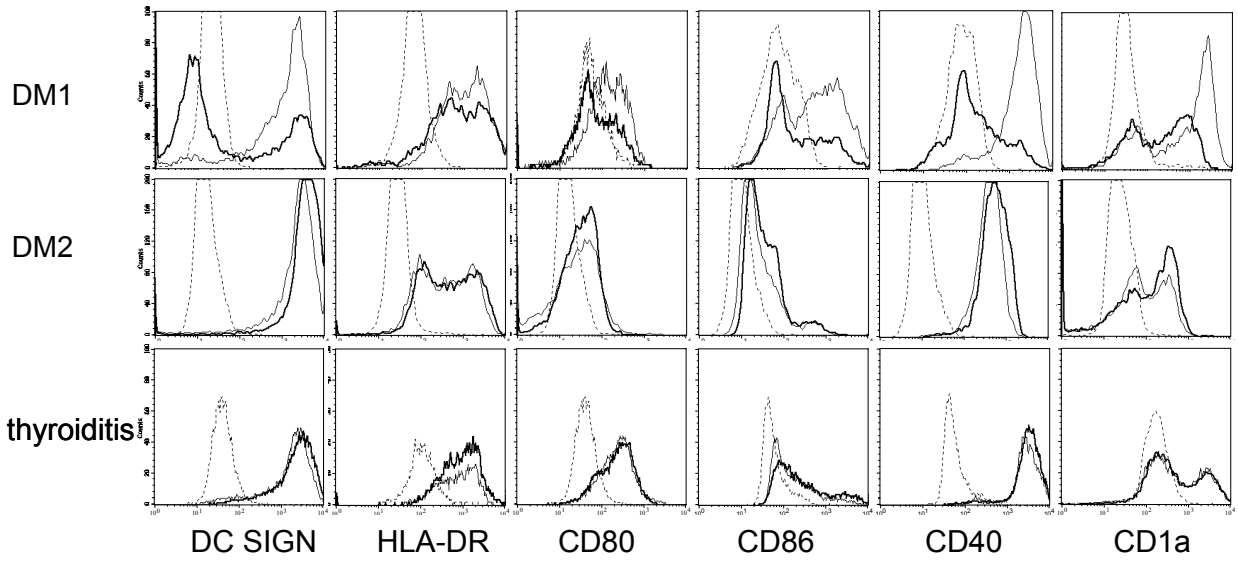
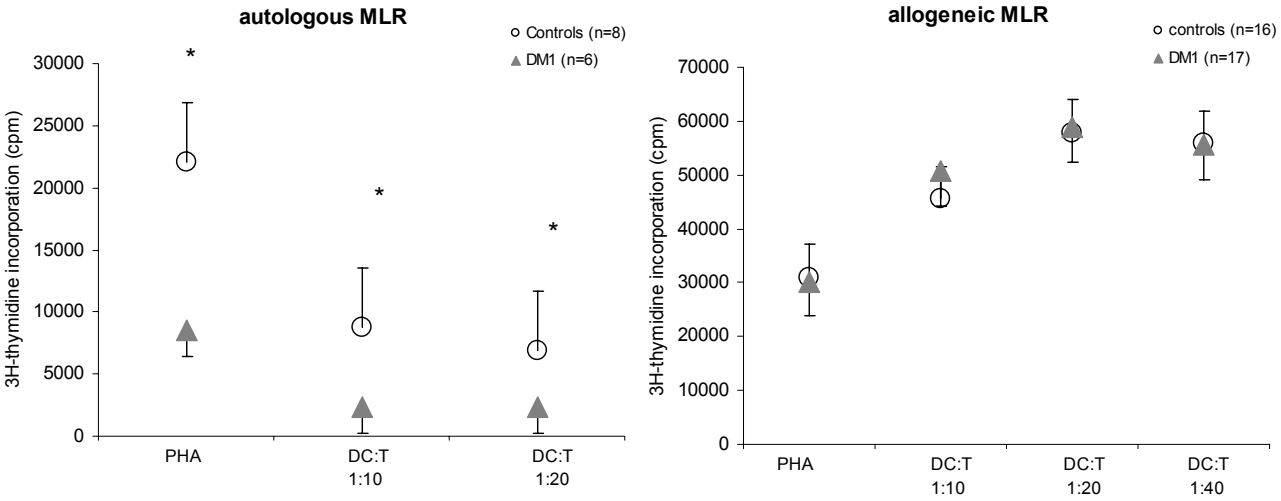


Fig. 1. Phenotype of immature DC derived from patients with DM1, DM2 and AI thyroiditis and control subjects. The phenotypes (DC markers and costimulatory molecules) of DC from patients (thick lines) are shown in histograms. The thin lines represent DC from control subjects and the dashed lines are DC stained with isotype control Abs. DC of DM1 patients have lower expression of DC-SIGN, CD80, CD86, CD40 and CD1a. DC of DM2 and AI thyroiditis patients are comparable to controls.

Fig. 2. Proliferation of T cells measured by ^{3}H-thymidine incorporation and expressed as counts per minute (cpm) are shown. Autologous T cells or pooled T cells from three different allogeneic donors were used for the autologous and allogeneic MLR respectively. DC of DM1 patients (grey triangles) have a lower autologous T cell stimulatory capacity compared to DC of controls (white circles), but have a normal capacity to stimulate allogeneic T cells. T cells of DM1 patients have significantly lower proliferation to PHA stimulus compared to T cells of controls. Data are shown as means and standard errors of cpm for triplicate samples per individual (* p=0.03).



Despite the similar yields, the percentages of DC positive for the following markers were significantly lower in DM1 patients: DC-SIGN (mean 28% ± SD 19%, vs. 54% ± 24% for young healthy controls, p=0.04), CD80 (5% ± 6% vs. 12% ± 10%, p=0.02), CD86 (22% ± 12% vs. 32% ± 13%, p=0.04), CD40 (30% ± 18% vs. 50% ± 21%, p=0.004) and CD1a (9% ± 11% vs. 27% ± 18%, p=0.003).

The percentages of HLA-DR^{+} DC were also lower for DM1 patients, yet not statistically significant (42% ± 19% vs. 53% ± 18%, p=0.14). With regard to the expression level, the mean fluorescence intensities (MFI) of the DC markers were significantly lower in DM1: DC-SIGN (1382 ± 1322 vs. 3613 ± 1998 in young healthy controls, p=0.02), CD86 (363 ± 276 vs. 534 ±319, p=0.05), CD40 (446 ± 448 vs. 916 ± 806, p=0.02) and CD1a (232 ± 318 vs. 406 ± 332, p=0.04). The percentages and expression levels of the afore-mentioned markers on the DC of DM2 patients and AI thyroiditis patients were comparable to those of the appropriate matched healthy controls (Fig. 1).

With regard to DC function, the T cell stimulatory capacity in autologous MLR of the phenotypically abnormal DM1 DC was significantly lower. For the DC:T cell ratio 1:10 and 1:20 DM1 patients showed a significantly lower incorporation of ^{3}H-thymidine (Fig. 2). When the T cells of the DM1 patients were stimulated with the mitogen PHA in the absence of DC, they also showed a lower proliferation as compared to healthy control T cells (8568 ± 7508 vs. 22099 ± 13550, p=0.03) (Fig. 2). When allogeneic T cells were stimulated with DC of DM1 patients the proliferation was normal (Fig. 2). The T cell stimulatory defect in autologous MLR is thus most

likely due to an intrinsically low proliferative capability of the diabetic T cells, and not to a low stimulatory capability of DM1-DC.

The expression of various integrins on monocyte-derived DC of DM1 patients is lower

The integrin profiles on the mo-DC were also studied in the various groups of patients and individuals. We found lower percentages and expression levels of several adhesion molecules on the DC of DM1 patients (n=17) as compared to those of the appropriate healthy controls (n=17). These patients had significantly lower percentages of DC positive for the integrins CD54 (25% ± 16% vs. 4% ± 18% in controls, p=0.001), CD18 (20% ± 15% vs. 40% ± 19%, p=0.002), CD11b (30% ± 17% vs. 51% ± 19%, p=0.003), CD49d (5% ± 5% vs. 10% ± 8%, p=0.02) and CD49e (20% ± 15% vs. 40% ± 21%, p=0.004). Also the MFI of these integrins was lower for DM1 DC: CD54 (609 ± 487 vs. 941 ± 435, p=0.02), CD18 (177 ± 136 vs. 351 ± 229, p=0.02), CD11b (853 ± 869, p=0.02), CD49d (52 ± 30 vs. 87 ± 51, p=0.02) and CD49e (177 ± 128 vs. 363 ± 250, p=0.03) (Fig. 3). The expression level was not significantly different in DM1 DC for the integrins CD29 (Fig. 3, p=0.12), CD49a and CD49b (data not shown).

The integrin profiles of DC of DM2 patients (n=13) and AI thyroiditis patients (n=12) were not different from those of healthy control DC (Fig. 3).

The percentage of $CD14^+$ monocytes expressing CD54 is lower in DM1 patients

To investigate whether the integrin expression on the actual precursors of the DC, i.e. the $CD14^+$ monocytes, was already aberrant in patients with DM1, we analysed the various adhesion molecules on these cells, using whole blood samples. Significantly lower percentages of $CD14^+$ monocytes of DM1 patients (n=24) were positive for CD54 (75% ± 19% in DM1 patients vs. 82% ± 14% for the appropriate healthy controls, n=41, p=0.03) and for CD49e (76% ± 21% vs. 87% ± 12%, p=0.003). Furthermore, we observed a tendency for a lower percentage of monocytes expressing CD49d in DM1 (77% ± 18% vs. 84% ± 10%, p=0.06). With regard to the expression level, the MFI of CD11b was significantly increased in DM1 monocytes as compared to healthy control monocytes (426 ± 308 vs. 257 ± 247, p=0.001). The MFI of CD18 was also increased in DM1 patients, however did not reach statistically significance (119 ± 58 vs. 102 ± 51, p=0.17) (Fig. 4). Other investigated integrins i.e. CD29 (Fig. 4), CD49a and CD49b (data not shown),

Increased soluble ICAM-1 (=CD54) level in serum

Since we found that CD54 expression was quite specifically disturbed on monocytes of DM1 patients, we wanted to confirm previous observations, that the level of sICAM-1 was higher in the serum of DM1 patients (26-30). We therefore investigated sICAM-1 levels in their serum (n=39). We indeed detected significantly increased sICAM-1 levels in our DM1 patients, i.e. 62 ± 21 ng/ml vs. 40 ± 15 ng/ml in young healthy controls, n=40, $p=1x10^{-6}$) (table 1). However DM2

patients (n=15) also showed higher sICAM-1 serum levels as compared to their appropriate controls (n=20), be it that the actual values were much higher in both patients and controls due to the usage of a more sensitive assay (457 ± 103 ng/ml vs. 286 ± 101ng/ml, p= $3x10^{-5}$). AI thyroiditis patients (n=28) also had slightly increased sICAM-1 levels (353 ± 208 ng/ml vs. 286 ± 101 ng/ml, p=0.02).

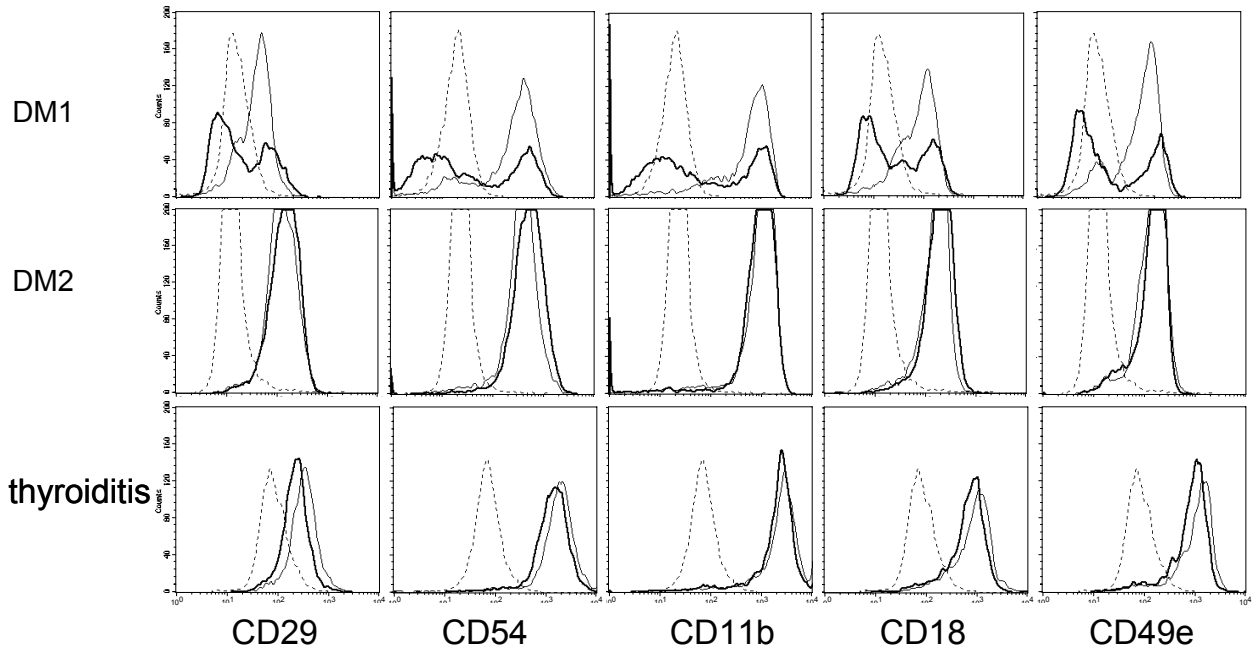
There was no correlation between the sICAM-1 level and the HbA1c level in DM1 patients, neither was there a correlation between the sICAM-1 levels and the TPO Abs levels and positivity in AI thyroiditis patients (data not shown).

Triggering of the CD54 molecule on monocytes using a stimulating anti-CD54 monoclonal Ab stimulates the generation and differentiation of DC.

To investigate the influence of integrin triggering on the differentiation of DC from monocytes, we added a stimulating anti-CD54 monoclonal Ab (mAb) to the DC cultures. As a possible other trigger we used rh-CD54 (see raised levels of sICAM-1 in the sera of patients) and another commercially available stimulating anti-integrin Ab, i.e. an anti-CD18 mAb.

Addition of anti-CD54 mAb (1:50) during the six days culture period of monocytes in GM-CSF and IL-4 resulted in an increase in the percentage CD86 positive DC (non-treated 34% ± 6% vs. Ab-treated 59% ± 14%, p=0.03, n=4 experiments), the MFI of CD86 rose from non-treated 696 ± 207 to Ab-treated 1380 ± 352 (p=0.03).

Fig. 3. Expression of adhesion molecules on immature DC of DM1, DM2, AI thyroiditis patients (thick lines) and control subjects (thin lines). Histograms of adhesion molecules on DC of are shown. The dashed lines isotype controls. Decreased expression of CD54, CD18, CD11b, and CD49e are visible in DM1 patients compared to controls, but not in DM2 or AI thyroiditis patients.



The anti CD54 mAb addition had no effect on the expression of DC-SIGN, HLA-DR, CD80, and CD40 on the generated DC (Fig. 5, upper panel).

When mo-derived "immature" (i) DC were exposed to the stimulating anti-CD54 mAb for 24 hours the clearest effects were seen. We cultured iDC at two cell concentrations (0.5 $x10^5$ and $1x10^5$ cells/ 200µl/ well) for 20 hrs and found that the presence of the mAb increased the numbers of clusters compared to the untreated condition, for 1:100 this was significant for both cell concentrations (0.5 $x10^5$ p=0.02 and $1x10^5$ p=0.006), and for 1:50 it was significant for $1x10^5$ cells/ 200µl/ well, p=0.02).

Thus, treatment of DC with anti-CD54 mAb enhanced homotypic cluster formation (Fig. 6). Compared to the untreated conditions, treatment with anti-CD54 mAb in addition increased the expression of HLA-DR significantly on the DC (from MFI of 628 ± 347 to 1244 ± 450, p=0.003, n=9). Anti-CD54 also increased the percentages of CD86 positive DC (from 40% ± 16 to 59% ± 14, p=0.03) and the MFI of CD86 expression (from 438 ± 348 to 832 ± 472, p=0.01) (see Fig. 5, middle panel). DC-SIGN, CD80 and CD40 expression were not influenced by the anti-CD54 treatment.

With regard to rh-CD54 (30ng/ml and 300ng/ml) and the anti-CD18 mAb treatments, these exposures had no effects on the generation of DC from monocytes or their further maturation and normal expression levels of DC-SIGN, HLA-DR, CD80, CD86, CD40 (Fig. 5, lower panel) and CD54 were found.

Table I. sICAM-1 levels in serum

subjects	numbers	sICAM-1 levels (mean ± s.d.)	p-value (vs. controls)
Controls	n=40	40 ± 15	
DM1 patients	n=39	62 ± 21	p <0.0001
Controls	n=20	286 ± 101	
DM2 patients	n=15	457 ± 103	p <0.0001
AI thyroiditis patients	n=28	353 ± 208	p=0.02

Soluble ICAM-1 levels in sera of DM1 patients, DM2 patients, AI thyroiditis patients and control subjects. In DM1 and DM2 patients, sICAM-1 levels are significantly higher compared to healthy controls. But also the sICAM-1 levels in AI thyroiditis patients are slightly increased. Data represent mean ± standard deviations of sICAM-1 levels and their respective p-values. Note that the mean sICAM-1 levels of DM1 patients and their controls are much lower than the other mean values. This is due to usage of a high sensitive ELISA in the latter.

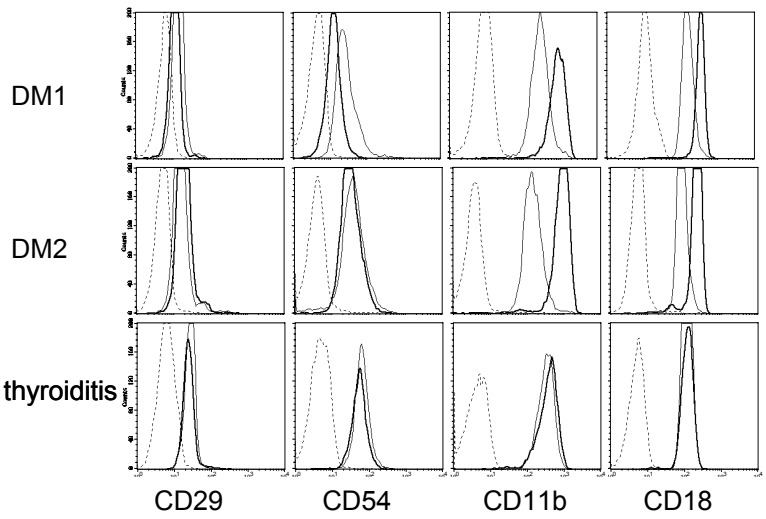
Discussion

Adhesion molecules can be divided into four sub-families: the selectins, the cadherins, the members of the immunoglobulin superfamily (including ICAM) and the integrins (31). Our study shows for the first time that a low expression level of an adhesion molecule, i.e. ICAM-1 (CD54) is *specific* for monocytes of DM1 patients; it did not occur on monocytes of DM2 and AI thyroiditis patients. Interestingly, a lowered expression of CD54 on monocytes was also found in another organ-specific autoimmune disease, viz. multiple sclerosis.
The cerebrospinal fluid of patients with relapsing multiple sclerosis contains lower numbers of $CD54^+$ monocytes and this abnormality was correlated to disease activity (32).

The here also reported low expression of CD49e and high expression of CD11b and CD18 on monocytes of DM1 patients was also seen on monocytes of DM2 patients. Hence these aberrant expressions were most likely due to the mild hyperglycaemic state present in both our DM1 and DM2 patients. Indeed Kim and colleagues showed that chronic high glucose increased the adhesion of monocytes to endothelium, which could be inhibited by antibodies against the ß2 integrin (=CD18) (33). Furthermore CD11b expression on monocytes has been reported to increase rapidly after acute glucose challenge in DM2 patients, as well as in healthy control subjects (34).

Triggering of adhesion molecules, i.e. triggering of integrins via an adhesion to extra cellular matrix (ECM) components, stimulates the development of DC from their precursors and their ultimate function (35;36). Human blood monocytes undergo differentiation to DC upon migration through an ECM rich environment, such as matrices containing FN, laminin or collagen (37).

Fig. 4. Expression of adhesion molecules on monocytes of DM1, DM2, AI thyroiditis patients (thick lines) and control subjects (thin lines). Representative histograms for expression of various adhesion molecules on blood monocytes are depicted. The dashed lines are the isotype controls. Monocytes of DM1 patients have lower percentages and expression levels of CD54. In monocytes of both DM1 and DM2 patients, the expression of CD11b and CD18 are increased compared to controls. No differences were observed between AI thyroiditis patients and their controls.



To study a possible role of CD54 in the differentiation and maturation of DC from monocytes we triggered this adhesion molecule on monocytes and DC with an anti-CD54 mAb during their generation from healthy control monocytes. We here demonstrate that this triggering resulted in an enhanced homotypic cluster formation and an enhanced differentiation and maturation of DC from their precursors. Our data are hence suggestive that the lower expression of CD54 on DM1 monocytes might be causally related to the previously described and here confirmed aberration in the generation and maturation of DC from monocytes in DM1.

