# Defects in Antigen-Presenting Cells in the BB-DP Rat Model of Diabetes

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# I

# General Introduction

# 1. 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, it is thought that such discrimination is made through a series of complicated and multi-step interactions between various cells and compounds 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 violently 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 neuro-endocrine character; hence this category of autoimmune diseases is often referred to as "neuro-endocrine autoimmune diseases". Important target tissues are the thyroid, the islets of Langerhans, the stomach, the adrenal and the ovary; important neurological target tissues are both the peripheral and central nerves. In this thesis emphasis is on the study of an animal model of endocrine autoimmunity, i.e. the Biobreeding (BB)-rat, an inbred animal in which thyroid and islet autoimmunity develop spontaneously in a large proportion of animals.

There are ethical and technical restrictions to studying the etiology and the pathogenesis of endocrine autoimmune diseases in man. Reliance on animal models is in part 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 prodromal 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. Biopsies can be taken and the animals autopsied. Their genome can be altered. Therapies to prevent or reverse the disease can readily be tested.

Over the past 50 years a number of animal models of various endocrine autoimmune diseases have been developed (Table I). These animal models have greatly contributed to the knowledge concerning the etiology and the pathogenesis of endocrine 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 and probably diverse human disorder. The animal disease is often studied in specifically inbred animals to generate homogenous and extreme forms of the 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. This is exactly what we will do in the discussion section of this thesis; we will describe in detail what is presently known on the abnormalities in dendritic cell (DC) generation and function linked to the endocrine autoimmune reaction in the BB-DP rat, and we will compare these to the ones known in the NOD mouse (another model of spontaneously developing endocrine autoimmunity) and to the ones found in patients.

**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 (*iddm2*) | normal | autoimmune diabetes | lymphopenic |
| | | | $RT1^u$ (*iddm1*) | high iodine diet | focal thyroid infiltrates | |
| | BB-DR rat | none | lacks *iddm2* | KRV virus | autoimmune diabetes | no lymphopenia |
| | NOD mouse | none | $H\text{-}2^{g7}$ (*idd1*) | normal | autoimmune diabetes | |
| | | | *Idd3*, *idd5* | | autoimmune sialoadenitis | |
| | | | toxic iodine dose | focal thyroiditis | manipulable by cytokines | |
| | NOD $H\text{-}2^{h4}$ | none | high iodine diet | focal thyroiditis | | |
| | OS chicken | none | at least 5 genes | normal | severe thyroiditis | HPA-axis disturbances |
| 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=hyroglobulin; FCA=Freund's complete adjuvant; MBP=myelin basic protein; TSH-R=thyroid stimulating hormone receptor; GP=gastric parietal cell; NP=nucleoprotein; KRV=Kilham rat virus; HPA=hypothalamus pituitary adrenal; LCMV=ymphocytic choriomeningitis virus. Adopted from Lam-Tse.

Recent studies have culminated in the awareness that endocrine autoimmune diseases must be regarded as polygenic diseases, in which the penetrance of a combination of genes is strongly influenced by environmental factors. Firstly, multiple gene polymorphisms determine part of the aberrant immune response towards the endocrine self. The most important polymorphisms are those in the MHC region, pointing to differences in autoantigen presentation.[2,3,4] However other polymorphisms are also involved, including those with a general role in the regulation of the immune response, e.g. in the CTLA-4 and PTPN22 genes.[5-9] There is also the involvement of polymorphisms and even mutations in genes controlling autoantigen presentation in the thymus, such as the VNT-INS gene[10] and AIRE gene[11] respectively, which force the thymocytes to develop into autoreactive T cells. However, genetic polymorphisms or mutations are clearly not explaining the etiology in total. Mono-zygotic twin studies, for example, have shown a concordance rate ranging from an 80% for thyroid autoantibody positivity,[12] via a 30-40% for type-1 diabetes[13] to a meager 20% for Graves' disease.[14] This demonstrates the important role of environmental eliciting factors in the development of endocrine autoimmune diseases. Despite this important role of the environment, this thesis will primarily focus on the dendritic cell abnormalities in the BB rat in relation to the genes found important in this animal model. But I will here start the introduction with a section on tolerance.