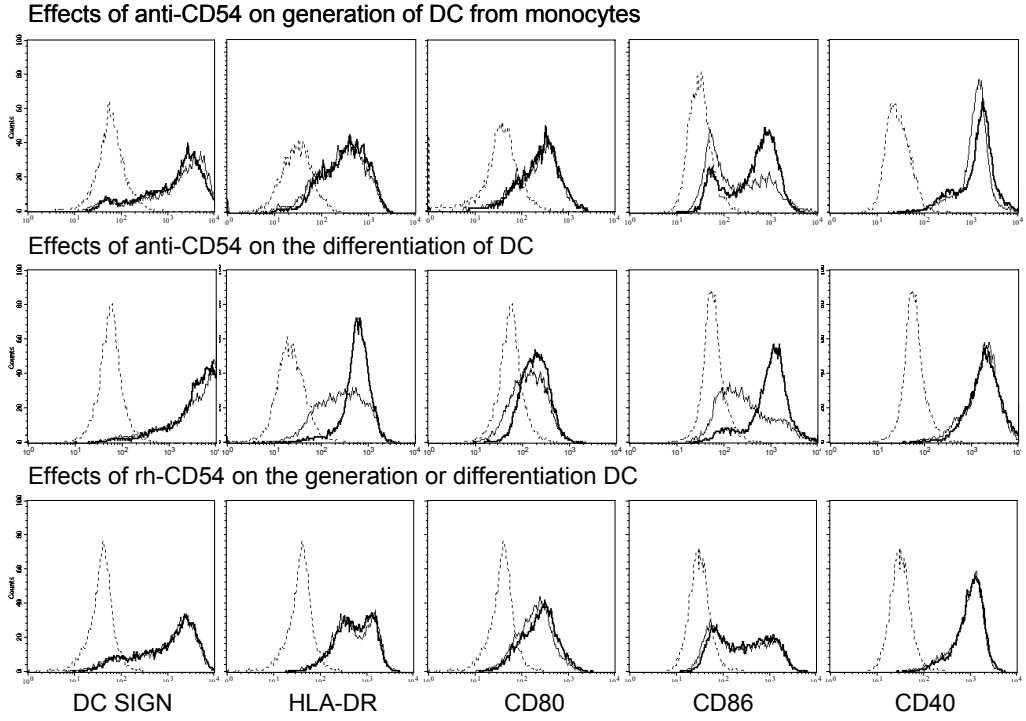
Our study is also novel in that it shows that the aberrantly developed mo-DC of DM1 patients also have a considerable lower expression of various adhesion molecules as compared to DC of DM2 patients and of healthy controls. CD11b, CD18, CD54, CD49d and CD49e were expressed on a lower level on DM1 DC, while CD49a and CD49b were normally expressed. It is known that DC activated by inflammatory stimuli (IL-1, TNFα, IFNγ and infectious agents) increase their surface expression of CD54 and CD49d (31;32;38). Our data of a low integrin expression on DC are in accord with a lower grade of maturation of the cells.

There are many previous studies that have shown that adhesion molecules are intricately involved in the inflammatory response in DM1 and that intervention via such molecules does influence disease development and progression. The early infiltrated DC in the insulitis of the NOD mouse are ICAM-1 and VCAM-1 positive (39;40) and a treatment with adhesion molecule blocking monoclonal antibodies (including anti-CD54mAb) prevents insulitis and diabetes development in this animal model (40-42). This is generally explained by the interference of the Abs in the adherence of leukocytes to the insular endothelium. Our data make it however possible that the Abs may additionally act via an effect on differentiation and maturation of DC.

Of interest are also the observations that CD54 polymorphisms may be involved in the pathogenesis of autoimmune diseases (43-45) and further studies on the CD54 expression on monocytes in DM1 individuals with the various distinct polymorphisms is indicated. So far, no associations were found between DM1 and ICAM-1 K469E polymorphism in Finnish and Danish study groups, however, a Japanese group found an association in adult onset DM1 patients (46-48).

Recombinant CD54, especially the CD54-Ig fusion product, has a protective effect on diabetes development in the NOD mouse, possibly via an induction of anti-CD54 antibody production and/ or a suppression of interferon-γ production by T cells (49;50). Moreover, rh-CD54 can interfere with antigen specific T cell proliferation in vitro, most likely via inhibition of T cell-APC interaction (28). It is well known that sICAM-1 is more abundantly produced in DM1 patients and we confirmed this observation by showing a higher level of sICAM-1 in the serum of DM1 patients as compared to healthy controls.

Fig. 5. Effects of anti-CD54 mAb and rh-CD54 on generation and differentiation of DC. Histograms for DC markers and costimulatory molecules are shown for DC treated with normal mouse serum (as a control, thin lines) and with anti-CD54 mAbs or rh-CD54 (as indicated, thick lines). The staining with isotype control Abs are represented in dashed lines. Addition of anti-CD54 mAbs during the DC development increases the CD86 expression (upper panel). Treatment of iDC with anti-CD54 affected the differentiation of DC: the expression of HLA-DR and CD86 were both upregulated (middle panel). Rh-CD54 has no effect on the phenotype when present during the DC development or after DC were generated (lower panel). Representative histograms are shown out of nine donors.

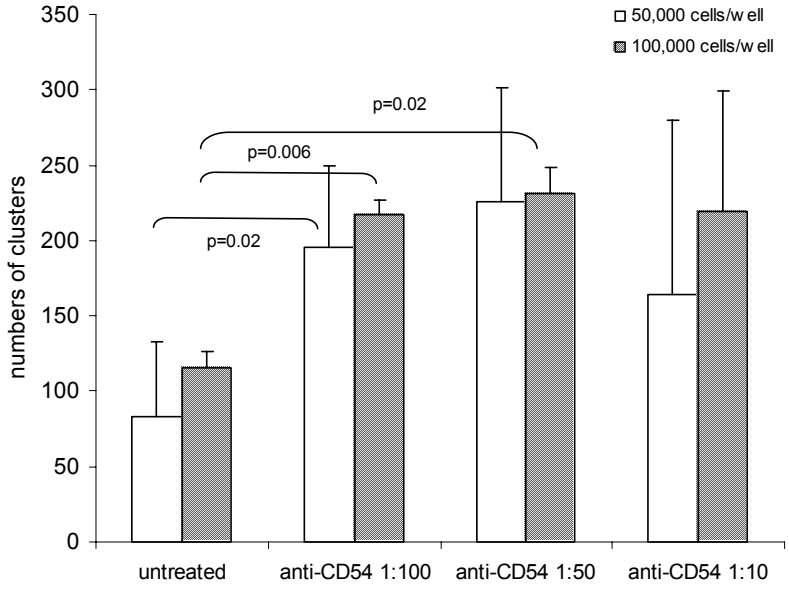


Moreover sICAM-1 levels were also raised in DM2 and AI thyroiditis patients, who do not show the aberrancies in DC development from monocytes.

With regard to the AI thyroiditis patients we could not find abnormalities in the expression of the here studied adhesion molecules on their monocytes and DC, nor did we find abnormalities in the generation of mo-DC. In a previous study we did find that monocytes of AI thyroiditis patients had a lower capability to arrange their cytoskeleton when stimulated with a chemo-attractant and were less capable in forming veiled accessory macrophages, particularly when FN-adhered monocytes were used (51). Apparently monocytes and monocyte-derived APC of DM1 patients show a wider spectrum of aberrancies as compared to monocytes and monocyte-derived APC of AI thyroiditis patients.

In conclusion, the here reported data on 1) a low expression of CD54 on DM1 monocytes and DC, 2) a role of CD54 in DC development and maturation and 3) an abnormal DC development in DM1 patients point to an important role of CD54 in specifically the DC aberrancies in the pathogenesis of DM1, the more since the abnormalities were not found in patients with DM2 and AI thyroiditis.

Fig. 6. The effect of anti-CD54 mAb on DC cluster formation. Number of clusters formed by DC of healthy donors (n=2) at cell concentrations of $0.5x10^5$ (white bars) and $1x10^5$ (dashed bars) for 20hrs in absence or presence (1:100, 1:50 and 1:10 dilutions) of anti-CD54 mAb. For both cell concentrations, the 1:100 dilution of anti-CD54 Ab significantly increased the numbers of DC clusters ($0.5x10^5$: p=0.02 and $1x10^5$: p=0.006). For 1:50 dilution, the numbers were also increased, but significantly for the condition of $1x10^5$ cells/well (p=0.02).



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CHAPTER 5

A COMPLEX IL-10 AND IL-12 PRODUCTION SET POINT OF ANTIGEN PRESENTING CELLS IN TYPE 1 DIABETES

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Abstract

Type 1 diabetes mellitus (DM1) is considered a T helper (Th) 1 mediated auto-immune disease. Monocytes and monocyte-derived dendritic cells (DC) are important regulators of the Th1/ Th2 balance via the production of interleukin (IL)-10 and IL-12.

The aim of the study was to investigate whether monocytes and monocyte-derived DC have an aberrant IL-10/ IL-12 production profile in DM1. Therefore, monocytes and monocyte-derived DC of 22 recent onset DM1 patients, 14 type 2 diabetic (DM2) diabetic patients, 21 auto-immune (AI) thyroiditis patients and 34 healthy controls were stimulated for optimal IL-10 production with Staphylococcus Aureus Cowan 1 strain (SAC). For optimal IL-12 production cells were stimulated with SAC plus interferon (IFN)-γ.

Firstly, we showed that monocytes of DM1 patients produced lower quantities of IL-10 as compared to monocytes of the various controls. The IL-12 production by type 1 diabetic monocytes was normal. Secondly, the generation of DC from monocytes was hampered in DM1 patients and these DC were poor producers of the Th1 type cytokine IL-12 (and also of IL-10). Autologous T cells stimulated by such DC were poor proliferators and producers of both Th1 type cytokines (IFN-γ) and Th2 type cytokines (IL-13 and IL-10). DM2 and AI thyroiditis patients did not show these aberrant DC functions.

In conclusion, our data showed a complex set point of antigen-presenting cells in DM1: While monocytes show a reduced production of IL-10 (a pro-inflammatory/ Th1 promoting sign), monocyte-derived DC show a reduced production of IL-12 (an anti-inflammatory/ Th2 promoting sign).

Introduction

Type 1 diabetes mellitus (DM1) is an organ specific auto-immune disease that results from a Th1 mediated destruction of the β cells in the pancreatic islets. Monocytes, dendritic cells (DC) and macrophages are the first cells accumulating in and around the pancreatic islets of Langerhans in animal models of the disease, such as the BB-DP rat and the NOD mouse (1,2). The T cell stimulatory function of these cells has been shown to be aberrant in these animal models (3-8) and in patients (9,10). Hence monocytes, macrophages and DC are presumed to wrongly orchestrate the T cell responses in the pancreas and pancreatic draining lymph nodes in DM1 ultimately resulting in the detrimental Th1 auto-immune response towards β cells. An abnormal production of regulatory type cytokines by monocytes and DC could clearly contribute to the wrong cross talk between these cells, macrophages and T cells in DM1. Monocytes and DC are important sources of Interleukin (IL)-10 and IL-12 (11,12). IL-10 is an important immune suppressive cytokine (13). IL-12 is a potent inducer of IFN-γ production by T cells and thus favours the development of a Th1 reaction (14,15). IL-12 is predominantly produced by DC during the final maturation steps of the cell (14).

We studied the IL-10 and IL-12 production by monocytes and monocyte-derived DC of DM1 patients and compared outcomes of the production profiles to those of healthy controls and type 2 diabetic (DM2) patients (as metabolic controls). We also studied the IL-10 and IL-12 production by monocytes and monocyte-derived DC of patients with another organ-specific auto-immune disease, i.e. auto-immune hypothyroidism (as autoimmune disease controls).

We observed that monocytes of DM1 patients had a reduced capability to produce IL-10 as compared to the capability of monocytes of healthy controls and DM2 patients. The IL-12 production capability of “DM1” monocytes was normal.

We also observed that DC derived from DM1 monocytes were poor producers of IL-12, which is in agreement with previous and here confirmed reports on a hampered differentiation and maturation of DC from DM1 monocytes. Monocyte-derived DC were also hardly capable of producing IL-10. Autologous T cells stimulated by the aberrantly differentiated DM1 DC proliferated poorly and were poor producers of both Th1 type (IFN-γ) as well as Th2 type cytokines (IL-13 and IL-10). However, DM1 DC were good stimulators of allogeniec T cells, indicating that DM1 T cells have intrinsically low proliferation capability.

Subjects and Methods

Patients and controls

Heparinized blood (60ml) was drawn from the following groups of individuals:

1. Recently diagnosed DM1 patients (n=22), who visited the outpatient clinics of different hospitals in the Netherlands. The patients were included according to the WHO criteria. The mean age was 16.3 ± 10.6 years, ranging from 4.4 – 39.8 years. The mean of HbA1c was 9.2 ± 2.3%.
2. DM2 patients (n=13), who visited the outpatient clinic of Department of Internal Medicine, Erasmus MC, Rotterdam, The Netherlands. The mean age was 56.6 ± 13.4 years, ranging from 36.4 to 83.3 years. The mean of HbA1c was 7.8 ± 1.4 %.
3. Recently diagnosed patients (n=21) with autoimmune (AI) thyroiditis, who visited the outpatient clinic of Department of Internal Medicine, Medical Center Rijnmond Zuid, Rotterdam, the Netherlands. The mean age was 49 ± 15.8 years, ranging from 17 to 74.1 years. All patients had high levels of thyroid peroxidase autoantibodies (> 400IU/ml).
4. Healthy controls (n=34) with an absent family history of autoimmune diseases, consisted of laboratory personnel and students. The mean age was 34.8 ± 12.7 years, ranging from 19.4 to 60.5 years.

Informed consent was obtained from all participants. The protocol has been approved by the Medical Ethical Committee of the Erasmus MC, Rotterdam, The Netherlands.

Monocyte isolation, generation of dendritic cells and the phenotype of DCs

The used methods for monocyte isolation, generation of dendritic cells and phenotyping of DC have extensively been described in Chapter 4 (Subjects and Methods).

Cytokine production by monocytes

Monocytes were suspended at a density of $0{,}5x10^6$ cells/ml in $RPMI^+$ containing UG, P/S and 10% iFCS and cultured on 24-wells plate (Nunc) or on human plasma fibronectin (FN, 20µg/ml, CLB, Amsterdam, The Netherlands) coated plate for 1hr at 37^0C in a 5%CO_2-95% atmosphere. Thereafter, the FN nonadhered cells were removed by washing two times with ice-cold PBS enriched by 3mM ethylene diamine tetraacetic acid (EDTA), pH 8 (Sigma-Aldrich, Steinheim, Switzerland). The nonadhered cells were counted. The untreated (non-FN) and FN adhered monocytes were cultured with staphylococcus aureus cowan 1 strain (SAC; 1:5000, Calbiochem, La Jolla, CA, USA) and the supernatants were collected after 24 hours for determination of the production of IL-10.

For the IL-12 production monocytes were cultured on 24 wells plate (Nunc) with SAC (1:5000) plus IFN-γ (1000 IU/ml, Biomedical Primate Research Centre, Rijswijk, the Netherlands) for 24 hours.

The quantity of IL-10 and IL-12 productions was measured by using an ELISA as indicated by the manufacturer (IL-10 ELISA Pelikine, CLB, Amsterdam, the Netherlands; IL-12 Eli-pair, Diaclone, Besançon, France).

Cytokine production by DC

DC were placed in 24-wells plates (Nunc) at a concentration of $0.5x10^6$ cells/ml and cultured for 24 hours in $RPMI^+$ and Serum Free medium supplement (Pepro Tech). For the IL-10 and IL-12 production the same culture conditions and ELISA's were used as for the production by monocytes.

Mixed Leucocyte Reaction (MLR).

See for detailed description of the used methods for the mixed leukocyte reaction: Chapter 4 (Subjects and Methods).

T cell skewing

Lymphocytes were isolated after the Percoll gradient as described above. After collecting the interphase, the rest was washed twice with PBS/ 0.1%BSA and the pellet was resuspended in $RPMI^+$ medium and counted. The cells were incubated with $20\mu l/10^7$ cells anti-CD3 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 minutes on ice. A magnetic cell sorting system (auto MACS sorter, Miltenyi Biotec) was used for the selection of CD3 positive T cells. The cells were refrozen until the autologous DC were generated. Autologous DC were cocultured with T cells at a ratio 1:10 for 2 days in flat-bottom 96-wells plate (Nunc). Thereafter the T cells were separated using the auto MACS sorter (Miltenyi Biotec) and additional stimulated in Yssel's medium/2.5% iFCS with 2ng/ml phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich, Zwijndrecht, The Netherlands) and 1µg/ml 4-bromo-calcium-ionophore (Sigma-Aldrich) at a cell concentration of $1x10^6$/ml for 24 hours. The supernatants were collected and frozen at -20^oC until determination of cytokine production. The levels of IL-10, IL-13 and IFN-γ in culture supernatants were determined by ELISA (all Pelikine) according to manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using SPSS version 11.0 for Windows. For differences between the groups the Mann Whitney U-test was used. Paired student's t test was used for comparison of values within the group for non-FN and FN adhered conditions. The values are given in mean $\pm$ standard deviation. P–values lower than 0.05 were considered significant.

Results

IL-10 and IL-12 production by monocytes

As reported in the literature (16) monocytes of healthy controls up regulate their IL-10 production after fibronectin (FN) adherence. Table 1 shows that we confirmed this effect: we found that the IL-10 production rose from 6168 ± 5872 pg/ml to 8547 ± 7031 pg/ml when we compared plastic (non-FN) adhered to FN-adhered monocytes of our healthy controls (means

of three separate series of experiments, n=32, p=0.04, it must be noted that there was a considerable inter-assay variation between the three series of experiments, we explain these considerable inter-assay variations as due to the use of different badges of culture additives, e.g. FCS). We therefore decided to concentrate on the data of FN-adhered monocytes and on comparison of data within each series of experiments.

Figure 1a gives the data of the optimal IL-10 production by FN-adhered monocytes of DM1 patients, DM2 patients and AI hypothyroid patients and their respective healthy controls in the various series of experiments carried out. FN-adhered monocytes of DM1 patients clearly produced lower quantities of IL-10 as compared to such monocytes of healthy controls (series 1) and values of 3679 ± 4467 pg/ml (n=12 patients) vs 8313 ± 4961 pg/ml (n=11 controls, p=0.02) were found respectively (figure 1a). Also when non-FN adhered monocytes were used this difference could be found, yet not statistically significant (n.s.): 3173 ± 3090 pg/ml for DM1s (n=13) vs 6693 ± 4221 pg/ml for healthy controls (n=11, p=0.06).

In the second series of experiments monocytes of DM2 patients were used to study possible influences of minor abnormalities in metabolic, i.e. glucose homeostasis, on the IL-10 production due to insufficient insulin control; the DM2 patients had as our DM1 patients raised HbA1c levels (see patients and methods).

We were unable to find any statistically significant difference in IL-10 production between the FN-adhered monocytes of DM2 patients and those of the healthy controls (Figure 1a) and values of 9457 ± 4914 pg/ml (DM2) vs. 13359 ± 8667 pg/ml (healthy controls) (n.s) were found. Also under non-FN adhered conditions significant differences could not be detected: DM2 patients, n=12, 8679 ± 5506 pg/ml vs. healthy controls, n=10, 11272 ± 7084 pg/ml (n.s.).

In a third series of experiments FN-adhered monocytes of AI hypothyroid patients also produced equal quantities of IL-10 as compared to their respective controls (Fig 1a) and values of 4370 ± 3593 pg/ml (AI hypothyroid patients, n=12) vs. 5153 ± 5545 pg/ml (healthy controls, n=12) (n.s.) were found.

Table 1. IL-10 production (mean ± s.d.) by monocytes of healthy controls

	Numbers	*IL-10 (pg/ml) non-FN*	*IL-10 (pg/ml) FN*	*p-value # non-FN vs FN*
Series 1	n=11	6693 ± 4221	8313 ± 4961	p=0.04
Series 2	n=9	11272 ± 7084	13359 ± 8667	n.s.
Series 3	n=12	2627 ± 3375	5153 ± 5545	p=0.02
All Series	n=32	6168 ± 5873	8547 ± 7031	p=0.04

FN= fibronectin; # paired student's t test

Fig.1 a) IL-10 production by monocytes.
Fibronectin (FN)-adhered monocytes were stimulated with Staphylococcus Aureus Strain Cowan (SAC) for 24 hours for optimal IL-10 production. The IL-10 production of FN-adhered monocytes of healthy controls (n=32), DM1 patients (n=12), DM2 patients (n=12) and autoimmune thyroid patients (AITD, n=13) in three separate series of experiments are shown in box plots with median and quartiles. The difference between DM1 patients and healthy controls was significant (p=0.02, Mann Whitney U test).

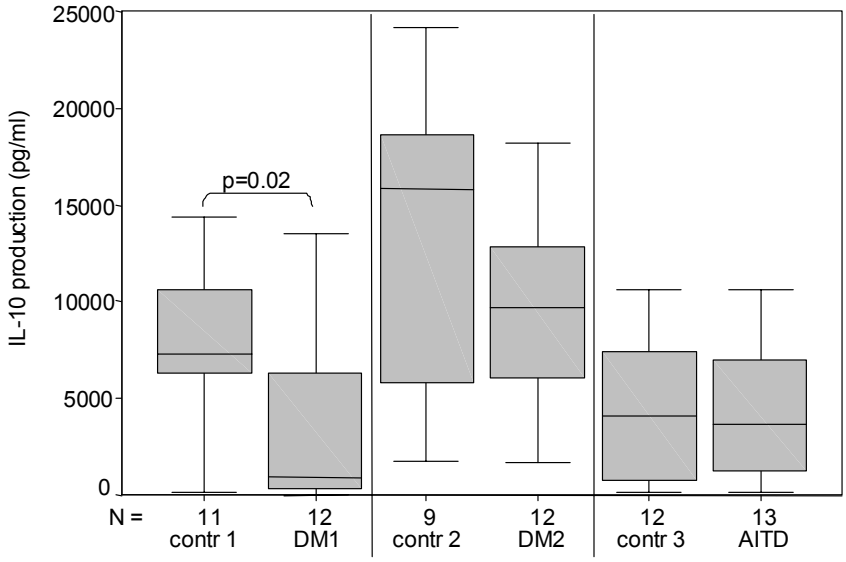
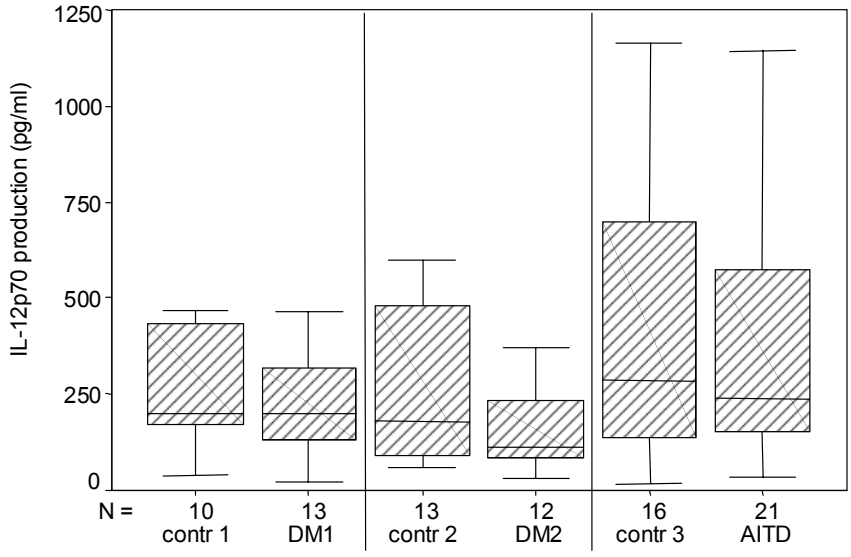


Fig.1 b) IL-12 production by monocytes.
Monocytes were stimulated with SAC plus IFN-γ for 24 hours for optimal IL-12 production. The IL-12 production of the monocytes of healthy controls (n=32), DM1 patients (DM1, n=12), DM2 patients (n=12) and autoimmune thyroid patients (AITD, n=13) in three separate series of experiments are shown in box plots with median and quartiles. There was no difference in the IL-12 production between patient groups and healthy controls.



Also under non-FN adhered conditions statistically significant differences could not be detected: 1517 ± 1600 pg/ml for non-FN adhered monocytes of AI hypothyroid patients (n=21) vs. 2475 ± 3161 pg/ml for healthy controls (n=14) (n.s.).

Figure 1b shows the IL-12 production by the monocytes of the various patient groups tested. Firstly, there was no difference in the IL-12 production between the IL-12 production of non-FN adhered monocytes and FN adhered monocytes (data not shown). Figure 1b therefore represents the values of non-FN-adhered monocytes: The IL-12 production by such monocytes of DM1 patients was not significantly different from that of healthy controls and values of 522 ± 927 vs 329 ± 291 pg/ml were found respectively. Also for the other patient groups (the DM2 and the AI thyroiditis patients) the IL-12 production was comparable to that of the controls (figure 1b).

Phenotype and IL-10 and IL-12 production of monocyte-derived DC

DC were generated from monocytes via standard protocols (culture in the presence of GM-CSF/ IL-4 for 6 days). The yields of DC, measured as the percentage of generated viable cells of the number of original monocytes, were comparable for DM1 patients, DM2 patients, AI thyroiditis patients and healthy controls (data not shown).

Prototypic immature monocyte-derived DC were generated in the case of our healthy controls with up-regulated DC specific markers as DC-SIGN and CD1a and up-regulated MHC class II and costimulatory molecules (Fig. 2).

The production of IL-10 by the monocyte-derived DC of the healthy controls was considerable lower than that of their original monocytes(compare Fig. 1a with Fig. 3a). This has been reported previously (17). The production of IL-12 by the monocyte-derived DC of our healthy controls and their original monocytes was comparable.

With regard to the characteristics of the monocyte-derived DC of the DM1 we firstly confirmed observations of others (10) and showed a hampered and aberrant differentiation of DC from monocytes in DM1. Significantly lower percentages of monocyte-derived DC of 17 DM1 patients were positive for the DC-specific markers DC-SIGN (28 ± 19% vs 54 ± 24% for healthy controls, p=0.04) and CD1a (9 ± 11% vs 27 ± 18%, p=0.003). Also the costimulatory molecules CD80 (5 ± 6% vs 12 ± 10%, p=0.02), CD86 (22 ± 12% vs 32 ± 13%, p=0.04) and CD40 (30 ± 18% vs. 50 ± 21%, p=0.004) came to expression on fewer DC generated from monocytes. The percentages of DC positive for HLA-DR were also lower, yet not statistically significant (42 ± 19% vs 53 ± 18%, p=0.14). The hampered generation of DC was also reflected by lower mean fluorescence intensities (MFI) of these molecules on the monocyte-derived DC: MFI's were significantly lower in DM1 DC in the case of DC-SIGN (1382 ± 1322 vs 3613 ± 1998 in healthy controls, p=0.02), CD1a (232 ± 318 vs 406 ± 332, p=0.04), CD86 (363

± 276 vs 534 ± 319, p=0.05) and CD40 (446 ± 448 vs 916 ± 806, p=0.02). Representative histograms are shown in Figure 2.

The monocyte-derived DC of DM2 patients and AI thyroiditis patients were comparable in marker expression to those of the healthy controls (Fig. 2).

With regard to the T cell accessory function of the monocyte-derived DC of the DM1 patients, the T cell stimulatory capacity in autologous MLR of the phenotypically abnormal DM1 DC was significantly lower. For a DC: T cell ratio 1:10 and 1:20 DM1 T cells showed a significantly lower incorporation of ^{3}H-thymidine (Fig. 4). It must be noted, however, that when the T cells of the DM1 patients were stimulated with the mitogen PHA in the absence of DC, they also showed a lower proliferation as compared to healthy control T cells (Fig. 4, 8568 ± 7508 vs. 22099 ± 13550, p=0.03). Using allogeneic T cells stimulated by DC of DM1 patients, T cells showed a normal proliferative capacity (Fig. 4). Hence the defective stimulation of autologous T cells by DM1 DC is most likely due to an intrinsically low proliferation capability of the T cells, and not to a low stimulatory capability of DM1-DC.

With regard to the IL-10 production of the DM1 monocyte-derived DC: the production of this cytokine was extremely low and almost negligable: 5 ± 14 pg/ml/ 0.5x10^{6} DC in DM1 patients, n=9, vs. 22 ± 49 pg/ml in healthy controls, n=14, series 1. This difference between healthy controls and DM1 patients was however not statistically significant (p=0.08) (Fig. 3a).

The quantity of IL-12 produced by the monocyte-derived DC of DM1 patients was statistically significantly lower than that of the healthy control DC: 14 ± 25 pg/ml in patients, n=8, vs. 285 ± 381 pg/ml in series 1, n=9 (p=0.04) (Fig. 3b).

The monocyte-derived DC of DM2 and AI thyroiditis patients produced equal quantities of IL-10 and IL-12 as those of the healthy controls (Fig. 3).

T cell skewing capacity of monocyte-derived DC of DM1 patients

Because of the abnormal IL-10 and IL-12 production of type 1 diabetic monocyte-derived DC we studied their T cell skewing capacities and co-cultured monocyte-derived DC of DM1 patients with autologous T cells for 2 days. Thereafter we stimulated T cells purified from such co-cultures with PMA and calcium ionophore for an additional 24 hours to maximize the intrinsic cytokine production acquired by the interaction with the DC. In accord with the low IL-12 production capability of the DM1 DC we found that the IFN-γ production of the T cells was lower as compared to that of the T cells stimulated by healthy control DC, i.e. values of 5730 ± 2681 pg/ml vs 9796 ± 8240 pg/ml were found respectively, but differences did not reach statistical significance (Fig. 5).

With regard to Th2-type cytokines produced by the DC-stimulated T cells: the IL-10 production of the DM1 DC-stimulated T cells was lower as compared to that of the T cells stimulated by healthy control DC (12 ± 8 pg/ml, n=5, vs 40 ± 40 pg/ml, n=7 respectively,

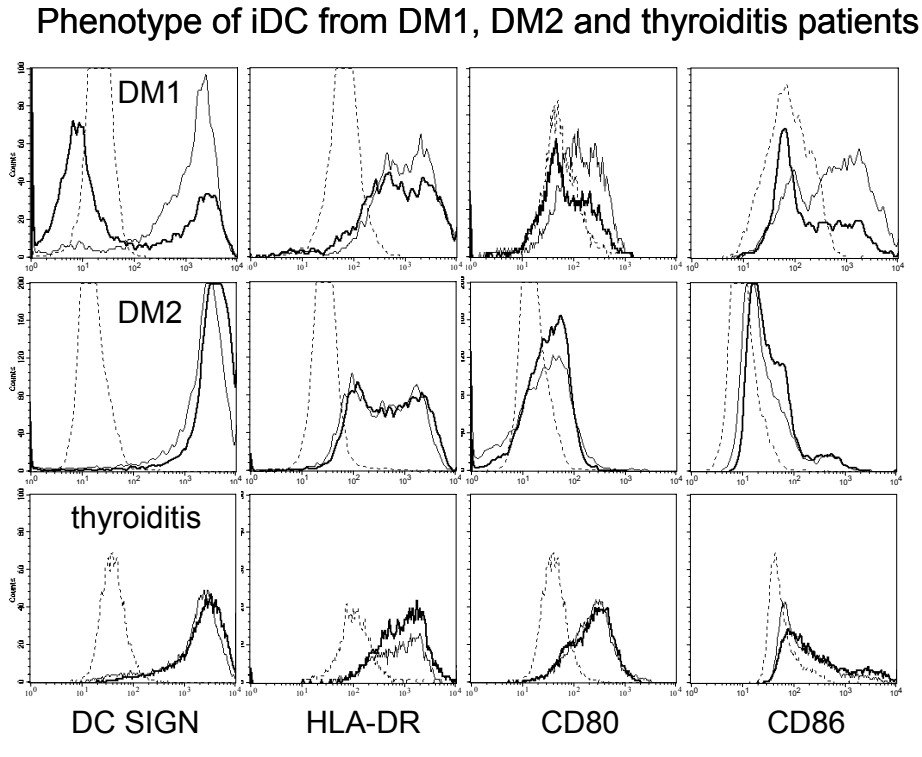
p=0.10, Fig.5). The IL-13 production was also slightly decreased: 23 ± 10 pg/ml vs. 32 ± 19 pg/ml (Fig.5), but again not statistically significant.

Discussion

Our data show that the IL-10 production capability of DM1 monocytes is diminished as compared to that of monocytes of DM2 patients and of healthy controls. Whether the polymorphisms reported for the IL-10 gene and linked to a proneness for auto-immunity (18-20) are linked to this diminished monocyte IL-10 production capability needs further study.

In a previous study on cytokine production by DM1 leukocytes a diminished production of IL-10 has been observed as well (21). However, the IL-10 production was measured in this study for unselected and PHA-stimulated peripheral blood mononuclear cells. Hence data were interpreted as due to a low production of IL-10 by DM1 T cells. Our data show that IL-10 production defects are not only detectable at the level of the T cells, but also at the level of the circulating monocyte pool.

Fig. 2: Phenotype of monocyte-derived DC. Representative examples of the phenotypes (DC specific markers and costimulatory molecules) of monocyte-derived DC of a patient with DM1, a patient with DM2 and an AI thyroiditis patient. The phenotypes are shown as histograms of the fluorescence intensities of the markers. Dashed lines represent DC stained with an isotype control Ab (negative staining), thick lines represent patient DC stained with the specific Ab, thin lines represent DC from a healthy control subject (matched for age) stained with the same specific Ab. Note that the DC of the DM1 patient has a lower expression of DC-SIGN, CD80, CD86, CD40 and CD1a. DC of DM2 patients and AI thyroiditis patients are comparable to those of healthy controls.



One of the major effects of IL-10 is the suppression of the production of pro-inflammatory and T helper (Th)1 related cytokines from lymphocytes and monocytes/ macrophages: IL-10 suppresses the production of interferon (IFN)-γ and IL-2 from Th1 cells and it also suppresses the production of IL-1, IL-6, TNF-α and G(M)-CSF from monocytes and macrophages (11,22-24).

The "pro-inflammatory" functions of antigen presenting cells (APC) are inhibited by IL-10 as well: the cytokine freezes the DC in their immature form by downregulating the expression of costimulatory molecules (25,26). IL-10 also inhibits the production of IL-12 by DC (25,26).

Our finding of a lower production of IL-10 by monocytes of DM1 patients is thus in agreement with the previously reported increased production rates of pro-inflammatory factors and cytokines (PGE2, TNF-α, IL-1β and IL-6) by monocytes and macrophages of DM1 patients, the increased expression of the inducible enzyme cyclo-oxygenase-2 by such cells and the higher levels of pro-inflammatory and Th1 type cytokines in the serum of DM1 patients (27-30). Collectively, these data and our data on a low IL-10 production by monocytes argue for of a shift in the cytokine balance in DM1s to a pro-inflammatory set point.

However our other findings reported here on the diminished IL-12 production by DM1 monocyte-derived DC show that such a view might be too simplistic. A complex role for the pro-inflammatory type cytokine IL-12 has been found before in the pathogenesis of DM1. Although IL-12 administered *in vivo* accelerates diabetes in NOD mice by inducing a massive infiltration of lymphocytes in the islets of Langerhans (31), neutralization of IL-12 by a monoclonal antibody *in vivo* has more complex effects in this diabetic animal model. When administered from 5 to 30 weeks of age, NOD mice exhibit a suppression of both insulitis and diabetes. In contrast, when injected into 2-week-old female NOD mice for 6 consecutive days, all mice showed a diminished IL-2 production, yet an enhancement of insulitis and diabetes. This suggests that depletion of endogenous IL-12 at a young age weakens tolerance induction in the NOD mouse model and that a sufficient IL12 production at young age is necessary to prevent or delay diabetes development (32). Although the low IL-12 production capability of DM1 monocyte-derived DC is thus in contradiction to a general shift towards pro-inflammatory / Th1 type mechanisms in DM1, it is in agreement with the previously described and here-confirmed findings on a hampered differentiation of DC from DM1 monocytes (9,10). Also in this series of experiments DC derived from DM1 monocytes showed an immature phenotype with a reduced expression of the DC specific markers DC-SIGN and CD1a and of the costimulatory molecules CD80, CD86 and CD40. The aberrant immature characteristics in the marker expression and cytokine production of the DM1 DC had their consequences for T cell stimulation and skewing. This report shows that autologous T cells stimulated by these DC had

a lower ability to proliferate and to produce both Th1 type cytokines (i.e. IFN-γ) as well as Th2 type cytokines (i.e. IL-10 and IL-13).

How is such an aberrant differentiation, a low IL-12 production and a poor T cell stimulation of DM1 monocyte-derived DC related to an enhanced β-cell auto-sensitization? Comparisons to the animal models of spontaneously developing autoimmune diabetes might be helpful to answer this question. Interestingly almost similar DC differentiation aberrancies exist in the NOD mouse and the BB-DP rat (4,6,33,34). In these animal models the poorly differentiated DC affect tolerance induction via a poor expansion of suppressor $RT6^+CD8^+$ T cells (BB-DP rat) or via a poor induction of Activation Induced T Death (AITCD) (NOD mouse). Also the above-cited (32) short-term treatment with the anti-IL-12 antibody in the young NOD mouse inhibiting IL-2 production was taken as an indication that the expansion and apoptosis of pathogenic T-cells was affected, resulting in the acceleration of autoimmune diabetes. It is tempting to speculate that similar mechanisms occur in DM1 patients. Perhaps the very low IL-10 production by the DM1 monocytes might play an additional role. A high levels of IL-10 in the context of APC has been reported to induce anergic and regulatory T cells (35,36).

Have our findings consequences for the treatment of DM1? On the basis of the here reported data a correction of the defective IL-10 production by monocytes might be envisaged. Administered in vivo IL-10 has clear immune suppressive effects. Systemic IL-10 administration inhibits the production of TNF-α and IL-1 and the expression of HLA-DR by monocytes and it shifts the Th1/ Th2 response in favour of the latter (13,37,38). In mouse models of DM1 and collagen induced rheumatoid arthritis systemic IL-10 administration prevents the progression of disease (37-39). In addition, treatment with viral IL-10 vectors have been shown to prevent disease in the NOD mouse model successfully (40,41). In humans, clinical trials with recombinant human IL-10 improve symptoms of rheumatoid arthritis, Crohn's disease and psoriasis (23,42-44).

However our here reported data and the above-cited ones of others (32) show that the timing of an IL-10 treatment is probably of importance: if IL-12 is important in the very early phases of the disease to eradicate pathogenic T cells, an IL-10 treatment should not be given too early to counteract the early beneficial effects of IL-12 (perhaps even an immune stimulation is indicated in these stages). It must also be noted that the adverse side effects of systemic IL-10 treatment are considerable (23). The approach to transfect monocytes transiently with viral IL-10 genes (45) might be viewed as a comprehensive late approach in the disease to restore the here-described IL-10 production defect of DM1 monocytes.

In a previous study (46) we found that monocytes of AI thyroiditis patients had a lower capability to arrange their cytoskeleton when stimulated with a chemo-attractant, this lower capability can also be found in DM1 (9).

Fig. 3: a) IL-10 production by monocyte-derived DC.
DC (6 days of culture with GM-CSF and IL-4) were collected, resuspended and stimulated with SAC for 24 hours. The IL-10 production of DC of healthy controls (n=14, n=9) and n=11 for the three respective separate series of experiments, of DM1 patients (n=9), DM2 patients (n=11) and AITD patients (n=11) was determined. Box plots with median and quartiles are shown. Note the lower IL-10 production of DC as compared to monocytes (Fig. 1a). The difference between DM1 patients and healthy controls was not yet significant (p=0.08, Mann Whitney U test).

A.

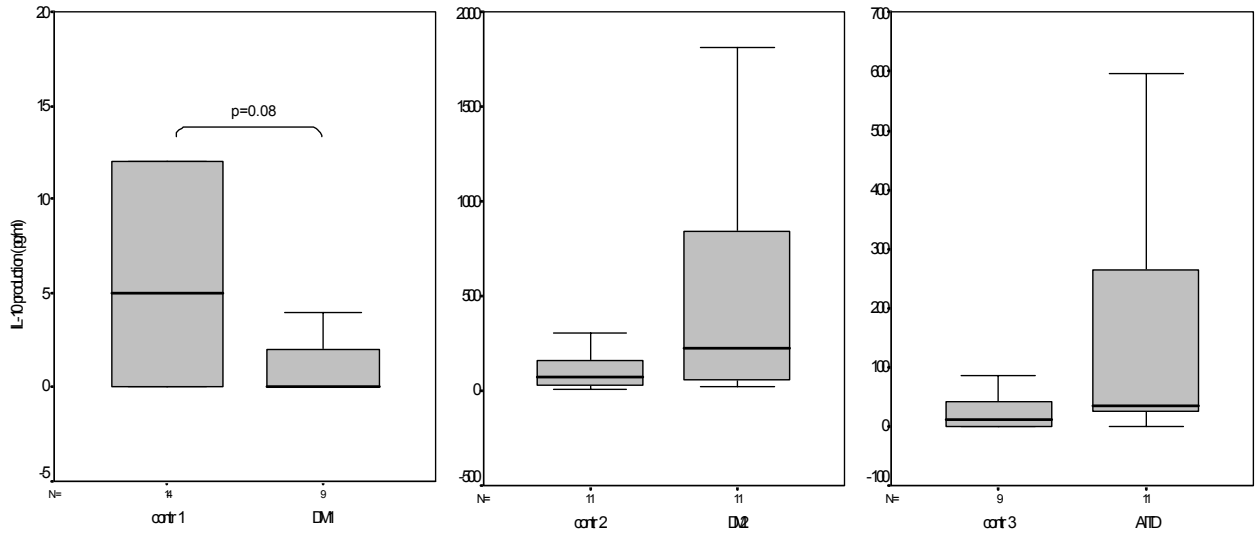
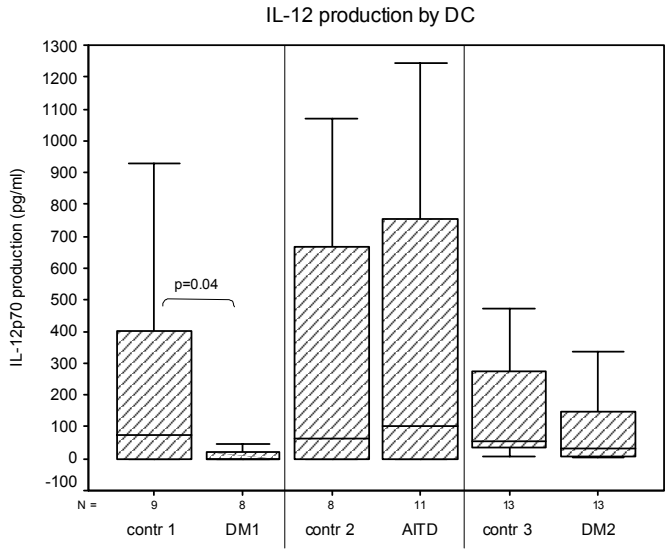


Fig. 3 b) IL-12 production by monocyte-derived DC.
The IL-12 production (SAC plus IFN-γ stimulated for 24 hours) by DC of the same groups as mentioned in figure 3a. DC of DM1 patients produced significant reduced quantities of IL-12 (p=0.04).

B.



In addition monocytes of thyroid autoimmune and DM1 patients share a diminished capability to differentiate into veiled accessory macrophages, particularly when fibronectin-adhered monocytes were used (9,46). However in this study we show that monocytes of AI

thyroiditis patients did not show the same defect in IL-10 production as the DM1 monocytes. They also had a normal generation of DC from their monocytes. Apparently monocytes and monocyte-derived APC of DM1 patients show a wider spectrum of aberrancies as compared to monocytes and monocyte-derived APC of AI thyroiditis patients.