## 2. TOLERANCE MECHANISMS

An essential prerequisite for the pathogenesis of autoimmune diseases is the breakdown of immune tolerance. Immune tolerance is defined as a state of unresponsiveness of the immune system against a specific antigen (Ag) or group of Ags. In the following section we will discuss the two main mechanisms of tolerance: central and peripheral tolerance.

Central tolerance in the process in which autoreactive lymphocytes are deleted from the repertoire, this occurs with regard to autoreactive T cells in the thymus. At the same time special subtypes of regulatory T cells (dampening autoreactive reactions) are created in the thymus in the central tolerance process.

Peripheral tolerance in the process in which autoreactive lymphocytes, which have escaped central tolerance mechanisms are kept under control in the periphery, e.g. by processes of Activation Induced T Cell Death (AITCD), negative signaling or by regulatory T cells expanded in the periphery from naïve T cells.

### 2.1. Central intrathymic tolerance

The thymus can spatially be subdivided into three main areas: cortex, medulla and cortico-medullary junction (Figure 1). The cortex consists mainly of thymocytes, cortical epithelial cells and, phagocytic 'tingible body' macrophages. The cortico-medullary junction mainly contains DC. The medulla contains more mature T cells, prominent medullary epithelial cells, Hassall's corpuscles, macrophages, dendritic cells and B cells.

The thymus being a prime central lymphoid organ acts as the T cell education center ensuring that T lymphocytes capable of reacting with foreign antigens are positively selected forming a recruitable population in our repertoire, while potentially strong autoreactive T effector lymphocytes are largely deleted from the repertoire (negative selection) or, when escaping negative selection are kept under control.

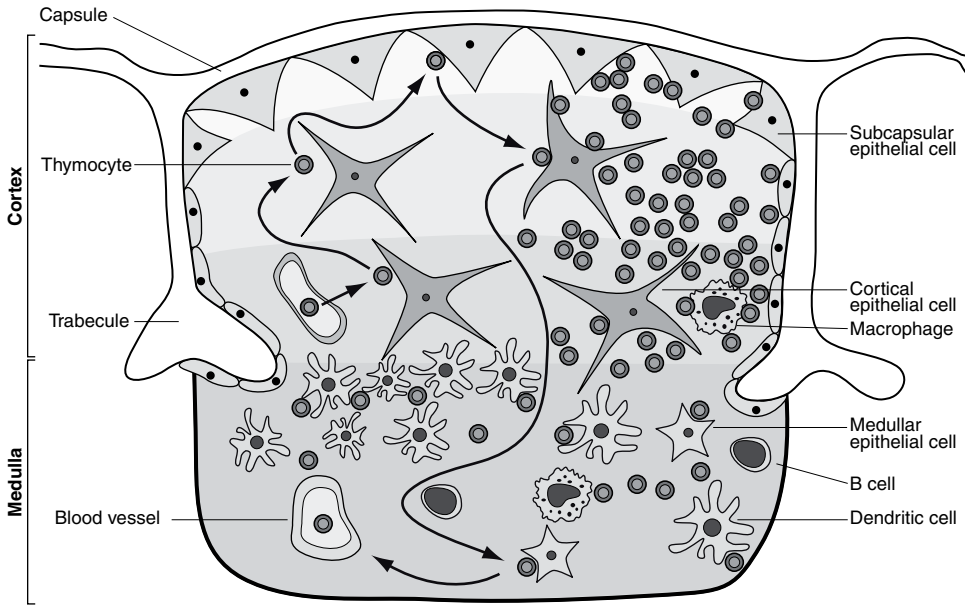


**Figure 1. Cellular composition of the thymus.**
The major cell types and the sequential cell-cell interactions along the development pathway of a thymocyte are depicted. Figure adopted from Kyewski et al. (2006).

The first checkpoint during T cell differentiation in the thymus is the so-called β-selection, which occurs upon pre-TCR signaling and ensures that only DN thymocytes which have successfully rearranged their TCRβ locus proceed to the DP ($CD4^+CD8^+$) thymocyte stage.[15] When the DP stage has successfully been reached TCRα is rearranged and all further selection of thymocytes is dictated by peptide-MHC class II (p-MHC) interaction on stromal cells within the thymus. Below I will discuss positive selection and negative selection.