In conclusion, we observed that monocytes of DM1 patients had a lower capability to produce IL-10 as compared to monocytes of healthy controls and DM2 patients. The IL-12 production by DM1 monocytes was normal. We confirmed that the generation of DC from monocytes was hampered in DM1 patients and such monocyte-derived DC hardly produced IL-10 and were poor producers of IL-12. This resulted in an aberrant T cell stimulating capacity of the cells, T cells stimulated by such DC were poor proliferators and poor producers of both Th1 cytokines (IFN- γ) and Th2 cytokines (IL-13 and IL-10).

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Fig. 4. T cell accessory function of DC of DM1 patients (grey triangles) and DC of healthy controls (white circles). Autologous T cells (left picture) or pooled T cells from three different allogeneic donors (right picture) were used. T cells stimulated by various concentrations of DC, measured by ^{3}H-thymidine incorporation and expressed as counts per minute (cpm)(vertical axis). The value of each individual is the mean of an experiment in triplicate. Data are shown as means and standard errors of experiments.
Note that DC of DM1 patients induce a lower autologous T cell stimulation as compared to the DC of healthy controls. However the T cells of DM1 patients also have a significantly lower proliferation to PHA (the positive control stimulus) compared to the proliferation of healthy control T cells. * represents p=0.03. DM1 DC are as good as control DC in stimulating proliferation of allogeneic T cells.

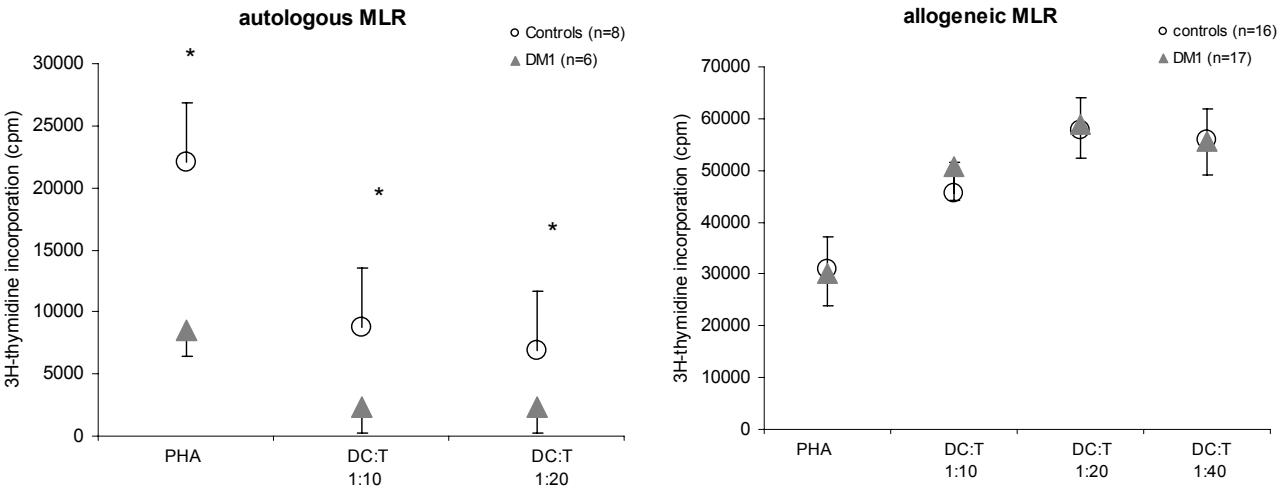
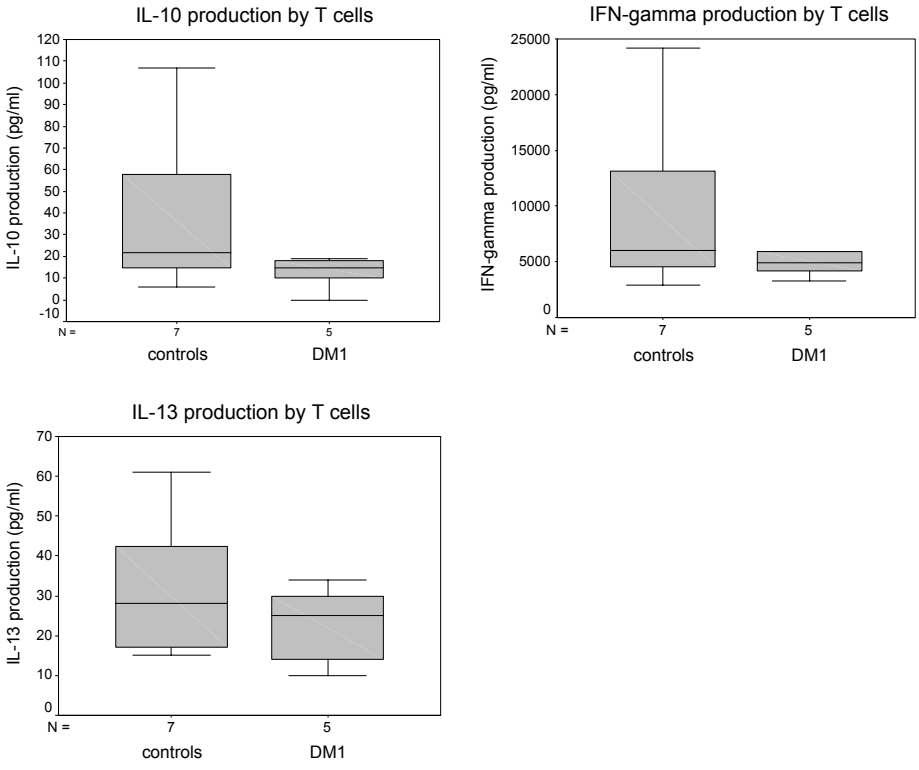


Fig. 5. T cell skewing capacity of monocyte-derived DC. The IL-10, IL-13 and IFN-γ production by T cells after they have been in MLR with autologous DC (1:10) for 2 days, isolated and stimulated with PMA and calcium ionophore for an additional 24 hours. The IL-10, IL-13 and IFN-γ production are given in pg/ml of healthy control DC and T cells (n=7) and those of DM1 patients (n=5). Box plots with median and quartiles are shown. Differences do not reach statistical significance.



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CHAPTER 6

ABERRANCIES IN MONOCYTES AND MONOCYTE-DERIVED DENDRITIC CELLS IN FIRST DEGREE RELATIVES OF TYPE 1 DIABETIC PATIENTS ARE MIRROR IMAGES OF THOSE FOUND IN OVERT TYPE 1 DIABETES.

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Submitted for publication

Summary

Dendritic cells (DC) are the initiators of naïve T cell responses and involved in the initiation of the islet autoimmune response in type 1 diabetes (DM1). DC are capable of skewing T cell responses (Th1 or Th2) and to induce T cell tolerance. There exists a hampered differentiation of DC from monocytes in DM1. The precursor monocytes also show various aberrancies.

Our aim was to study monocyte and DC abnormalities in first-degree relatives (FDR) of DM1 patients. We studied the expression of HLA-DR, CD1a, CD80, CD86, CD40, and CD54 on monocyte-derived DC of 13 FDR. One of the FDR was islet cell antibody positive. In addition we studied the T cell stimulatory capacity and the interleukin (IL)-10 and IL-12 production of monocytes and DC. Serum levels of soluble (s) ICAM-1 were studied as well. Outcomes were compared to those of 39 recent-onset DM1 patients and 24 healthy controls.

Like DM1 patients the FDR had increased serum levels of sICAM-1, yet the FDR did not show the lower monocyte CD54 expression, the lower monocyte IL-10 production, the hampered DC development from monocytes and the lower DC function found in recent-onset DM1 patients. On the contrary, DC development from monocytes was stimulated in the FDR while their monocytes and monocyte-derived DC showed an increased IL-10 production.

In conclusion, low risk FDR of DM1 patients show various aberrancies of monocytes and monocyte-derived DC, which are mirror images of those found in recent-onset DM1 patients.

Introduction

Soluble intercellular adhesion molecule (sICAM)-1 levels are increased in the serum of DM1 patients (1-3) and these high levels of sICAM-1 are assumed to be due to an increased shedding of this adhesion molecule from activated endothelium (4). Indeed adhesion molecule expression is increased on endothelial cells of islet blood vessels in DM1 (5-7). Such higher expression plays a role in the diapedesis of leukocytes from the blood stream into the islets.

An interaction of these infiltrated leukocytes with connective tissue and adhesion molecule positive islet epithelial cells subsequently leads to an accumulation and activation of the infiltrated leukocytes. With regard to the integrin expression on leukocytes we recently showed a reduced expression of the adhesion molecule CD54 (=ICAM-1) specifically on monocytes and monocyte-derived dendritic cells of DM1 patients. Signalling via CD54 is necessary for a full differentiation and maturation of DC. We therefore speculated that the reduced expression of CD54 on monocytes and DC is causal -at least in part- to the earlier found hampered differentiation and maturation of DC from monocytes in DM1. In the animal models the hampered differentiation and maturation of DC from precursors is already present in the pre-diabetic phase prior to the islet auto-antibody production and the insulitis, indicating that this disturbance is rather a cause than a consequence of the autoimmune process.

The reduced expression of CD54 on monocytes and monocyte-derived DC and the reduced ability of monocytes to differentiate into DC are not the only abnormalities of the monocyte-DC compartment in DM1. There are various reports on a raised production of IL-1, TNF-α and other pro-inflammatory cytokines and factors by monocytes of DM1 patients (8-10). We recently extended these findings on an abnormal cytokine production of diabetic monocytes and showed that monocytes of patients produced lower quantities of the immune-suppressive cytokine IL-10.

Since abnormalities in the monocyte-DC system can already be detected in the animal models of type 1 diabetes in the pre-diabetic phase, we asked ourselves the question whether first degree relatives (FDR) of DM1 patients already show such abnormalities. We therefore evaluated the serum sICAM-1 level, the DC development from monocyte-precursors, the T cell stimulatory capacity of the DC, the IL-10 and the IL-12 production of monocytes and monocyte-derived DC and the CD54 expression on these cells in 13 FDRs and 24 healthy controls. We compared outcomes with findings in 39 recently-diagnosed cases of type 1 diabetes.

Subjects and Methods

Subjects

Family members of DM1 patients were asked to participate in the study at the time of diagnosis of the DM1 proband. Informed consent was obtained from all participants and the protocol has been approved by the Medical Ethical Committee of the Erasmus MC, Rotterdam, The Netherlands. We collected 13 first degree relatives, with no clinical signs of disease. Their mean age was 29.2 years, standard deviation (s.d.) 16.6 years (range from 5.3 to 49 years). Data were compared to those of 39 recently diagnosed DM1 patients including the index cases, these data have been published separately elsewhere. The patients had a mean age of 28 years, s.d. 6.6 years (range from 19.4 to 42.5). All patients were treated with insulin. The mean of their glycosylated haemoglobin level was 9.2% ± 2.3% (s.d.). We also included two groups of healthy controls: a control group for the FDR (a total of n=24) with a mean age of 36.1 ± s.d. 12.1 years, (range from 21.7 to 60.5) and a control group for the DM1 patients with a mean age of 13.7 ± s.d. 6.5 years (range 6.5 to 33.2). Venous heparinized and non-heparinized blood was obtained from patients, FDR and healthy controls.

Serum

Serum samples were collected and stored at -80^{0}C until the analysis for soluble (s) ICAM-1. For this determination two separate commercially available ELISA methods were used. For the patients and their controls we had used the Bio-source ELISA KH5401; for the FDR and their controls we used a newer, more sensitive assay: the Bio-source KH5412 high sensitive, (BIO-source, Camarillo, CA, USA).

In the serum of the FDR we also determined the presence of islet cell antibodies (ICA). These antibodies were detected with a standard indirect immunofluorescence method using blood group O human pancreas.

Monocyte isolation and generation of dendritic cells

The Ficoll (Pharmacia, Uppsala, Sweden; density 1.077 g/ml) and Percoll (Pharmacia; density 1.063 g/ml) density gradient centrifugation were used to isolate monocytes from heparinized blood. The monocytes were cultured at a concentration of 0.5x10^6 cells/ml on 24-wells culture plates (Nunc, Rosklide, Denmark) for six days under plastic-adherent conditions in RPMI 1640 with 25mM HEPES and L-glutamine (BioWhittaker Europe, Verviers, Belgium) (hereafter referred to as RPMI$^+$) containing ultraglutamine (UG) (2mM, BioWhittaker), penicillin/ streptomycin (P/S) (100U/ml penicillin, 100µg/ml streptomycin, BioWhittaker) and 10% inactivated FCS (iFCS) (BioWhittaker) in the presence of GM-CSF 400U/10^6 cells and IL-4 500U/10^{6}cells (both Pepro Tech EC, London, England). The cells were incubated at 37^{0}C and 5% CO_2. On day three, half of culture fluid was refreshed with GM-CSF 400U/10^{6}cells, IL-4 500U/10^{6}cells. After six days DC were collected by resuspending and washing the wells

thoroughly with cold phosphate buffered saline (PBS) pH 7,4 (BioWhittaker), with 0,1% bovine serum albumin (BSA) (Bayer, Kankakee, IL, USA) and 3mM ethylene diamine tetraacetic acid (EDTA), pH 8 (Sigma-Aldrich, Steinheim, Switzerland). The cells were counted with 0.1% trypan blue (Sigma Chemical co, St Louis, USA) to assess cell viability.

Phenotype of monocytes and DC

The following monoclonal antibodies (mAbs) were used for flowcytometry: anti-IgG1 Fluorescein isothiocyanate (FITC, 1:10, Becton Dickinson (BD), San Jose, CA, USA), anti-IgG1 Phycoerythrin (PE, 1:10, BD), anti-CD14 FITC (1:250, Beckman Coulter, Hialeah, FL, USA), DC-SIGN PE (1:10, R&D systems, Minneapolis, MN, USA), anti-HLA-DR PE (1:200, BD), anti-CD80 PE (10µl/10^5 cells, BD), anti-CD86 FITC (10µl/10^5 cells, Pharmingen, San Diego, USA), anti-CD1a PE (1:100, Beckman Coulter), anti-CD40 FITC (10µl/10^5 cells, Serotec, Oxford, England), anti-CD54 PE (1:4, BD), anti-CD3 FITC (1:20, BD), anti-CD19 PE (1:25, BD) and 7AAD (1:250, Molecular probes, Eugene, Oregon, USA). For monocytes quadruple staining was performed (anti-CD14 allophycocyanin (APC), 1:25 BD) and gated on 7AAD negative and CD14 positive population. Cell suspensions were incubated in polypropylene tubes with mAbs for 15 minutes, then washed twice with PBS/ 0,1% BSA. The cells were measured immediately following cell staining using a FACScan flowcytometer and analyzed using CellQuestPro (BD, Mountain View, CA, USA). Routinely 10,000 events were collected. Debris and dead cells were gated out on basis of their light scatter properties. In addition, we stained the cells with trypan blue and 7AAD. The gated population consist no $CD3^+$ and $CD14^+$ cells. Data were expressed as mean ± s.d. of percentage of positive cells and mean ± s.d. of mean fluorescence intensity (MFI). The values of IgG isotype controls were subtracted.

Mixed Leucocyte Reaction (MLR).

Lymphocytes were isolated after the Percoll gradient as described above. After collecting the interphase, the rest was washed twice with PBS/ 0.1% BSA and the pellet was resuspended in $RPMI^+$ medium and counted. The cells were incubated with 20µl/10^7 cells anti-CD3 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 minutes on ice. A magnetic cell sorting system (auto MACS sorter, Miltenyi Biotec) was used for the selection of CD3 positive T cells. The cells were refrozen until the autologous DC were generated. DC were irradiated with 20Gy and placed in flat-bottom 96-wells-plates (Nunc, Roskilde, Denmark) in $RPMI^+$ medium containing UG, P/S and A^+-serum. We added 100µl of the concentration of $3x10^5$, $1.5x10^5$, $0.75x10^5$ cells/ml of DC and an equal volume of autologous T-cells at concentration of $15x10^5$ cells/ml to the wells resulting in a total volume of 200µl per well. As positive control these T-cells were stimulated with phytohemagglutamin (PHA) (Sigma). Proliferation was determined after 18 hours of 0.5µCi/ well ^{3}H-thymidine addition on day 5.

Cells were harvested on filter papers and radioactivity was counted in a liquid scintillation analyzer (LKB Beta plate, Wallac, Turku, Finland). The values are the mean of triplicates.

Cell culture for cytokine production

Monocytes were suspended at a density of $0{,}5x10^6$ cells/ml in $RPMI^+$ containing UG, P/S and 10% iFCS and cultured on 24-wells plate (Nunc) or on human plasma fibronectin (FN, 20µg/ml, CLB, Amsterdam, The Netherlands) coated plate for 1hr at 37^0C in a 5%CO_2-95% atmosphere. Thereafter, the FN nonadhered cells were removed by washing two times with ice-cold PBS enriched by 3mM ethylene diamine tetraacetic acid (EDTA), pH 8 (Sigma-Aldrich, Steinheim, Switzerland). The nonadhered cells were counted. The untreated (non-FN) and FN adhered monocytes were cultured with staphylococcus aureus cowan 1 strain (SAC; 1:5000, Calbiochem, La Jolla, CA, USA) and the supernatants were collected after 24 hours for determination of the production of IL-10. For the IL-12 production monocytes were cultured on 24 wells plate (Nunc) with SAC (1:5000) plus IFNγ (1000 IU/ml, Biomedical Primate Research Centre, Rijswijk, the Netherlands) for 24 hours. DC were placed in 24-wells plates (Nunc) at a concentration of $0.5x10^6$ cells/ml and cultured for 24 hours in $RPMI^+$ and Serum Free medium supplement (Pepro Tech). For the IL-10 and IL-12 production the same culture conditions were used as for the production by monocytes.

Cytokine determination

The quantities of IL-10 and IL-12 productions by monocytes and DC were measured by using an ELISA as indicated by the manufacturer (IL-10 ELISA Pelikine, CLB, Amsterdam, the Netherlands; IL-12 Eli-pair, Diaclone, Besançon, France).

Statistics

Statistical analysis was carried out using SPSS version 11.0 for Windows. For differences between the groups the Mann Whitney U-test was used. Paired student's t test was used for comparison of values within the group for non-FN and FN adhered conditions. The values are given in mean ± standard deviation. P–values lower than 0.05 were considered significant.

Results

Soluble ICAM-1 levels in serum and monocyte expression of adhesion molecules

Figure 1a shows that the sICAM-1 levels in serum were significantly increased in 11 ICA negative FDR tested (mean 389 ± s.d. 132 ng/ml) as compared to the levels in 20 healthy controls (286 ± 101 ng/ml, p=0.01). Also the ICA positive relative had an increased serum sICAM-1 level (444 ng/ml).

In a separate and previous series of experiments (using another ELISA for sICAM-1) recently-diagnosed DM1 patients also had significantly increased levels of sICAM-1 in their serum as compared to the levels found in healthy controls (62 ± 21 ng/ml in 40 patients vs. 40 ± 15 ng/ml in 39 controls, p<0.0001) (Fig. 1b).

We previously showed a reduced CD54 expression in DM1 patients (Lam-Tse et al., submitted). In this series of experiments on FDR we double stained peripheral blood mononuclear cells for CD14 and several adhesion molecules to investigate whether the expression of such monocytes was abnormal in monocytes of FDR. As shown in table 1 and fig. 2, we did neither find differences in expression levels of CD54, nor in those of CD29, CD11b and CD18 between monocytes of 8 tested FDR and 8 of age-matched healthy controls.

Differentiation of DC from monocytes

Figure 3 shows that the generation of DC from monocytes of 12 tested ICA negative FDR resulted in cells with a higher expression of (co)-stimulatory molecules as compared to the DC differentiated from monocytes of healthy controls: The monocyte-derived DC of the ICA negative FDR had a significantly higher expression of CD86 as compared to 12 healthy control DC (mean fluorescent intensity (MFI) of 250 ± 60 (s.d.) in FDR vs. 170 ± 72 in healthy controls, p=0.007).

Fig. 1. Serum levels of sICAM-1 (ng/ml) are shown for **(a)** 11 ICA negative FDR and 20 healthy controls and **(b)** 39 DM1 patients and 24 healthy controls. FDR and DM1 have an increased serum sICAM-1 compared to their controls (p=0.01 and p<0.0001, resp.). Box plots with median and quartiles are shown. The difference in the mean between (a) and (b) is due to the use of two different assays (see methods).

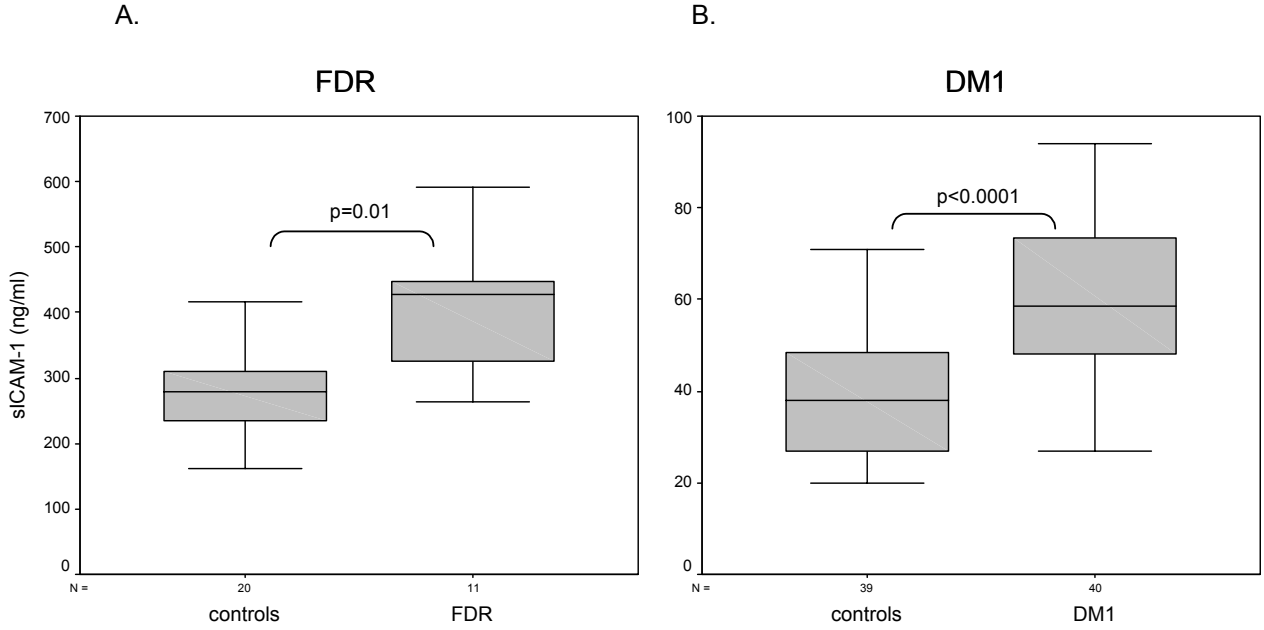
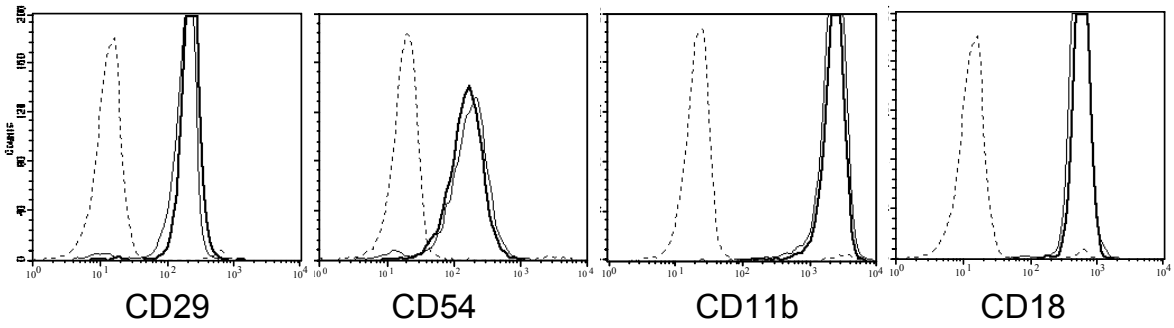


Table 1. Adhesion molecule expression on $CD14^+$ monocytes (mean ± s.d.)

	CD29	CD54	CD11b	CD18
% of cells positive				
Healthy controls	96 ± 3	88 ± 11	98 ± 1	97 ± 2
FDR	97 ± 1	81 ±23	98 ± 1	98 ± 1
MFI of the cells				
Healthy controls	180 ±25	145 ± 42	2319 ± 615	498 ± 100
FDR	202 ± 45	121 ± 54	2428 ± 224	513 ± 99

n=8 each group, all values not significantly different
FDR= first degree relatives, MFI= mean fluorescence intensity

Fig. 2. Representative histograms of adhesion molecule expression on CD14+ monocytes are shown of 8 FDR and 8 healthy controls. FDR is represented in thick lines, healthy controls in thin lines and the isotype in dashed lines. The expression of the adhesion molecules CD29, CD54, CD11b and CD18 on monocytes are comparable between FDR and controls.



Moreover, the expression of HLA-DR on the DC of the ICA negative FDR was increased too, yet not statistically significantly (753 ± 270 in FDR vs. 600 ± 214 in controls, p=0.16). The CD54 expression was similar to that of DC generated from healthy control monocytes (Fig. 3).

The data in the ICA negative FDR contrast to our findings in recently-diagnosed DM1 patients. Monocyte-derived DC of 17 tested recently-diagnosed DM1 patients had a significantly lower expression (MFI) of the costimulatory molecule CD86 (363 ± 276 vs. 534 ±319, n=17, p=0.05) and of the adhesion molecule CD54 (609 ± 487 vs. 941 ± 435, p=0.02). In general DC maturation from monocytes was hampered in DM1 patients and a lower expression of the DC specific marker CD1a (232 ± 318 vs. 406 ± 332, p=0.04) was found too (Fig. 3). These abnormal expressions were specific for type 1 diabetes and were not found in DM2 patients (data not shown).

The one single FDR positive for ICA had monocyte-derived DC with a lower CD86 expression, yet a higher HLA-DR and normal CD54 expression.

Fig. 3. Mean fluorescence intensities are shown for various markers on monocyte derived DC from **(a)** 12 FDR and 12 healthy controls **(b)** 17 DM1 patients and 17 healthy controls. Means and standard errors are given. DC of FDR have an increased HLA-DR (p=0.16) and CD86 expression (p=0.007). DM1 patients had a significantly reduced expression of CD86 (p=0.05), CD40 (p=0.02), CD1a (p=0.04) and CD54 (p=0.02).

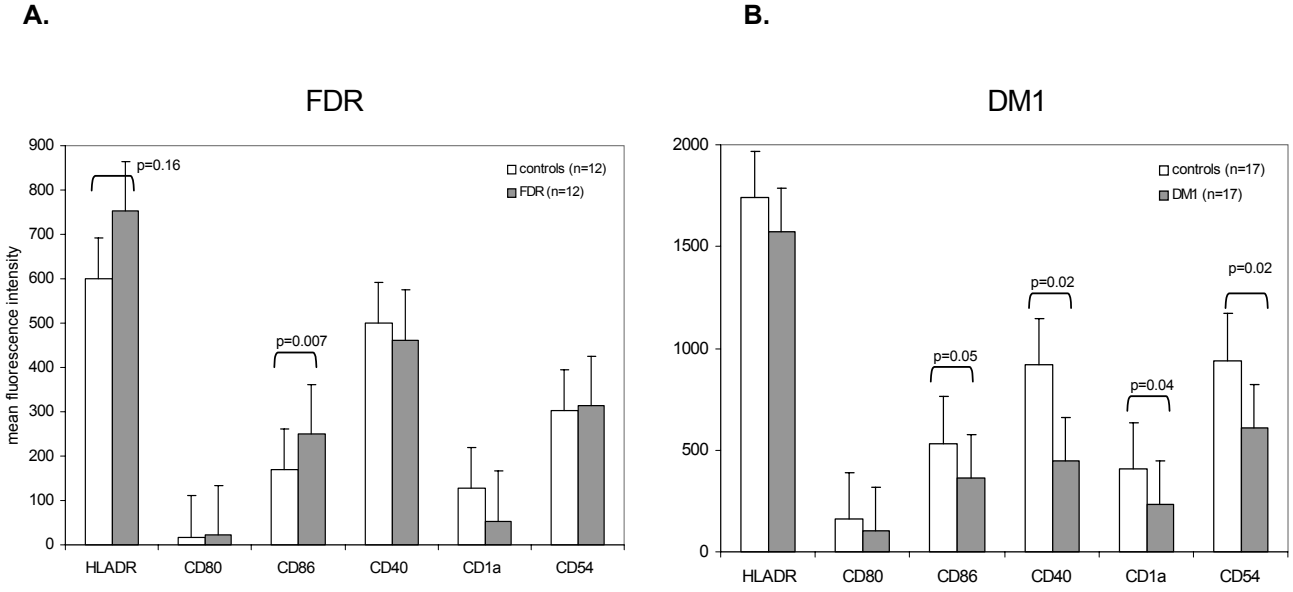
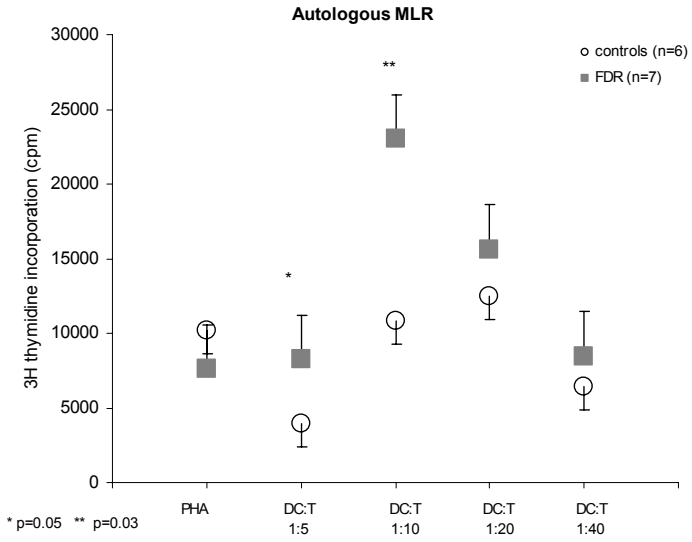


Fig. 4. The T cell stimulatory capacity (i.e. proliferation: 3H-thymidine incorporation) in the autologous mixed leucocyte reaction (MLR) of DC of controls (n=6, white circles) and FDR (n=7, grey squares). For the DC:T cell ratio's 1:5 and 1:10, DC of FDR induce a significantly higher T cell stimulation compared to controls (* p=0.05 and ** p=0.03). Means count per minute (cpm) and standard errors are given of the indicated conditions.



The stimulatory capacity of DC for autologous T cells

To assess the T cell stimulatory capacity of DC, DC were co-cultured with autologous T cells in various ratio's (Fig. 4). We found a significantly increased T cell proliferation measured by ^{3}H-thymidine incorporation in 7 tested FDR compared to 6 healthy controls for the DC:T cell

ratio of 1:5 (FDR 8245 ± 3278 cpm vs. controls 3982 ± 2155 cpm, p=0.05) and 1:10 (FDR 23018 ± 9734 cpm vs. controls 10848 ± 7593cpm, p=0.03).

IL-10 and IL-12 production of monocytes

Figure 5 shows the IL-10 and IL-12 production of the monocytes of 11 ICA negative FDR and of the recently-diagnosed DM1 patients as an IL-10 / IL-12 production ratio. The IL-10 / IL-12 production ratio of the monocytes of these FDR was increased, and values of 579 ± 678 were found for FDR (n=10) vs. 284 ± 722 for healthy controls (n=11), yet statistical significance was not reached (p=0.07) (Fig. 5a). This effect was mainly due to an increased IL-10 production by monocytes of the FDR (76939 ± 54767 pg/ml, n=10 vs. 35095 ± 42815 pg/ml, n= 11, p=0.09). The monocytes of the one single FDR positive for ICA had a low IL-10 production of 13684 pg/ml.

The data in the ICA negative FDR contrast again to our findings in the recently-diagnosed DM1 patients. The monocytes of the DM1 patients had a significantly decreased monocyte IL-10 / IL-12 ratio as compared to healthy control monocytes. DM1 patients (n=12) had a mean IL-10 / IL-12 ratio of 18 ± 30 as compared to a value of 33 ± 34 in appropriate healthy controls (n=9), p=0.05 (Fig. 5b). This was mainly due to a lowered IL-10 production and values of 3679 ± 4467pg/ml in 12 patients and 8313 ± 4961 pg/ml in 11 controls were found (p=0.02).

IL-10 and IL-12 production of the monocyte derived DC

Figure 6 shows the IL-10 and IL-12 production of the monocyte derived DC of 24 healthy controls, 12 ICA negative FDR and 9 recently-diagnosed DM1 patients. Firstly it must be noted that the capability of the monocyte-derived DC to produce IL-10 was markedly reduced as compared to that of the original monocytes, in some DM1 patients the monocyte-derived DC produced undetectable levels of IL-10. We therefore could not use the IL-10 / IL-12 production ratio as with the monocytes (see Fig.5).

Fig 6a shows that the IL-10 production by the monocyte-derived DC of the ICA negative FDR was increased, whereas it was decreased in the recently-diagnosed diabetic patients as compared to the values found in the healthy controls. DC of the FDR produced significantly higher quantities of IL-10 (43 ± 36 pg/ml in 11 FDR vs. 31 ± 70 pg/ml in 24 controls, p=0.02), while the IL-10 production by the DC of the patients was significantly decreased (5 ± 14 pg/ml in 9 patients vs. 31 ± 70 pg/ml in 24 controls, p=0.03) (Fig. 6a).

The monocyte-derived DC of the one single case positive for ICA showed a high IL-10 production of 120 pg/ml.

With regard to the IL-12 production of monocyte-derived DC Fig 6b shows that the IL-12 production by the DC of the recently-diagnosed DM1 patients was significantly reduced, but

that this was not the case in the ICA negative FDR. The mean IL-12 production by the DC of 21 healthy controls was 359 ± 36 pg/ml, of the 12 ICA negative FDR 104 ± 159 pg/ml (p=0.40) and of 8 DM1 cases tested 14 ± 25 pg/ml (p=0.002).

The monocyte derived DC of the one single case positive for ICA showed a high IL-12 production of 139 pg/ml.

Discussion

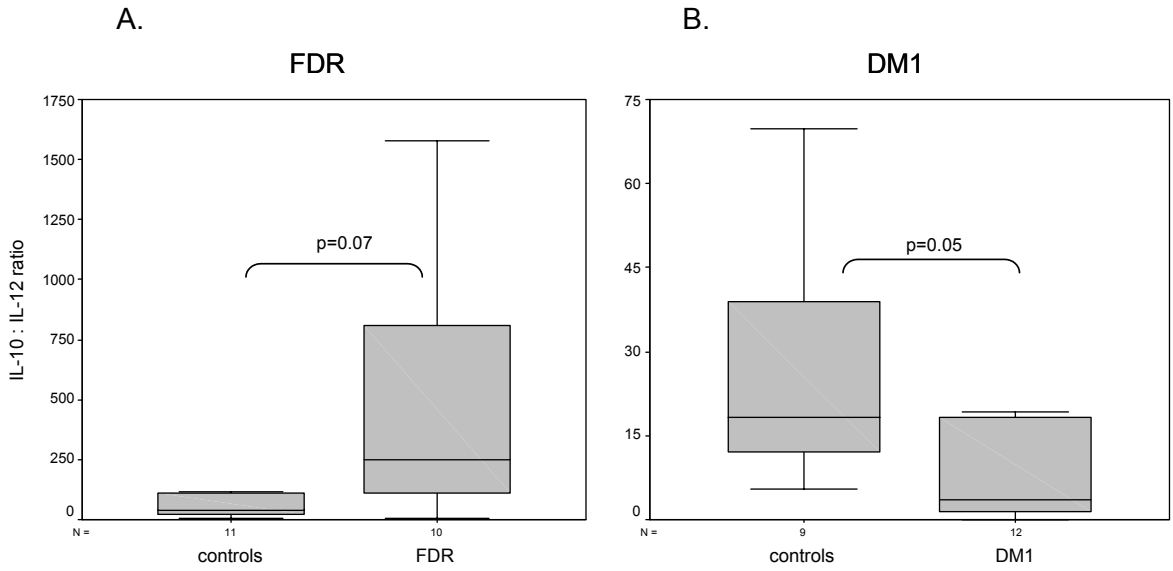
Although the number of included FDR is small, we observed similarly raised serum levels of sICAM-1 in FDR as in recently-diagnosed DM1 patients, yet the FDR did not show the lower monocyte CD54 expression, lower monocyte IL-10 production, hampered DC development from monocytes and lower DC function that we found in recently-diagnosed DM1 patients. On the contrary, DC development from monocytes was stimulated in the FDR while their monocytes and monocyte-derived DC showed an increased IL-10 production.

Our sICAM-1 data in the FDR are in accord with the data of three other studies which also found increased levels of serum sICAM-1 in FDR of DM1 patients. One of these studies reported –as we do– that the levels of sICAM-1 were also raised in ICA negative FDR (1). The two other studies, however, did not investigate the sICAM-1 levels in ICA negative FDR versus those in healthy controls and can therefore not give definite conclusions on the sICAM-1 level in ICA negative FDR of DM1 patients (3, 11).

These studies reported positive correlations between islet specific auto-antibody titers and sICAM-1 serum levels. There were however no indications found for an HLA-DR association with the increased sICAM-1 levels in those reports (1, 11). Also, the individual s-ICAM levels in FDR do not predict an outbreak of type 1 diabetes (12). Nevertheless, taking the data of these reports together, there is little doubt that the pre-diabetic phase of type 1 diabetes (not necessarily resulting in overt type 1 diabetes) is characterized by an elevated serum level of sICAM-1.

Elevated serum levels of sICAM-1 are thought to represent the presence of ongoing tissue damage and/ or inflammation, since sICAM-1 is also increased in the serum of patients characterized by other chronic inflammatory (auto-immune) processes, such as myasthenia gravis, Graves' disease and rheumatoid arthritis (1, 2, 13, 14). The molecule is thought to be derived from activated endothelium, leukocytes and certain immune-activated epithelia (4). sICAM-1 is involved in processes of leukocyte adhesion to vessel walls and to tissue structures and hence in the migration, accumulation and differentiation of leukocytes in such areas of inflammation (15, 16, 17). In addition sICAM-1 is thought to counteract inflammatory and auto-immune reactions.

Fig. 5. The IL-10/ IL-12 production ratio of monocytes. **(a)** Monocytes of FDR (n=10) had a higher ratio compared to monocytes of healthy controls (n=11), p=0.07. **(b)** Monocytes of DM1 patients (n=12) had a significantly lower ratio compared to monocytes of healthy controls (n=9), p=0.05. Box plots with median and quartiles are shown.

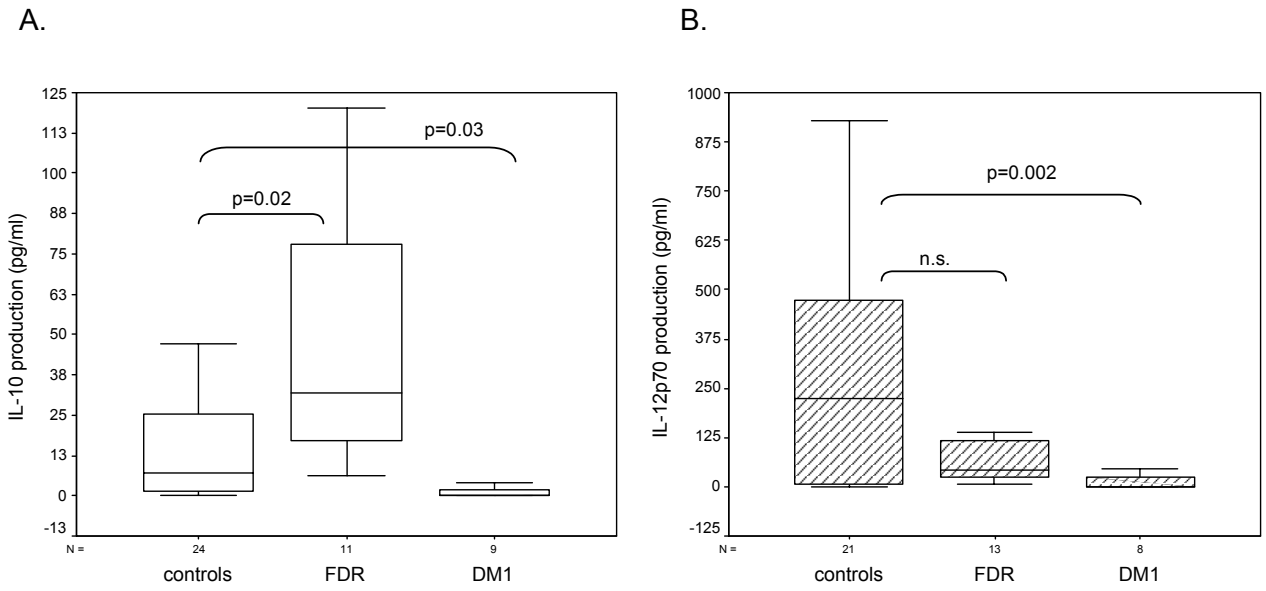


The molecule is able to inhibit autoreactive T cell proliferation in DM1 patients (18) and treatment of prediabetic NOD mice with recombinant ICAM-1 delays disease by active immunoregulation by inhibiting pro-inflammatory Th1 responses (19). Elevated levels of sICAM-1 in FDR may thus be instrumental in down regulating already existing inflammatory processes and pro-inflammatory Th1 cells. Our data -though limited, as stated earlier - also show that monocytes and monocyte-derived DC of ICA negative FDR of DM1 patients have an increased production of the immuno-suppressive cytokine IL-10. This contrasts to patients with recently-diagnosed DM1, whose monocytes and monocyte-derived DC show a diminished IL-10 production. Patients with overt diabetes also show a hampered differentiation of DC from monocytes, resulting in DC with a poor expression of essential adhesion, stimulatory and costimulatory molecules on their cell surface, an abnormal low stimulation of the proliferation of autologous T cells and a low capability to skew autologous T cells in either a Th1 or Th2 direction (20, 21, Lam-Tse et al, *submitted*).

This was not the case in the ICA negative FDR of DM1 patients. On the contrary, their monocyte-derived DC showed an increase in the expression of CD86, an important costimulatory molecule and an increased T cell proliferation in autologous MLR.

We have previously listed our arguments to consider the monocyte and DC aberrancies in DM1 as a sign of a poor capability of the DM1 immune system to preserve tolerance (Lam-Tse et al, *submitted*).

Fig. 6. The IL-10 production and IL-12 production by monocyte derived DC. **(a)** DC of FDR (n=11) had a higher IL-10 production compared to DC of healthy controls (n=24), p=0.02, whereas of DM1 patients (n=9) had a significantly lower production compared to monocytes of healthy controls (n=24), p=0.03. **(b)** DC of FDR (n=12) had a lower, but not significant, IL-12 production compared to healthy controls (n=21). DM1 patients (n=8) had a significantly lower IL-12 production compared to controls (n=21), p=0.002. Box plots with median and quartiles are shown. Here, we did not use the ratio, because in some DM1 patients the monocytes produced undetectable levels of IL-10.



Since we found mirror-image aberrancies in the monocyte and DC functions in the FDR it may come as no surprise that we consider these as an indication that the monocyte-DC system is levelled at an anti-inflammatory / pro-tolerance set point in practically all the FDR of DM1 patients. Interestingly the vast majority of these FDR was negative for ICA and thus are at low risk to develop overt type 1 diabetes.