*2.1.1. Positive selection*

Positive selection refers to the active process of rescuing MHC-restricted thymocytes from programmed cell death. Positive selection of thymocytes occurs when the thymocyte TCR interacts with p-MHC in a certain window of affinity/avidity. If this is interpreted as an unnecessary specificity, meaning a lack of self-MHC restriction (non-recognition), the result will be so-called "death by neglect" of thymocytes and the thymocytes will go into apotosis via an intrinsic death program. The loss of thymocytes during positive selection is mitigated by two mechanisms. First, TCRs normally randomly generated have an intrinsic affinity for their polymorphic MHC ligands. Second, the TCRα locus is able to recombinate several times within the 3-4 day life span of a DP thymocytes, allowing different TCR specificities to be generated and tested. This increases the chance of an appropriate TCR/p-MHC match and thereby thymocyte survival and the creation of a meaningful repertoire. Nonetheless most of

the thymocyte apoptosis is attributed to death by neglect, leading to a loss of thymocytes of approximately 90-95% and thus only 5-10% of developing thymocytes recognize a meaningful p-MHC on antigen-presenting cells in the cortex. Positive selection is generally thought to be mainly mediated by the MHC expressing thymus epithelial cells (TEC) in the cortex,[16] though we will give evidence in this thesis for a special sub-set of macrophages in the thymus cortex of the rat (the so-called branched cortical macrophages), which are also involved in positive selection.

*2.1.2. Negative selection*

Negative selection refers to the deletion or inactivation of potentially strong autoreactive effector thymocytes by interaction of these thymocytes with thymus APC expressing relevant autoantigens.

The medulla and cortico-medullary junction are thought to be the prime sites of getting rid of potentially autoreactive effector thymocytes. Three strong arguments support this view. First, at the double-positive stage the TCR is not fully up-regulated in the cortex. This low TCR is beneficial in the cortex, since this causes a bias towards low-avidity interactions with p-MHC, tipping the balance towards positive selection. Second, during transition into the medulla, full TCR up-regulation (T cell intrinsic) and co-stimulatory expression by DCs and medullary TECs (T cell extrinsic) provide a bias towards high-avidity signals. Third, the medulla provides the ideal environment, since self-antigens are expressed here. Negative selection depends a.o. on the tissue-specific antigen expression governed by the transcription factor *Aire* (highly expressed in medullary TECs). Because negative selection is imperfect, normal individuals harbour potential autoreactive effector T lymphocytes.

Next to the TECs thymic DC are thought to play a role in negative selection. Thymic DC are mainly localized at the cortico-medullary junction and in the medulla of the thymus. The first evidence for a role of bone-marrow derived cells (not being epithelial cells) in negative selection came from studies using radiation induced bone-marrow chimeras.

Presently several experiments suggest DC to be involved in negative selection: DC introduced in thymus organ cultures induce tolerance to alloantigen.[17] Furthermore selective expression of MHC class II I-E molecules on DCs under the control of the CD11c promoter resulted in the deletion of I-E-reactive $CD4^+$ T cells.[18]

In the mouse thymus two myeloid DC lineages contribute to the DC in the thymus. The $CD11c^+CD8\alpha^+CD11b^-$ sub-set develops within the thymus from early thymocyte precursors (the lymphoid lineage) and is the most predominant population, whereas the $CD11c^+CD8\alpha^-CD11b^+$ sub-set (the myeloid lineage) derives from myeloid DC precursors most likely circulating in the blood.[19] In the human thymus three distinct thymic DC are existing: the plasmacytoid DC and two myeloid DC sub-sets. The two myeloid DC are the $CD11c^+CD11b^-$ sub-set and the $CD11c^+CD11b^+$ sub-set.[20] The human lymphoid DC lineage probably also occurs in the thymus, but specific markers for this sub-set are lacking.[21] The role of the different thymic DC sub-sets is at present ill-defined.

*2.1.3. The expression of autoantigens in the thymus*

Antigens are expressed intrathymically by thymic APCs, i.e. the cortical and medullary thymic epithelial cells (cTECs and mTECs), thymic DCs, macrophages, and B cells.