Similar data have been reported previously with regard to the T cell system. With regard to human Th1/ Th2 studies Kallman et al (22) showed that cultured whole blood of an ICA negative co-twin, and not an ICA positive co-twin, produced increased amounts of IL-10 and IL-4 as compared to the diabetic co-twin. Hussain et al (23) showed that twins of diabetic patients who remained non-diabetic in a 6 year follow-up had a Th2 type profile; twins who progressed to overt DM1 had a mixed Th1/ Th2 profile. The same author however found – in fact dissimilar to our data -a Th1 deviation in FDR of DM1 patients (8). The ICA status was not taken into account in this latter study. When the ICA status is taken into account, there are ample data that FDR of DM1 patients who are ICA negative do not show a Th1 deviation (24, 25), but are even in a Th2 mode (22). In ICA positive FDR a Th1 deviation has been shown (24; 26, 27-31). In the NOD mouse a Th2 type / anti-inflammatory profile prevails in the pre-diabetic stage, shifting towards a Th1 type / pro-inflammatory profile at the time of destructive insulitis and the development of disease (32, 33).

Except for one case, all our tested FDR were ICA negative. The ICA positive individual had a raised serum level of sICAM-1, but a profile of monocyte and monocyte-derived DC abnormalities intermediate between the ICA negative FDR and the recently-diagnosed DM1 patients, i.e. a low IL-10 production by monocytes as the overt DM1 patients, but a normal DC generation from monocytes as the ICA negative FDR (except for a lower expression of CD86 on these cells). Needless to say, that many more of such ICA positive FDR need to be studied to give solid conclusions on monocyte and DC set points in ICA positive FDR. It must be noted that Takahashi et al did find DC abnormalities similar to those found in overt DM1 patients in islet-Ab positive individuals (21).

In conclusion, ICA negative FDR of DM1 patients show various aberrancies in their monocytes and monocyte-derived DC which are mirror images of those found in overt DM1 patients. We favour a view that such aberrancies represent an anti-inflammatory and tolerogenic set point of the immune system instrumental to counteract already existing harmful deviations in the immune system that heighten the risk for islet autoimmunity in FDR.

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CHAPTER 7

DENDRITIC CELLS IN AUTOIMMUNE THYROID DISEASE

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Introduction

In the early 1980s it was reported that thyrocytes aberrantly express MHC class II molecules in Hashimoto and Graves' disease (normal thyrocytes do not) and that these thyrocytes are able to stimulate T cells *in vitro*. This led to the hypothesis that an aberrant expression of MHC class II molecules on thyrocytes is the basic abnormality in thyroid autoimmunity and that an immune response to thyroid auto-antigens is initiated by an erroneous local presentation of such antigens to intra-thyroidal accumulated T cells (1).

Another mechanism of initiation of the thyroid autoimmune reaction was also proposed (2). A class of antigen presenting cells (APC), the so-called dendritic cells (DC), was reported to be present in the thyroids of Hashimoto and Graves' patients. DC are known to be the most potent professional APC of the immune system. Therefore these cells are the most likely candidates for the initiation of the thyroid autoimmune response.

Up to the early 1990's a plethora of studies has been concentrating on the first hypothesis. However progress in immunology over the last 10 years point to the DC as one of the most important APC in the initiation of autoimmune reactions. There is perhaps a small role for thyrocytes aberrantly expressing immune molecules in later phases of the thyroid autoimmune reaction.

Dendritic cells (DC) and the physiology of the normal thyroid

DC and the normal thyroid

DC are present in low numbers in normal thyroids composing 2-3% of the interstitial cell population (3, 4, 5). There are indications that such DC are able to proliferate (6), which means that not all of the thyroid DC need to be recently immigrated from the blood stream. Malignancies of thyroid DC have been described (thyroid Langerhans cell histiocytosis) (7).

Thyroidal DC are often in close contact with thyrocytes. The thyroidal DC are in a clear immature state and often show monocyte marker characteristics (8). It is thought that soluble factors produced by TSH-stimulated thyrocytes, such as GM-CSF, TGFβ, IL-6 and osteoprotegerin, keep intra-thyroidal DC in their immature state (6, 9, 31). TNFα on the other hand induces maturation of immature thyroidal DC (13). Also thyroid hormones and related iodinated compounds are known to influence the maturation of immature DC and the differentiation of related APC (10, 11).

DC as thyroid regulators

DC are capable to regulate the growth of thyroid follicles in vitro. Simons et al (12) showed that the co-culture of isolated thyroid or splenic DC with thyroid follicles resulted in

intense interactions of the DC with the thyroid follicles and a dampening of TSH-induced proliferation of thyrocytes composing the follicles. The thyroid hormone release from the follicles was suppressed to a limited extent. Cytokines (IL-1 and IL-6) secreted by the DC, and not adhesive interactions are important in this regulatory function of DC (12).

Interestingly spleen DC and pituitary folliculo-stellate cells (which are in part pituitary interstitial DC) express functional TSH-receptors (13, 14). Upon stimulation with TSH, spleen DC up-regulate their c-AMP, phagocytic capability and production of IL-1 and IL-12 (13). If such TSH-receptor expression and cytokine production also occurs at the level of the thyroidal DC this would imply that the T4-TSH feed back mechanism does not only targets thyrocytes, but also thyroid DC via which thyrocyte growth can be regulated.

DC and iodine deficient goiters

In view of the above-reviewed evidence for a thyrocyte regulatory role of DC, it is noteworthy that DC accumulate and form homotypic clusters in iodine deficient goiters both in man as well as in an animal model (15, 16). The accumulation of the DC might be taken as a sign that the cells are instrumental in a growth regulation of the iodine-deficient goiter, yet direct evidence for this has not been given. The homotypic cluster formation of DC is a sign of maturation of the cells (17), and indeed during the formation of iodine-deficient goiter there is an activation of Tg-specific T and B cells (16).

Dendritic cells (DC) and the autoimmune diseased thyroid

Early accumulation and clustering of DC in the thyroids of animal models of autoimmune thyroiditis

A small increase in the number of DC and a homotypic clustering of the cells in the interstitium of the thyroid is one of the first signs of a developing thyroid autoimmune reaction in the bio breeding diabetes prone (BB-DP) rat, an important animal model of spontaneously developing autoimmune thyroid disease (3). These signs precede the T cell expansion and the production of autoantibodies in the thyroid draining lymph nodes and the actual infiltration of the rat thyroid with lymphocytes. At this early phase of the autoimmune reaction BB-DP thyrocytes are negative for MHC class II (3). In another autoimmune thyroiditis rat model, the neonatal thymectomized Buffalo Strain rat and in the non obese diabetic (NOD) mouse (a mouse model of spontaneous autoimmune thyroiditis), similar findings have been reported (18, 19). After isolation of BB-DP thyrocytes from these “early phase” thyroids, the cells were very poor stimulators of T cell expansion in vitro. In contrast, isolated thyroidal DC were excellent in this function equalling splenic T cells (8). These arguments provide, at least in the

animal models, sufficient proof to reject the idea that an aberrant expression of MHC class II molecules is a prime event in thyroid auto-immunity initiating the process.

With regard to the human, thyrocytes are not capable of expressing costimulatory molecules to a noteworthy extent (20, 21), there is however some evidence that they might in Hashimoto's thyroiditis (22). In addition, while the co-culture of thyrocytes and T cells alone, resulted in a relatively weak T cell proliferation, addition of low numbers of monocytes or APC to the culture led to a clear enhancement of the T cell proliferative response (22). These observations point in the direction that also in the human professional APC play a prime role in the induction of autoimmunity.

Thyroid factors instrumental in the early attraction of DC to the thyroid of animal models of autoimmune thyroiditis

In the majority of the NOD strains the incidence of autoimmune thyroiditis is in general very low, but it varies from colony to colony (19). Certain dietary iodine regimens, however, have a triggering effect on the development of autoimmune thyroiditis in the low-incidence NOD strains. In such strains an iodine-induced necrosis of thyrocytes is a clear factor leading to an intra-thyroidal accumulation of various inflammatory cells, amongst which the DC. This early inflammatory influx is followed by a reaction of the draining lymph nodes and an initiation of an auto-immune thyroiditis (19).

In the BB-DP rat and obese strain (OS) chicken (both other animal models of autoimmune thyroiditis) there are however no signs of an early iodine-induced necrosis of thyrocytes attracting the DC in an inflammatory reaction. Interestingly intrinsic disturbances in the growth and the differentiation of thyrocytes have been shown in both models, in the BB-DP rat leading to a high incidence of ultimo-brachial cysts and an altered production of IL-6 by thyrocytes (10, 24). Whether such alterations do lead to a higher influx of DC is not known.

Late accumulation of DC in the thyroid of the BB-DP rat

After the appearance of Tg autoantibodies in the circulation, T and B cells start to infiltrate the thyroid and at the same time as this infiltration there is a remarkable sharp increase in the number of thyroid DC (3). The T cells, B cells and DC accumulating in the thyroid do not form destructive infiltrates, but are organized as peripheral lymphatic tissue (3, 26). The diapedesis of lymphocytes from the bloodstream to this tissue is facilitated through the formation of specialized "high endothelial venules" (HEV) (25). Intra-thyroid lymphatic tissue probably serves a further expansion of autoreactive T cells and a further production of Tg auto-antibodies. Thyrocytes adjacent to areas of intra-thyroid lymphatic tissue start to express MHC-class II molecules (3, 24, 25), probably as a consequence of the cytokines produced in the intra-thyroid lymphatic tissue. Perhaps such MHC class-II positive thyrocytes,

but more likely auto-antigen specific B cells (27) play a role as APC in this late phase of autoimmune reactivity.

DC in the thyroids of patients with Graves' disease and Hashimoto's disease

Thyroids of patients (predominantly Graves' goiters) are available for microscopical investigation only in a late phase of the disease, when one has decided to a surgical intervention. The glands show areas of intra-thyroid lymphatic tissue and areas in which the thyroid follicles are largely intact with significantly elevated numbers of perifollicularly located DC (9, 33, 34). Such perifollicular DC have an immature phenotype (28).

Phenotypically mature DC are predominantly present in the larger areas of intra-thyroid lymphatic tissue and co-localized with activated CD4+ T cells (28). In such areas HEVs are present with an up-regulated integrin expression on their endothelial cells (25, 30).

Abnormal DC differentiation in spontaneous animal models of thyroid autoimmune disease. A role in defective tolerance induction?

Since DC are critically involved in the initiation of the autoimmune process (see above), it is important to note that there is accumulating evidence that the differentiation of DC from precursors is abnormal in the BB-DP rat and the NOD mouse. In the BB-DP rat precursors were more abundant than DC in the very early infiltrates of the thyroid (8), and lymph node and spleen DC were in a relatively immature (interstitial) state (32, 33).

In the NOD mouse, studies have concentrated on the *in vitro* development of DC from bone-marrow precursors. This development was found to be hampered leading to a low yield of DC with a low grade of maturation and a low capability to stimulate T cells (34). However opposite findings have been made (35) and differences in results are probably dependent on the culture conditions used (Leenen, personal communication). Functional studies on interstitial DC are lacking in the NOD mouse. Spleen and lymph node DC of the NOD mouse however have a normal state of maturation and are perfectly capable of stimulating T cells (36). In fact there is an excessive proliferation reaction of the NOD T cells *in vitro*, when stimulated with DC. This is most likely due to a defect other than the DC maturation defects in the immune system of this animal, but a defect in the mechanisms of apoptosis of T cells leading to a hampered AITCD (36).

Although it is not clear if and how differentiation defects of DC in the animal models play a role in their defective ability to mount tolerance to auto-antigens, there are a few indications that they might. The immature lymph node and spleen DC of the BB-DP rat were in particular less capable of expanding an important suppressor T cell population of the rat, the

so-called $RT6^+$ T cells (32), while in the NOD mouse, transfers of *in vitro* maturated DC prevented the development of DM1 in this animal (37).

An abnormal DC differentiation in DM1 patients, but not in thyroid autoimmune patients

In analogy to the data on differentiation and maturation defects in the animal models there are reports including our own data as described in chapter 4 and 5 accumulating on similar defects in DM1 patients. We described that this is not the case for monocytes of DM2 patients or patients with autoimmune thyroiditis.

However, defects other than differentiation defects of monocyte-derived DC are noticeable in the monocytes of patients with autoimmune thyroiditis, such as an altered expression of integrin molecules, a hampered ability to arrange the actinomyosin cytoskeleton after chemotactic stimulation, and a lower potency to differentiate into a population of APC other than the classical DC, namely the motile veiled macrophages (38, 39). Although this suggest that adhesive, motile and migratory functions of monocytes are hampered in thyroid autoimmune disease, it is not known if and how such defects have any effect on the apparent hampered tolerance for thyroid auto-antigens in these patients.

Future prospects: DC as putative tools in the treatment of autoimmune diseases

Although the role of the above-described defective APC function is far from clear in the development of type 1 diabetes and autoimmune thyroiditis, it is clear that DC form a potent group of cells to modulate immune responses. DC vaccination protocols are presently under design to elicit strong immune reactions (40) including autoimmune reactions to eradicate tumors, also thyroid malignancies (41). Such vaccination protocols aim at constructing DC potent to elicit strong effector immune responses. The present state of the art points to mature DC expressing important tumor antigens and producing IL-12 as the most likely candidates to perform this job.

Since DC are also involved in tolerance induction, it is not a far-fetched idea to construct DC to induce or restore tolerance. Very immature steady state DC, expressing important auto-antigens and producing IL-10, are thought to be able to perform this job (42). However the differentiation and maturation defects of DC in the BB-DP rat and the NOD mouse suggest that there is no shortage of such very immature DC in these animals. The disease preventing effects of the transfer of artificially matured DC in the NOD mouse point in another direction, namely that mature DC are more critical to elicit or restore tolerance in

conditions of endocrine autoimmunity (43, 44). Such DC might be critical for a deletion of autoreactive T cells in the periphery via AITCD.

Conclusions

Interstitial thyroid DC regulate the growth and hormone production of thyrocytes. DC and not aberrantly MHC-class II expressing thyrocytes are the cells initiating thyroid autoimmune reactivity.

The differentiation of immature and mature DC from precursors is abnormal in the animal models of spontaneously developing endocrine autoimmune disease, but consequences are not clear for the defective state of tolerance in these animals.

However, the differentiation of immature and mature DC from precursors is normal in patients with autoimmune thyroiditis, apart when co-occurring with type 1 diabetes in the setting of an APS type 3. For the latter, further research is required. Whether DC are good candidates for a novel form of treatment for auto-immune thyroiditis, i.e. vaccinations to induce tolerance, is doubtful.

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CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

Main conclusions regarding monocytes and monocyte-derived dendritic cells in type 1 diabetic patients

This thesis describes major aberrancies in monocytes and monocyte-derived dendritic cells of type 1 diabetic (DM1) patients. One of these is that monocytes of DM1 patients had an abnormal expression of adhesion molecules. Monocytes of DM1 patients specifically expressed lower levels of the adhesion molecule ICAM-1 (CD54), while monocytes of DM2 patients did not *(Chapter 4).* Monocytes of DM1 patients did also express lower levels of the integrin CD49e and higher levels of CD11b and CD18 and their serum levels of sICAM-1 were raised. These latter aberrancies were however shared with DM2 patients, and are hence likely – at least in part - consequences of the abnormal metabolic status of diabetes.

Monocytes of DM1 patients also produced lower quantities of the immunosuppressive cytokine IL-10 *(Chapter 5).* This aberrancy was again specific for DM1 patients, it was neither found in DM2 patients (metabolic controls) nor in patients with autoimmune thyroid disease (organ-specific autoimmune controls).

Dendritic cells (DC) generated from the aberrant monocytes of DM1 patients were themselves also aberrant *(Chapters 4 and 5)*: the DC generated by culture in GM-CSF and IL-4 had a lower expression of various adhesion molecules (CD54, CD11b, CD18, CD49d and e), of MHC class II molecules and of the important costimulatory molecules CD80, CD86 and CD40. The DC also produced lower quantities of IL-10 and IL-12 and had a reduced capacity to stimulate autologous T cells, i.e. the stimulated T cells showed a poor capability to proliferate and also a reduced potency to produce IFN-γ. However, DM1 DC were normally capable of stimulating the proliferation of allogeneic T cells. This indicates that the DM1 T cells are aberrant as well.

We also tested 13 non-selected first-degree relatives (FDR) for above described characteristics and functions of their monocytes and DC. One of these FDR was positive for ICA, hence virtually all these FDR were at low risk to develop DM1. Interestingly the monocytes and monocyte derived cells of the ICA negative FDR did show aberrancies, but these were the mirror images of those found in overt DM1 (*Chapter 6*). The monocytes of FDR produced higher quantities of IL-10 as compared to healthy control monocytes and showed a development into highly functional dendritic cells. Monocytes of FDR expressed normal levels of ICAM-1. Yet the soluble ICAM-1 was already raised in the serum of FDR. Similar to the levels of recently diagnosed DM1 patients. This cannot be due to the abnormal metabolic status.

The abnormal CD54 expression on the monocytes of DM1 patients, a sign of a disturbed adhesion and migration of monocytes in DM1?

One study other than ours also showed that the expression of CD54 on monocytes was reduced in recent onset diabetic patients, although not significantly (1). However in this study DM2 patients were not included as controls.

The low CD54 expression on monocytes likely implicates a disturbed adhesive potential of the cells in DM1. Adhesive processes allow cell to cell interaction, binding to extra cellular matrix proteins, and play a role in the activation and maturation of cells (2-7). As an example of the latter we demonstrated that a triggering of CD54 by stimulating monoclonal antibodies stimulated the differentiation and maturation of DC from monocytes. This process also resulted in an increased formation of homotypic cell clusters. This is in accord with other studies on the influence of signalling via adhesion molecules on DC and the role of homotypic cluster formation in this process (6;8;9).

Adhesive processes are also important in the diapedesis of monocytes from the bloodstream into the tissues. It is thus tempting to speculate that our data of a lower percentage of monocytes expressing high levels of CD54 in DM1 represent the disappearance of specifically CD54 positive monocytes from the circulation. Indeed pro-inflammatory circulating monocytes with a raised tendency to infiltrate tissues have a high expression level of various integrins and an enhanced adherence to extracellular matrix (ECM) proteins (10). Such pro-inflammatory monocytes are easily trapped in inflamed peripheral tissues. Indeed MΦ and DC, infiltrating the pancreata of the NOD mouse and DM1 patients are clearly CD54 positive (11;12). Interestingly various interventions aimed at blocking adhesion molecules, including CD54, prevent or delay autoimmune diabetes in the animal models (13-17). These interventions include the administration of monoclonal antibodies against the adhesion molecules or the administration of recombinant adhesion molecules or transgenic manipulations (13;14;16;18). However, if our data represent a preferential disappearance of pro-inflammatory monocytes from the circulation in DM1 patients, we should also expect a lower number of CD11b and CD18 positive monocytes in the circulation of DM1 patients and this was not the case.

The specifically lowered CD54 expression on DM1 monocytes might also be an indication of an intrinsic alteration in the structure of the CD54 molecule. There are polymorphisms of the CD54 molecule and these gene polymorphisms have been investigated in DM1. An association between these polymorphisms and DM1 could not be shown (19-21). There are to our knowledge no studies on these gene polymorphisms and the level of CD54 expression on peripheral blood monocytes. It is also possible that the lower CD54 expression on DM1 monocytes is due to the metabolic status and/ or the specific treatment with insulin of

the DM1 patients. Aljada et al showed that insulin is able to down regulate the expression of ICAM-1 on human umbilical endothelial cells via inhibition of the NFkB pathway (22). However a considerable proportion of our DM2 controls were treated with insulin as well and these did not show lower numbers of blood monocytes expressing CD54.

Hyperglycemia is also known to influence the adhesion molecule metabolism: high glucose levels increase CD54 expression on endothelial cells (23) and its ligand CD11b/ CD18 on monocytes in vivo (24). We however did not find a higher, but a lower expression of CD54 on circulating monocytes specifically of DM1 patients. We consider it therefore unlikely that a chronic hyperglycemia explains this phenomenon. The chronic hyperglycemia may however serve as a good explanation for the increased CD11b/ CD18 expression on the monocytes of both DM1 and DM2 patients described in this thesis. It might also explain the increased levels of sICAM-1 in DM1 and DM2 patients, since it is shed from activated endothelium (yet we also found a raised sICAM-1 in normoglycemic FDR of DM1 patients).

Last but not least our data on a low CD54 expression on monocytes in DM1 patients may be explained by representing a differentiation disturbance of monocytes from bone-marrow precursors and/or a recruitment disturbance of sufficiently differentiated monocytes from the bone-marrow to the peripheral circulation. Data from Nikolic studying the NOD mouse model in our laboratory (personal communication) show that the circulating monocyte pool is aberrant in this model of DM1 and that there is a shift in the balance from immature to more mature forms of monocytes in the circulation of the NOD mouse. These data will be described extensively in a thesis from her hand.

The lowered IL-10 production by DM1 monocytes and monocyte-derived dendritic cells, a sign of a disturbed immune suppression and a pro-inflammatory set point in DM1?

It is the generally held view that the actual insulitis process with the destruction of the β cells is a T cell-mediated process due to Th1 cells and dominated by Th1 type cytokines, such as IFN-γ. The Th2 type cytokines IL-4 and IL-10 are under-represented. Many studies in the BB-DP rat and the NOD mouse support this view, and although human studies on the cytokine expression profile in pancreatic islets are scarce, increased IFN-γ and reduced IL-4 expression have been reported in established cases of DM1 (25).

In the NOD mouse model there clearly is a pre-phase to the actual destructive Th1 mediated insulitis. In this pre-phase there is an extensive pancreatic infiltration of T cells, DC and macrophages, but the cells remain strictly around the islets (“peri-insulitis”) and the β cells in the islets are not attacked. The peri-insulitis process is dominated by the Th2 type cytokines IL-4 and IL-10 (26-28). Interestingly these cytokines were also found increased in the islets

during diabetes protective treatments (27;29). In addition, manipulation in the Th1/ Th2 cytokine balance in the NOD mouse by transgenic expression, virus vector induced expression, direct administration of cytokines or antibodies against cytokines all influence disease course.

Particularly DC and other APC (monocytes and macrophages) contribute significantly to the polarizing influence on T helper cell differentiation. (30). The APC are able to exert strong Th1 differentiation signals by the production of the pro-inflammatory cytokine IL-12 (31;32). IL-10 on the other hand (abundantly produced by APC) is a critical immunosuppressive cytokine. It is able to inhibit the cytokine production, including the IL-12 production by APC and the cytokine down regulates the expression of MHC class II and costimulatory molecules on APC (33-36). Supporting the view that autoimmune insulitis is a Th1 phenomenon one has found IL-12 increased in the β cell destructive insulitis of the animal models (27;37-39). The increased IL-12 expression preceded the IFN-γ expression in the islets (38). IL-10 administration to NOD mice protects from disease development (29;40-42).

Hence it is important to be informed on the IL-12 and IL-10 production capability of APC populations in DM1 patients. Recently pancreatic biopsies under laparoscopy have been performed safely and in situ cytokine phenomena might perhaps be studied (43). Yet up till now cytokine studies on peripheral blood have only been practicle. Cytokine analysis of serum and leukocytes in DM1 patients has not provided consistent results (44-53). As described in *chapter 5*, we found that isolated monocytes and monocyte-derived DC of DM1 patients produced reduced levels of IL-10, favouring a view that APC in overt DM1 patients allow a Th1 reaction to occur.

However, as in the case of the reduced numbers of CD54 positive monocytes (see before) this finding might be explained by a preferential outflow of IL-10 producing APC from the blood stream to the islets in DM1 patients. We nevertheless favour a view that intrinsic disturbances in the generation and differentiation of APC from the bone-marrow explains the defective IL-10 production by these APC in DM1 patients. In parallel investigations in our laboratory on the BB-DP rat bone marrow derived DC of the BB-DP rat are aberrant in differentiation and have a reduced capability to produce IL-10 (Sommandas, personal communication).

Interestingly IL-10 is also required for the induction of the differentiation of certain types of regulatory T cells by DC (35;54). Are our data hence indicative of an important intrinsic defect in the Il-10 production capability of “DM1” APC leading to a poor induction of regulatory T cells and /or an inability to dampen down inflammatory responses? A pro-inflammatory status is also shown by Litherland et al, who found higher percentages of monocytes of DM1 patients and their relatives expressing cyclooxygenase 2 (55). Also with regard to the production of the pro-inflammatory cytokines IL-1 and TNFα, Hussain et al found a higher

production of IL-1 and TNFα in serum and supernatants of PBMC´s of DM1 patients and relatives (45;46). It must be noted however that we were unable to find difference in the production of IL-1β and TNFα by monocytes between DM1 patients and control subjects (data not described in this thesis).

The aberrant differentiation of DC from monocytes in DM1, a sign of a lower potency to induce tolerance of the T cell system?

We found the development of DC from monocytes disturbed in recent onset DM1 patients *(Chapters 4 and 5).* The DC had maturational aberrancies, expressed lower levels of integrins, produced lower quantities of IL-10 and IL-12 and had a lower potency to stimulate autologous T cells. In chapter 4 we discussed the possibility that the aberrant development of the DC from monocytes might be due to the earlier discussed decreased expression of CD54 on the DM1 monocytes, since the CD54 signal is involved in the differentiation/ maturation of DC.

Apart from being due to the low expression level of MHC class II molecules and of costimulatory molecules, the defective T cell stimulatory capacity of the monocyte-derived DC of DM1 patients might also in part be due to the low expression level of integrins, since the binding of integrins between DC and T cells provides a strengthening of the “immunological synapse” between the DC and T cells. This “immunological synapse” supports an appropriate binding between MHC class II and TCRs as well as between the costimulatory molecules CD80/ CD86 and CD28 and CTLA-4 (5) (see Fig. 2 in the Introduction).

In the NOD mouse and the BB-DP rat similar defects in the generation of DC from precursors and in the function of spleen and lymph node DC have been found. Spleen and lymph node DC of the BB-DP rat have a reduced expression of MHC class II, CD80 and CD86, a lower homotypic cluster capability and a lower T cell stimulatory capacity (56-58). Spleen and lymph node DC and accessory macrophages of the NOD mouse show a reduced expression of costimulatory molecules, a reduced IL-12 production and a reduced stimulatory capacity for (autologous) T cells (57-59). Both in the BB-DP rat and the NOD mouse aberrant myeloid bone marrow precursors (mainly pro-monocytes) generate lower yields of DC with an aberrant low expression of MHC class II and costimulatory molecules, a low IL-12 and IL-10 production and a reduced capacity to stimulate T cells (56-58;60-62).
In sum, a defective differentiation and function of DC is obvious in DM1 and the animal models of autoimmune diabetes and must therefore represent an important aberrancy.

How should a defective generation and function of DC –so obvious in the NOD mouse, the BB-DP rat and the DM1– lead to a defective tolerance?

As stated in the *Introduction* of this thesis DC are not only instrumental as accessory cells in the generation of effector immune responses, but also in the induction of tolerance. Steinman et al consider DC in transit from the periphery to the draining lymph node under physiological conditions ("steady state" DC) as capable of maintaining tolerance (63). Also recognition of an auto-antigen in the periphery in combination with a triggering of costimulatory molecules is required for the maintenance of regulatory T cells in the NOD mouse model (64). CD80/ CD86 knock-out NOD mice have a profound decrease of their immuno-regulatory $CD4^+CD25^+$ T cell subset and show an accelerated diabetes development (64). It is known that elimination of this T regulatory subset results in development of autoimmune diseases, such as inflammatory bowel's disease, insulitis and thyroiditis (65;66).

DC are however not only instrumental in the induction of T cell tolerance via the generation of regulator T cells. DC are also instrumental in the induction of T cell tolerance via the induction of Activation Induced T Cell Death (AITCD) in already activated T cells and/or via a down regulation of activated T cells via a CD80/ CD86-CTLA-4 signalling. As stated before spleen DC and accessory macrophages of NOD mice show a reduced CD86 expression and have a lower capacity to stimulate T cells. They however also have a lower capacity to up regulate CTLA-4 in these poorly activated T cells (67). CTLA-4 activation has been proven to down regulate autoimmunity, since CTLA-4 knock out mice spontaneously develop severe autoimmune diseases (68;69). CTLA-4 is exclusively expressed on activated $CD4^+$ and $CD8^+$ T cells and binds with a much higher affinity to CD80/ CD86 than CD28. CTLA-4 triggering not only down regulates T cell function, it also mediates T cell apoptosis apart from the involvement of Fas-FasL interaction in this process (68;70). We previously showed in a co-culture of NOD mouse spleen and lymph node DC and autologous T cells, that the T cells showed a reduced apoptosis. This prolonged the duration of (be it a lower) effector T cell immune response (71).

Up till now evidence is lacking in the DM1 patient (the "human model") on a relationship between a defective DC development/ function and a defective development or maintenance of regulatory T cells and/ or a disturbed CTLA-4 function/ AITCD. Interestingly CTLA-4 polymorphism have been reported in DM1 patients, as well as Fas-induced T cell apoptosis disturbances (70;72-75). Obviously more experiments are needed here.

How do these findings of aberrant monocytes and monocyte-derived DC in human DM1 patients relate to our model of the pathogenesis of autoimmune diabetes in the NOD mouse and the BB-DP rat?

In *Chapter 3* we reviewed the literature on the value of the animal models for studying human endocrine autoimmune disease. Studies on the very early phases of the endocrine autoimmune diseases in humans are difficult to carry out. Therefore, animal models are good alternatives to study the initial aberrancies leading to autoimmunity. Chapter 3 describes in detail the various aberrancies in the animal models.

In sum, the studies in the animal models have shown that the ultimate autoimmune destruction of the glandular cells is a multi-step process, requiring several genetic and environmental aberrancies (or variants) to converge before full-blown disease develops. Two major groups of (early) aberrancies can be detected (figure 1):

I. Inborn and pre-autoimmune aberrancies in the immune system

Animals at risk to develop endocrine organ-specific autoimmune diseases show various aberrancies in their:

DC and MΦ

As stated before BB-DP rats and NOD mice show pre-autoimmune defects in the development and maturation of DC from their precursors and a high pro-inflammatory set-point of MΦ with a high production of IL-1 and prostaglandins (56;76-78) .

T cells

In the BB-DP rats and the Tx mice there is a lack of suppressor T cell populations, i.e. in $RT6^+$ and $CD4^+CD25^+$ T cells respectively. In the BB-DP rat this defect is genetically determined (a mutation in the Ian5 gene), in these thymectomised mice it has been surgically induced. The NOD mouse and the OS chicken have hyper-proliferative T cells. In the NOD mouse model this is due to apoptotic disturbances of the T cells, and stimulation of apoptosis, for example by administration of a Fas agonist, can reverse the disease in this model (79). In the OS chicken the defective reaction to steroids may play a role in the hyper-proliferation of its T cells (80).

There are indications that the interaction between the aberrant APC and aberrant T cells lead to a defective T cell tolerance due to an insufficient induction of regulatory T cells and/ or AITCD (Fig. 1).

II. Eliciting local aberrancies in the target glands

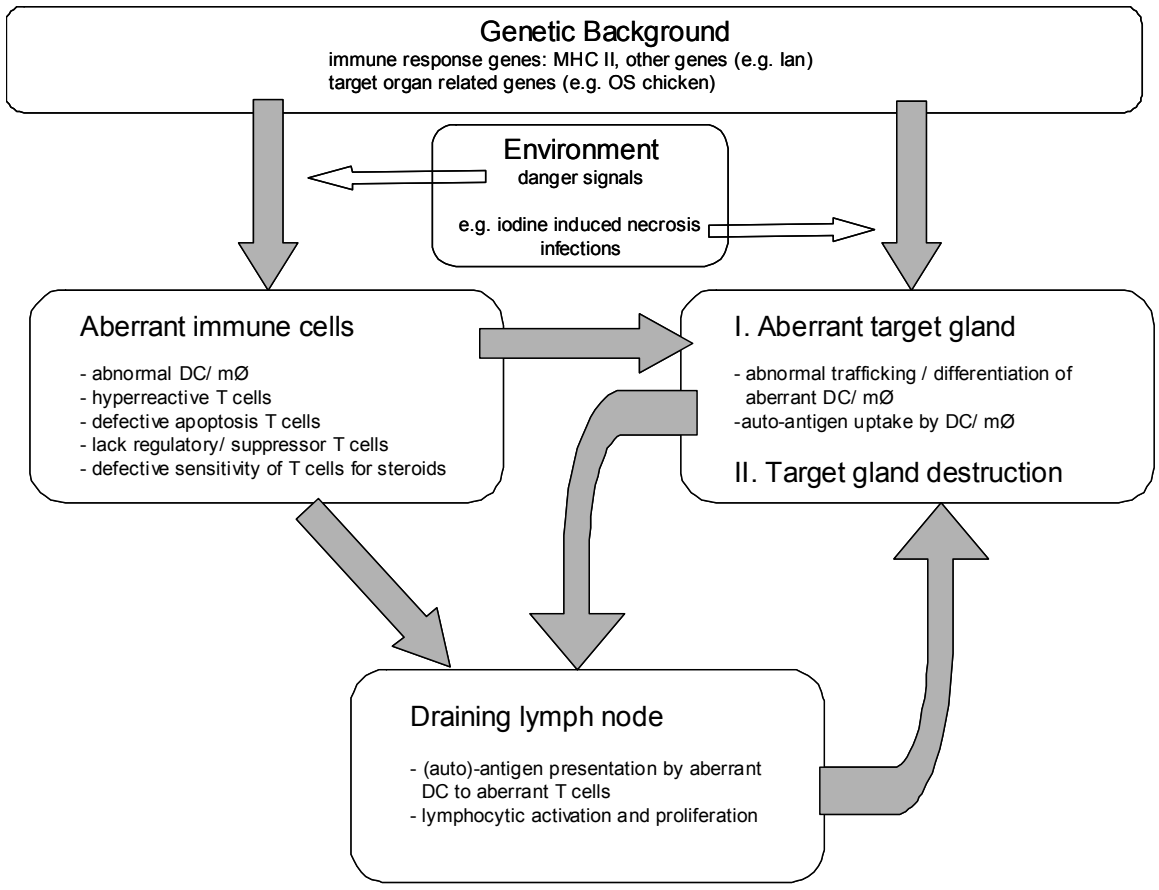
These local aberrancies might firstly be of inborn character. An example is the abnormal morphogenesis and stromal composition found in NOD islets. This abnormal architecture of the NOD islets goes together with a relatively high local accumulation of DC and macrophages (81). Interestingly the growth of BB-DP thyrocytes and OS-chicken thyrocytes is abnormal too, in the latter model even in the fetal stage (82), again pointing in

the direction of an abnormal morphogenesis of the thyroid in important animal models of thyroid autoimmune disease.

The local glandular aberrancies may also be induced or aggravated by environmental factors. A (toxic) high iodine-intake induces thyrocyte necrosis in the NOD mouse and the BB-DP rat resulting in an aspecific inflammatory influx of DC and macrophages. Whether there are environmentally inducing agents regarding the initiation of autoimmune insulitis in animal models is not completely clear. The insulitis due to high load of EMC-D virus in a mouse model might hint to an influence of microbial factors in the initiation of an autoimmune insulitis (83).

In this thesis we describe various aberrancies in monocytes and monocyte-derived DC of DM1 patients, which are very similar to the macrophage and dendritic cell aberrancies in the animal models of autoimmune diabetes.

Fig. 1 This scheme shows that susceptible genetic features and environmental triggering factors lead to a dys-regulated immune system. An autoimmune reaction will be initiated, when there is a trigger for an influx of APC in the target-gland-to-be. The APC will take up auto-antigens, travel which these to the draining lymph nodes, present the auto-antigens to naïve lymphocytes and initiate an autoimmune response. The clonally expanded autoreactive lymphocytes will start to accumulate in the target gland as long as the auto-antigens are presented there by APC. Depending on the phenotype of the infiltrated lymphocytes (CD4 helper T cells, CD8 cytotoxic T cells) and the cytokines produced (IFNγ) immune destruction of the glandular cells will be initiated.



If the model of the pathogenesis of animal endocrine autoimmune diseases is also of value for human DM1, it is clear that the monocyte and monocyte-derived dendritic cell aberrancies are probably not sufficient to elicit full-blown DM1 in humans. Local glandular aberrancies and defects in T cell function are additionally needed for that.

There is however also an interesting dissimilarity between our data on human monocyte and monocyte-derived DC and data on such cells in the animal models. In both the NOD mouse and the BB-DP rat the dendritic cell and macrophage abnormalities can be found from birth onwards or actually weaning onwards. When we take the data found in the FDR of our DM1 patients into consideration, this does not seem the case.

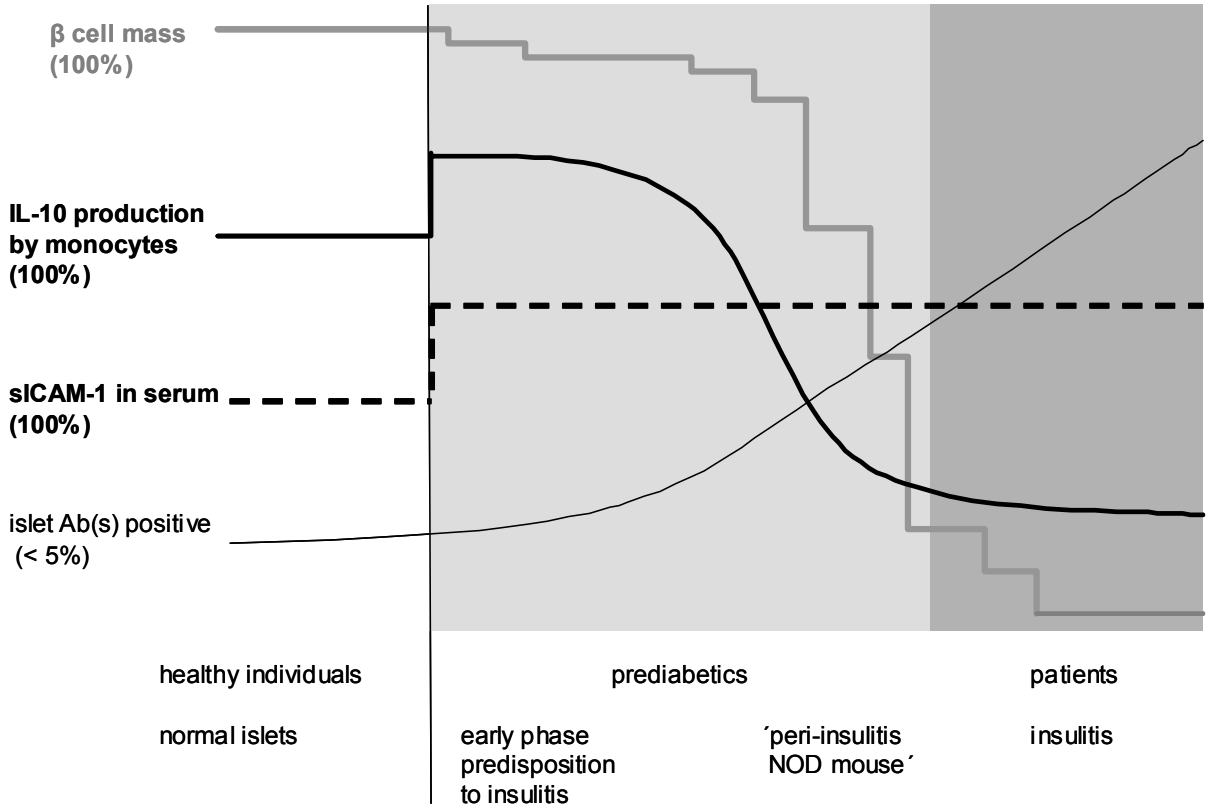
Aberrancies in monocytes and monocyte derived DC in first degree relatives of DM1 patients, do they represent compensatory mechanisms?

It must first be noted that the tested FDR were few in number and virtually all negative for ICA and hence at low risk for the development of DM1. Interestingly the monocytes and monocyte-derived DC of the FDR of DM1 patients are not defective as in the case of the overt DM1 patients and in the animal models prior to the development of autoimmune diabetes. On the contrary, the monocytes of the FDR showed a raised production of anti-inflammatory IL-10 and their monocyte-derived DC showed an enhanced differentiation from monocytes and had an increased expression of HLA-DR and the costimulatory molecule CD86. The monocyte-derived DC of the FDR also stimulated autologous T cells to an enhanced proliferation. In addition these monocyte-derived DC showed an increased IL-10/ IL-12 production profile, which contrasted to the cytokine production profile of the DC of overt diabetics, which were poor simulators of IL-10 and IL-12. As stated before, the pre-diabetic NOD mouse has dendritic cell maturation defects from birth (weaning) onwards. However it also shows similar phenomena regarding the IL-10 production as the FDR. A peak of serum levels of IL-10 can be found in pre-diabetic animals and such peak was evident in animals with an already existing autoimmunity, i.e. a peri-insulitis. The subsequent progression to infiltrative insulitis in the NOD mouse is marked by a decrease of serum IL-10 and an increase of serum IFN-γ, i.e. a shift towards a Th1 type inflammation (84). These data suggest that in the NOD mouse APC might be set at an anti-inflammatory set point compensating an underlying immune abnormality leading to infiltrative insulitis.

We therefore assume that the monocytes and monocyte-derived DC of the FDR of DM1 patients (also when they are at low risk) are set at an anti-inflammatory set point probably to compensate an underlying immune abnormality already existing in the FDR and leading to a proneness for autoreactivity and/ or inflammation. As a sign of such proneness we

found an increased sICAM-1 level in the serum of our low risk FDR. Others have also found a raised sICAM-1 in FDR of DM1 patients, be it in high risk FDR (85). sICAM-1 has been associated with various inflammatory diseases and might indeed represent a proneness for inflammation or an already existing inflammatory process in the FDR. However, one has also speculated on a tolerogenic role for sICAM-1. Reports show that s-ICAM inhibits the proliferation of autoreactive T cells and shifts the immune response to a Th2 cytokine profile (15;16;86). As such it would support the view of an anti-inflammatory set-point in our low risk FDR.

We like to construct the following hypothetic model for the development of human DM1:



1. In *healthy individuals* there is no elevation of sICAM-1 and the differentiation and function of monocyte and monocyte-derived dendritic cell functions and cytokine production are normal.

2. In an *early phase of predisposition to diabetes* (first degree family members) there is an elevation of sICAM-1, as a sign that there is an endothelial dysfunction and a tendency for leukocyte infiltration into tissues. Yet monocytes and DC are set at an anti-inflammatory set

point and counteract the tendency to develop autoimmunity. The majority of these individuals will never progress to pre-diabetes and overt diabetes.

3. In *an early pre-diabetic phase* pancreas infiltration starts and some islet-reactive antibodies may appear in the circulation. This infiltration is however of Th2 type and not destructive to islet cells (the peri-insulitis in the NOD mouse), since monocytes and DC are still levelled at an anti-inflammatory set point. A proportion of these individuals will never progress to destructive insulitis. Interestingly in the NOD mouse the generation of DC from precursors is already compromised in this phase (actually already from birth onwards). It seems not to be the case in the human.