Another way that antigen gains access to the thymus is via the circulation. The most prominent cell type expressing antigens is the mTEC. mTECS have recently been attributed a great deal of attention, since they express and present a vast array of tissue-restricted antigens. Therefore they are excellently equipped to perform a main task, the negative selection of potentially autoreactive effector T cells. *Aire* is presently the only known gene expressed in the thymus governing autoantigen expression, but the molecular and gene expression in the thymus regarding the expression of autoantigens is at present poorly understood.[11] Within the thymus *Aire* is highly expressed in mTECs, but also in DC, be it at much lower levels.[22] *Aire* is mutated in the rare autoimmune disorder autoimmune polyglandular syndrome type 1 (APS-1). Studies in *Aire*$^{-/-}$ mice have demonstrated that the Aire protein induces thymic-specific transcription of tissue-specific genes, which are involved in the establishment of central tolerance, such as oxytocin, insulin-like growth factor 2, insulin and neuropeptide Y.[23]

Interestingly, members of the major autoantigen in T1D, the insulin family, are all expressed in the thymus stroma according to a specific hierarchy and cellular topography. Insulin-like growth factor 2 (IGF-2) expression by TECs is the highest followed by IGF-1 expression by macrophages and INS expression by mTEC and/or DC.[24-26] Considering this specific hierarchial gene expression pattern, IGF-2 is better tolerated than INS. These observations are important in view of the fact that thymic expression of autoantigens increases self-tolerance and increases resistance against autoimmunity.[23,27]

### *2.1.4. The CD4$^+$CD25$^+$ T cells*

Apart from playing a role in positive and negative selection shaping the T cell repertoire, the thymus also creates naturally occurring regulatory T cells preventing autoimmunity. This is essential since negative selection is imperfect and autoreactive T effector cells have escaped to the periphery. We will here briefly discuss the CD4$^+$CD25$^+$ T cells, the ART2$^+$ regulatory T cells of the rat and the Invariant Natural Killer T (i-NKT) cells.

The recent interest in CD4$^+$CD25$^+$ T cells as a specific sub-population of thymus-derived regulatory T cells has a historical association with the day-3-mouse thymectomy model.[28,29] 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 TCRα 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, IL-15 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.[30,31]

Naturally occurring CD4$^+$CD25$^+$ Treg cells are defined by the constitutive expression of the high-affinity IL-2 receptor α chain (CD25) and IL-2 is indeed required for triggering their suppressive function.[32,33]

In human fetuses CD4$^+$CD25$^+$ Tregs can already be detected at an gestational age of

13 weeks and are already fully functional, able to maintain homeostasis at this early life-stage.[34] The differentiation of the majority of Tregs in the thymus commences upon induction of Foxp3 in a sub-set of thymocytes expressing αβ TCR having increased affinity for self p-MHC complexes.[35] The transcription factor forkhead box p3 (Foxp3), a gene coding for a member of the forkhead/winged-helix family of transcriptional regulators has been shown to be a specific marker for naturally occurring $CD4^+CD25^+$ Treg, playing a role as a key regulator in the programming of nTregs.[36,37,38] The continuous expression of Foxp3 is necessary to actively maintain a suppressive phenotype.[39] Additional signaling through the common γ chain (γc) and CD28 facilitates the Foxp3 dependent Treg development.[40] When analyzing $Foxp3^+$ cells histologically, they are primarily localized within the thymic medulla, a region containing mature thymocytes.[38,41] In accordance with this more than 90% of the $Foxp3^+CD4^+$ SP cells express low levels of CD24 ($HSA^{lo}$), a marker for mature thymocytes.[35] Next to CD25 expression naturally occurring Tregs are characterized by high surface expression of CTLA-4 and GITR.

Recently, an IL-7 like cytokine (TSLP, thymic stromal lymphopoietin), expressed by epithelial cells of the Hassall's corpuscles (HSC), is suggested to play a role in Treg selection. The TSLP-R (IL-7-like R) expressed by thymic DC has been suggested to positively select $CD4^+CD25^+Foxp3^+$ Tregs in the human thymic medulla.[42] A recent report in mice confirms the role of TSLP in Treg induction: TSLP strongly promotes $Foxp3^+$ Tregs in mouse thymic organ cultures.[43]

*2.1.5. $ART2^+$ regulatory T cells*

$ART2^+$ cells (formerly known as $RT6^+$) are known for quite some time to be important in the prevention of autoimmune diabetes in the BB rat model, since the adoptive transfer of these cells in BB-DP rats (lacking $ART2^+$ cells) prevented autoimmune diabetes, while ART2 depletion induced autoimmune diabetes in BB-Diabetes Resistant (DR) rats, a sub-line of BB rats having $ART2^+$ cells and without diabetes. Hillebrands et al. recently showed that there are two distinct potent $ART2^+$ Treg populations: $CD4^+ART2^+CD25^+Foxp3^+CD45RC^-PD\text{-}1^+$ and $CD4^+ART2^+CD25^-Foxp3^-CD45RC^-PD\text{-}1^+$ T cells.[44] The former overlaps with the natural occurring Treg cells being hyporesponsive and suppressive both *in vivo* and *in vitro*, while the latter is suppressive *in vivo,* but is not hyporesponsive and not suppressive *in vitro*. Both $ART2^+$ Treg populations were capable of preventing diabetes in BB-DP rats.

Until now not much is specifically known about the induction of these $ART2^+$ cells in the rat. Future work must uncover the exact mechanisms responsible for the induction of these important Treg populations in the rat.

*2.1.6. Invariant Natural Killer T cells and other naturally occurring Tregs*

Invariant (i)-NKT cells comprise a unique sub-set of T cells that co-express an invariant (i) TCR and natural killer (NK) cell related surface marker, including NK1.1.[45] i-NKT cell development is thymus dependent or independent and the liver serves as a candidate site for the extrathymic development of i-NKT cells, although this issue is controversial.

The strongest evidence in support of a role for i-NKT cells in the regulation of autoimmunity is provided by studies of autoimmune diabetes in humans,[46] NOD mice[47,48,49] and BB rats.[50] Considerable evidence suggests that NOD mice and T1D patients suffer from numerical and

functional deficiencies in i-NKT cells. In BB rats i-NKT cells numbers are reduced to 10% as compared with Diabetes Resistant rats.[50] It is thought that i-NKT cells can stimulate the maturation of dendritic cells, indirectly leading to Treg induction.[51]

Other Treg sub-sets include the $CD4^+CD25^+CD27^+CD70^-CD62L^+CTLA4^{high}$ (CD27+Treg) and $CD4^+CD25^-CD27^-CD70^+CD62L^-CTLA4^{intermediate}$ (CD27-Treg) cells. CD27 is a member of the tumor necrosis factor-receptor family.[5] Among $CD8^+$ T cells regulatory T cells sub-sets are also existing, like the $CD8^+CD28^-$.[53] This sub-set was effective in prevention of experimentally induced EAE[54] and Inflammatory Bowel Disease.[55]

## 2.2. Peripheral tolerance

### *2.2.1. Tolerogenic DC and Treg cell maintenance and induction*

Recently more insight has been gained on the importance of tolerogenic DC. Several tolerogenic DC populations and tolerogenic DC states have been described (Figure 2).

Although most early reports suggest that ‘’immature DC’’ act as tolerogenic DC by virtue of their anergy induction, more recent reports show that in particular also so-called steady state and semi-mature DC (i.e. naturally traveling DC not activated by “danger signals”) are particularly tolerogenic and the main inducers of Tregs, while DC activated by danger signals via their Toll-Like Receptors (TLR, e.g. TLR4 via LPS stimulation) are the ones instrumental in the expansion of effector T cells from naïve T cells.[56,57,58] The nomenclature of immature DC vs. mature DC is thus an oversimplification. There exist even mature DC stimulated by bacterial products that are tolerogenic. In fact DC encounter during their differentiation and depending on their localization (skin, liver, gut, lungs, etc.) and milieu (neuro-endocrine/