4. *A late pre-diabetic phase*. High risk pre-diabetic FDR with positive antibodies to a panel of islet antigens show a pro-inflammatory Th1 set point (46;48;51;52;87;88). This would indicate that in this phase there is a shift from anti-inflammation characteristic of the early pre-diabetic phase (high IL-10 production by monocytes, appropriate DC, overrepresentation of Th2 cells) to pro-inflammation (low IL-10 production by monocytes, a defective dendritic cell function and generation, overrepresentation of Th1 cells). This shift to pro-inflammation would eventually lead to the actual islet cell destruction.

5. *Infiltrative insulitis and overt diabetes* develops, when monocytes and monocyte-derived DC have completely broken down and have totally lost their capacity to produce sufficient IL-10 and to develop into appropriately functioning and tolerizing DC.

A dissimilar pathogenesis of DM1 and autoimmune thyroid disease at the level of the immune system?

DM1 and autoimmune thyroid disease (AITD) are both organ specific autoimmune endocrine diseases and frequently coexist as so-called APS type 3b. This co-existence is reviewed in *chapter 2* and is particularly evident in the spontaneous animal models of DM1, the NOD mouse and the BB-DP rat. Thyroid autoimmunity is evident in up to one third of patients with DM1. Also relatives of DM1 patients, particularly their mothers, have a high frequency of AITD.

The literature on DC and AITD is reviewed in *chapter 7*. Apart from being involved in a physiological regulation of growth and function of thyrocytes, there also exists evidence that DC are involved in the early stages of autoimmune thyroid disease. Local thyroid DC start to form clusters and increase in number in the very early phases of the thyroid autoimmune reaction in the animal models of the disease (89). This local reaction of the intra-thyroidal DC is followed by the swelling of the regional lymph nodes and the production of anti-thyroglobulin

antibodies in these lymph nodes. Increased numbers of DC are also present in the thyroid glands of patients with Graves' disease and Hashimoto's thyroiditis (90;91).

Recently, our group described aberrancies in the expression of adhesion molecules on monocytes of AITD patients. Monocytes of such patients could not efficiently be activated by adherence to a fibronectin surface: the cells had a lower upregulation of adhesion molecules after such adherence as compared to monocytes of healthy controls, and had a reduced capacity to rearrange their cytoskeleton ("polarize") upon chemotactic stimuli. The fibronectin-adhered monocytes of AITD patients also had a reduced capacity to transform into actively moving accessory veiled macrophages (92).

In this thesis we describe that the expression of CD54 and that of other adhesion molecules on non-fibronectin adhered monocytes of patients with AITD was normal. We did detect a slight increase in serum sICAM-1.

With regard to the aberrancies of monocytes and monocyte-derived DC, which are characteristic of DM1 (the lower IL-10 production, the hampered differentiation, see before), we were unable to find such aberrancies in these cells of the AITD patients.

This means that the pathogenesis of DM1 and AITD (when occurring as separate diseases) differs at the level of the aberrancies in the immune system, although both disorders are closely related as organ specific autoimmune diseases and show a tendency to co-occur (see Chapter 2). Studies on monocytes and monocyte-derived DC in APS type 3b are therefore indicated.

Possible therapeutic strategies in DM1 based on the aberrancies described in this thesis.

There are a few possibilities to explore interventions in DM1, focussing on a correction of the here-described aberrant IL-10 production of DM1 monocytes and the aberrant accessory functions of the DM1 monocyte-derived APCs.

Drug treatment

The phosphodiesterase inhibitor pentoxifylline inhibits disease development in the BB-DP rat and NOD mouse model (93;94) and suppresses TNFα production and restores IL-10 production. It has minimal side effects. A decade ago, pentoxifylline has been used for treating new onset DM1 patients and was shown to decrease insulin requirement (95). Hence this drug is an interesting drug to be used in prediabetics at the time their monocytes loose the capacity to produce high quantities of IL-10.

Plain IL-10 administration might be effective as well under such circumstances. IL-10 is effective in psoriasis (96) and Crohn's disease (97). A disadvantage of a direct administration

Plain IL-10 administration might be effective as well under such circumstances. IL-10 is effective in psoriasis (96) and Crohn's disease (97). A disadvantage of a direct administration of the cytokine is its side-effects: transient neutrophilia, monocytosis, thrombocytopenia and T cell suppression (98;99).

Vaccination protocols

Although the role of the above-described defective APC function is not entirely clear in the development of DM1 and autoimmune thyroiditis, it is clear that DC form a potent group of cells to modulate immune responses. DC vaccination protocols are presently under design to elicit strong immune reactions (46) including autoimmune reactions to eradicate tumors, also thyroid malignancies (47). Such vaccination protocols aim at constructing DC potent to elicit strong effector immune responses. The present state of the art points to mature DC expressing important tumor antigens and producing IL-12 as the most likely candidates to perform this job in tumor eradication.

Since DC are also involved in tolerance induction, it is not a far-fetched idea to construct DC to induce or restore tolerance. Very immature steady state DC, expressing important auto-antigens and producing IL-10, are thought to be able to perform this job (5). A more focused approach is perhaps the vaccination with auto-antigen loaded DC which are in addition virus-transfected with IL-10. Since the IL-10 is defective in monocytes and mo-DC of DM1 patients, an artificially transient increased IL-10 expression in such cells might perhaps restore the counteracting regulatory T cell or Th2 responses. Transient expression is important, because continuous elevated production of IL-10 by APC is thought to increase susceptibility for infections (100).

However the differentiation and maturation defects of DC in the BB-DP rat and the NOD mouse suggest that there might in fact be no shortage of such very immature DC in these animals. The disease preventing effects of the transfer of artificially matured DC in the NOD mouse point in another direction, namely that mature DC are more critical to elicit or restore tolerance in conditions of endocrine autoimmunity (48, 49). Such DC might be critical for a deletion of autoreactive T cells in the periphery via AITCD.

Overall, we conclude that the development and function of monocyte-derived DC of DM1 patients are aberrant. One of the causes for the poor development of DC from monocytes probably is the low CD54 expression on monocytes of DM1 patients. Although this has to be investigated in more detailed experiments and in causal way. Moreover, the aberrantly developped DC in DM1 show a poor capability to skew T cells in either a Th1 or Th2 type response.

FDR show almost mirror images of the aberrancies in monocyte-derived DC. We hypothesize that the raised soluble ICAM-1 levels are a sign of proneness for inflammation

and that the mirror images of the aberrancies in monocyte derived DC serve to compensate for the pre-existing vulnerability for autoimmunity. When a FDR decompensates from an anti-inflammatory setpoint to a pro-inflammatory setpoint, the pathway to excessive insulitis and β cell destruction is taken. Treatments with anti-inflammatory agents, such as IL-10, could not only be usefull strategies in diabetic patients, but also FDR under such circumstances as preventative strategies.

The aberrancies found in DC of DM1 patients were not detectable in autoimmune thyroiditis patients. However, disturbances have been found in monocytes of AI thyroididtis patients, although not as outspoken as in DM1 patients.

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SUMMARY

Type 1 diabetes mellitus (DM1) and autoimmune thyroid disease (AITD) are organ specific autoimmune diseases in which the immune system is directed against the β cells and the thyrocytes respectively. The etio-pathogenesis of organ-specific or endocrine autoimmune diseases is complex, polygenic and heavily dependent on various environmental influences. The exact etiology of these diseases remains to be clarified, the pathogenesis is strongly associated with autoimmune phenomena. None of current treatment approaches provide a cure, but represent replacement therapies.

In *Chapter 2* we review the literature on the co-existence of DM1, AITD and autoimmune gastritis (AIG). These diseases often co-occur forming the so-called autoimmune polyendocrine syndrome (APS) type 3. In our own large study on DM1 families, we indeed showed a clear association between the presence of DM1 and AITD. We were not able to show an association between DM1 and AIG, probably due to the small numbers of subjects. Overall, it was concluded that thyroid autoimmunity and gastric autoimmunity are more frequent in patients with DM1 and in relatives of DM1 patients, particularly females. Therefore, screening DM1 patients and their relatives (particularly females) for thyroid (and to a lesser extent gastric) autoimmunity is recommended. If positive, excess iodine should be avoided and thyroxine treatment considered. There is not sufficient support to recommend screening for islet Ab in patients or relatives of patients with autoimmune thyroiditis and autoimmune gastritis.

In *Chapter 3* we discuss the usefulness of animal models for the understanding of precisely the very early stages of endocrine autoimmune diseases. To prevent the outbreak of endocrine organ-specific autoimmune diseases, detection of individuals at risk for such diseases and development of intervention strategies are crucial. An exquisite knowledge of the early stages of these diseases is therefore required. Experiments in human subjects at risk to develop such diseases can only be carried out to a limited extent. Animal models might be helpful in this problem. From the studies into the early stages of the pathogenesis of endocrine organ-specific autoimmune diseases in these animal models we have been able to construct a general blueprint for the etio-pathogenesis, which might lead the way for human studies. The animal models show various pre-autoimmune aberrancies in their target glands, T cells, macrophages (MΦ) and dendritic cells (DC). The aberrant target cells, T cells, DC and MΦ need to interact abnormally before the autoimmune disease can fully develop. The various aberrancies are partly genetically determined by a variety of separate genes (particularly MHC-related genes and other immune genes) and partly environmentally induced (e.g. via viruses, a

high iodine diet, and experimental manipulations). In this thesis we have concentrated on aberrancies in the monocyte and monocyte-derived DC compartment in humans with endocrine organ-specific autoimmune diseases, which are similar autoimmune abnormalities in the animal models already occurring in the pre-diabetic phase.

In *Chapter 4* we describe the role of monocyte and DC in endocrine organ-specific autoimmune diseases. The function of DC and their development from precursors have been shown defective in the animal models of DM1 and AITD as well as in DM1 patients. In this study we confirm that the generation of DC from DM1 monocytes results in populations of DC that are relatively immature. We also found that the expression of the adhesion molecules was considerably decreased on monocytes and DC of DM1 patients and that these molecules are involved in the differentiation and maturation of DC. We conclude that the low expression of CD54 on DM1 monocytes and immature DC likely plays a role – at least in part - in their defect to mature into fully potent APC. The aberrancies in adhesion molecule expression on monocytes and DC were not found in metabolic controls (DM2 patients) and patients with autoimmune thyroid disease (AITD).

In *Chapter 5* we analyse the cytokine production profile of monocytes and monocyte-derived DC of 22 DM1, 14 DM2, 21 AITD patients and 34 healthy controls and we showed that DM1 patients have an aberrant IL-10 / IL-12 production profile. Monocytes of DM1 patients had a lower capability to produce IL-10. DC were poor producers of IL-12. Autologous T cells stimulated by such DC were poor proliferators and poor producers of both Th1 type cytokines (IFN-γ) and Th2 type cytokines (IL-13 and IL-10). These results are compatible with the view that monocytes are in a pro-inflammatory state in DM1. The generation of DC from such monocytes is hampered and results in DC with an aberrant immature marker pattern (see also *Chapter 4*) and a low IL-12 production capability. Such aberrant DC are unable to stimulate T cells properly. We argue that such DC are in particular unable to induce tolerance towards autoantigens.

In *Chapter 6* we investigate whether the aberrancies we found in DM1 patients were also already detectable in 13 first degree relatives of DM1 patients. These relatives were except for one case all islet cell antibody negative. Like DM1 patients the relatives had increased serum levels of sICAM-1, yet the FDR showed a normal monocyte CD54 expression, an increased monocyte IL-10 production, an enhanced DC development from monocytes and these DC had an increased stimulatory capacity of T cell proliferation. Thus this study showed various aberrancies in the monocytes and DC of the FDR, which were mirror images of those found in overt DM1 patients. We argue that such aberrancies represent an anti-inflammatory and tolerogenic set point of the immune system in FDR of DM1 patients that is instrumental to counteract already existing harmful deviations in the immune system that heighten the risk for islet autoimmunity.

In *Chapter 7* we review the literature on the role of DC in the development of AITD. DC are normally present in the interstitium of thyroids and such DC regulate the growth and hormone production of thyrocytes. DC increase in number and form homotypic clusters in the very early phases of the thyroid autoimmune response in the animal models of endocrine autoimmune disease and in the thyroids of Hashimoto and Graves' patients. Therefore DC are the most likely candidates for the initiation of the thyroid autoimmune response. Unlike the situation in DM1 patients, however, the differentiation of DC from precursors is normal in patients with AITD as well as their monocytic IL-10 production. Some minor abnormalities in monocyte functioning and functioning of monocyte-derived APC have been detected previously in AITD patients. Apparently monocytes and monocyte-derived APC of DM1 patients show a wider spectrum of aberrancies as compared to monocytes and monocyte-derived APC of AITD patients.

In *Chapter 8* the results of chapter 2-7 are integrated and discussed to try to understand the etio-pathogenesis of organ specific autoimmune diseases in relation to the found aberrancies of monocyte and DC. Also suggestions for future therapies based on a correction of the described aberrancies are given.

SAMENVATTING VOOR NIET INGEWIJDEN

Type 1 diabetes mellitus (DM1) en autoimmuun schildklierziekten (AITD) zijn chronische orgaan specifieke autoimmuunziekten. Autoimmuunziekten zijn ziekten waarbij het afweersysteem van een individu zich tegen lichaamseigen cellen of stoffen keert en waardoor die cellen/ stoffen niet goed meer functioneren. DM1 en AITD onstaan als een groot deel van de insuline producerende cellen (β-cellen) in de alvleesklier en respectievelijk het schildklierweefsel gedestrueerd zijn. De behandeling is levenslang en bestaat uit toediening van hormoonvervangers. De complicaties en gevolgen van deze ziekten zijn ernstig. Tot op heden zijn er geen curatieve therapieën voorhanden en is het niet bekend waarom het afweersysteem zich tegen deze lichaamseigen cellen keert. In dit proefschrift beschrijven we de rol van het afweersysteem bij het ontstaan van deze ziekten.

Het is bekend dat AITD vaak voorkomt bij DM1 patiënten. In *hoofdstuk 2* hebben we onderzocht of dat ook geldt voor eerstegraads familieleden van DM1 patiënten. We hebben in een groot cohort van bijna 700 eerstegraads verwanten de aanwezigheid van AITD en atrofische gastritis (een andere orgaan specifieke autoimmuunziekte) bekeken. Atrofische gastritis is het gevolg van een destructieve autoimmuun reactie tegen de slijmproducerende cellen in de maag. We hebben de aanwezigheid van auto-antistoffen bepaald. Auto-antistoffen zijn eiwitten die specifieke lichaamseigen cellen en stoffen herkennen en ertegen gericht zijn en zij komen vaak voor in het bloed van patiënten met autoimmuunziekten. Uit dit onderzoek kwam naar voren dat de eerstegraads verwanten van DM1 patiënten frequenter auto-antistoffen tegen β-cellen en schildkliercellen hebben dan de algemene bevolking. Zij hebben dus naast een verhoogd risico op het ontwikkelen van DM1, ook een verhoogde kans op AITD. Bij dit onderzoek was er echter geen verhoogde frequentie van de aanwezigheid van auto-antistoffen tegen maagcellen gevonden.

Er bestaat een correlatie tussen DM1 met het vaker voorkomen van genen die verantwoordelijk zijn voor verhoogde gevoeligheid op autoimmuunziekten. Ook in deze studie zijn deze verbanden gevonden. Het is dus zinvol om eerstegraads verwanten van DM1 patiënten op auto-antistoffen tegen schildkliercellen te screenen. Indien deze auto-antistoffen aanwezig zijn, dient de schildklierfunctie bepaald te worden. Zo kan AITD vroegtijdig herkend en behandeld worden.

Omdat er bij de mens nog geen veilige niet invasieve methoden voor handen zijn om de initiatie en het verloop van de autoimmuunziekten te bestuderen, wordt er veel gebruik gemaakt van proefdiermodellen. In *hoofdstuk 3* beschrijven we de verschillende proefdiermodellen voor DM1 en AITD. Uit deze modellen blijkt dat reeds in de vroege fase

van de ziekten er afwijkingen zijn in de betreffende organen. Ook zijn al afwijkingen te vinden in de afweercellen (o.a. dendritische cellen (DC), macrofagen en T cellen). Deze afwijkingen kunnen zowel genetisch bepaald zijn als door omgevingsfactoren uitgelokt worden. De modellen hebben elk hun voordelen, maar ook nadelen voor de interpretatie naar de mens. Elk model kan staan voor de pathogenese van elke patiënt afzonderlijk, en meerdere modellen kunnen in een patiënt voorkomen. De extrapolatie van de uitkomsten van deze studies dient dan ook met uiterste voorzichtigheid te geschieden.

In dit proefschrift is de nadruk gelegd op de (vroege) afwijkingen in DC in DM1 en AITD. DC zijn de belangrijkste afweercellen voor het opgang zetten van een goede en effectieve afweerreactie. DC kunnen bacteriën en virussen opnemen, deze op een zodanig modificeren en presenteren dat deze herkend kunnen worden door andere afweercellen (met name T cellen). Daarnaast zijn DC ook essentieel voor het herkennen en tolereren van het eigen lichaam. DC zorgen voor een goede aansturing van T cellen. In proefdiermodellen voor DM1 en AITD zijn er aanwijzingen gevonden van gestoorde functies van DC waardoor de T cellen de lichaamseigen cellen aanvallen en zodoende de ziektebeelden kunnen ontstaan. In *hoofdstuk 4 en 5* bestuderen we de DC en haar voorlopercel, de monocyt, op afwijkingen in DM1 en AITD patiënten. We vonden dat monocyten van DM1 patiënten minder adhesiemoleculen op hun celoppervlakte hebben. Dit heeft gevolgen voor de adhesie en migratie van cellen uit de bloedbaan naar de plaats van ontsteking. Bovendien kan het gevolgen hebben voor de ontwikkeling van een monocyt tot DC en hun functies. DC van DM1 patiënten zijn minder goed ontwikkeld waardoor zij niet in staat zijn een goede afweerrespons te geven cq. T cellen te stimuleren. Tevens kunnen monocyten van DM1 patiënten minder interleukine (IL)-10 produceren, een ontstekingsremmende eiwit. IL-10 productie door deze cellen is belangrijk voor het induceren en behouden van tolerantie. Deze afwijkingen in DC en monocyten kunnen (mede) verantwoordelijk zijn voor het ontstaan van DM1. In AITD patiënten waren hierin geen afwijkingen gevonden.

Vervolgens onderzoeken we in *hoofdstuk 6* in een pilotstudie of ook eerstegraads verwanten afwijkingen hebben van hun DC en monocyten. Deze verwanten hebben geen auto-antistoffen tegen β-cellen en eerder onderzoek heeft uitgewezen dat dan het risico op het ontwikkelen van DM1 laag is. Hun DC zijn zeer goed ontwikkeld en hebben een hoge productie van IL-10, wat wijst op een afweeronderdrukkende reactie. Deze verwanten zouden, alhoewel ze genetisch gevoelig kunnen zijn om DM1 te ontwikkelen en zich aan dezelfde omgevingsfactoren blootsstaan als hun diabetische familielid, door de juiste afweeronderdrukkende reactie de ziekte niet ontwikkelen. Bij verwanten mét auto-antistoffen tegen β-cellen (hoog risico op het ontwikkelen van DM1) moet nog onderzocht worden of deze afweeronderdrukkende reactie aan het falen is. Bij deze personen zouden preventieve

therapieën toegepast kunnen worden. Het toedienen of stimuleren van de IL-10 productie is logischerwijs een mogelijke interventie.

In *hoofdstuk 7* beschrijven wij wat in de literatuur reeds bekend is over DC in AITD. Alhoewel wij geen duidelijke afwijkingen in DC en monocyten bij AITD patiënten hebben gevonden in *hoofdstuk 4*, zijn er in de literatuur (voornamelijk proefdiermodellen) toch aanwijzingen dat DC een rol spelen in de initiatie van de ziekte. Het is mogelijk dat deze op een ander niveau in het systeem plaatsvindt, zoals in de schildklier zelf of volgens een ander mechanisme. Het is ook mogelijk dat de afwijkingen alleen in de zeer vroege fase van de ziekte aanwezig zijn. Onderzoek hiernaar moeten nog plaatsvinden in eerstegraads verwanten van patiënten met AITD.

Samengevat is in dit proefschrift aangetoond dat DC een (centrale) rol spelen bij de initiatie van het autoimmuun proces bij DM1. Bovendien dragen de bevindingen bij aan mogelijke strategieën voor behandeling correctie van de defecten van DC bij de verwanten van DM1 patiënten.

LIST of ABBREVIATIONS

AIG	autoimmune gastritis
AITD	autoimmune thyroid disease
APC	antigen presenting cell
APS	autoimmune polyglandular syndrome
BB DP	biobreeding diabetes prone
BB DR	biobreeding diabetes resistant
CD	cluster of differentiation
cpm	counts per minute
CTLA-4	cytotoxic T lymphocyte antigen-4
DC	dendrtitic cell
DC-SIGN	DC-specific ICAM-grabbing non integrin
ECM	extracellular matrix
ELISA	enzyme linked immuno sorbent assay
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FN	fibronectin
GAD(A)	glutamate decarboxylase (antibody)
GD	Graves' disease
GM-CSF	granulocyte macrophage-colony stimulating factor
GPA	gastric parietal cell antibody
HLA	human leukocyte antigen
HT	Hashimoto's thyroiditis
ICA	islet cell antibody
ICAM	intercellular adhesion molecule
IFNγ	interferon γ
Ig	immunoglobulin
IL	interleukin
IP-10	interferon inducible protein-10
LFA	lymphocyte function associated antigen
mAb	monoclonal antibody
MHC	major histocompatibility complex
Mφ	macrophage
NOD	nonobese diabetic
OS	obese strain
PE	phycoerythrin
PERCP	peridin chlorophyl protein
poly I:C	polyionisinic-polycytidilic acid
RIP-LCMV	rat insulin promoter- lymphocytic choriomeningitis virus
SAC	staphylococcus aureus cowan strain 1
SD	standard deviation
TCR	T cell receptor
Th	T helper
TNFα	tumor necrosis factor α
Tx	thymectomy
VLA	very late antigen

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