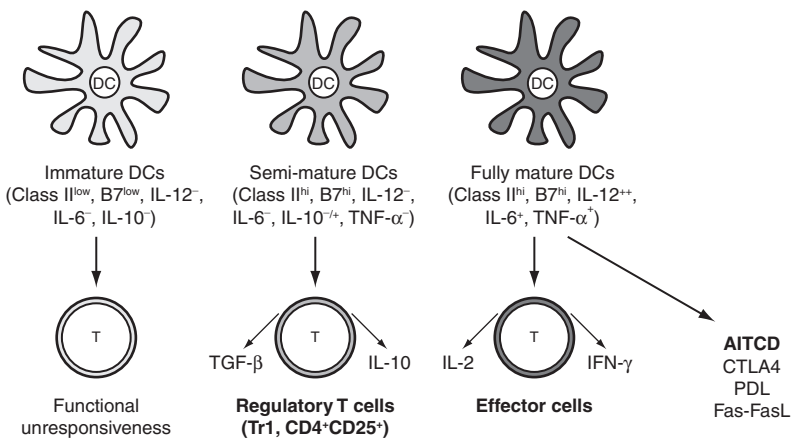


**Figure 2. The maturation state of a DC determines the T cell response.**
Immature DC induce unresponsiveness of T cells, while semi-mature DC induce regulatory T cells (naturally occurring $CD4^+CD25^+$ T cells and the adaptive regulatory Tr1 cells). Mature DC induce effector T cells and Activation Induced T Cell Death (AITCD) via CTLA-4, PDL and Fas-FasL interaction. Adopted from Lutz et al. (2002).

hormonal and microbial) various signals via their large array of surface receptors; these signals are then integrated resulting in either an immunogenic or tolerogenic DC phenotype.

One of the first populations of tolerogenic DC described *in vitro* and *in vivo* is the semi-mature DC which arise after maturation of immature DC (iDC) with TNF-α.[59,60] These *in vitro* generated semi-mature DC resemble steady state migratory DC[61,62] transporting self-antigens for peripheral tolerance *in vivo*. These semi-mature DC are able to induce $CD4^+$ Tr1 cells.[63]

Next to myeloid DC, plasmacytoid DC are capable to act as tolerogenic DC. Plasmacytoid DC can induce both $CD4^+$ and $CD8^+$ Tr1 cells. Particularly in the lung environment plasmacytoid DC have been shown to be important in the maintenance of tolerance. In the lung environment plasmacytoid DC are able, in an ICOS-L dependent way, to directly stimulate the formation of Treg and are able to suppress the potential of mDC to generate effector T cells.[64]

Also the mature myeloid DC populations, in particular, have been shown to be effective in expanding Tregs especially in an antigen-specific *in vitro* setup.[56,57]

Some populations of tolerogenic DC express IDO (indoleamine 2,3-dioxygenase), an enzyme degrading tryptophan, which is an essential amino acid. The mechanism of action of IDO expressing DCs is the inhibition of T cell proliferation by depleting the T cells of the essential amino acid tryptophan. Apart from that, IDO also generates quinolinic acid, while degrading tryptophan. This compound is thought to have a direct effect on the generation of adaptive Treg cells from naïve T cells.

Suffice now to say, that the book on tolerogenic DC has not been closed and that many more populations/states are and will be described influencing Treg cell development and tolerance induction.

### *2.2.2 Adaptive Tregulator cells*

Adaptive Tregs, unlike naturally occurring $CD4^+CD25^+$ Tregs, originate from uncommitted naïve or memory Th cells upon activation by tolerogenic APC. We will discuss just two well-known populations, the Tr1 and Th3 cells.

#### 2.2.2.1. Tr1 cells

The hallmark of Tr1 cells is their high production of IL-10. Tr1 cells are inducible in an antigen-specific manner via an IL-10 dependent process in the periphery from naïve $CD4^+$ T cells (but it has also been suggested that they are able to arise from Th1 and Th2 cells) and can therefore be considered adaptive Tregs. Studying Tr1 cells has been hampered by a lack of a specific marker for this particular sub-set. Tr1 cells, unlike nTregs, do not constitutively express Foxp3.

Specific IL-10 producing DC and plasmacytoid DC play an important role in the induction of Tr1 cells, the latter for the induction of $CD8^+$ Tr1 cells. An important growth/survival factor for Tr1 cells is IL-15. The mechanism of suppression by Tr1 cells is by the secretion of the cytokines IL-10 and TGF-β. IL-10 can mediate tolerance directly and indirectly. IL-10 with or without IFN-α can directly stimulate development of Tregs from human naïve T cells *in vitro*.[65] IL-10 indirectly suppresses cytokine production and proliferation of $CD4^+$ T cells by inhibiting the antigen-presenting capacity of APCs (DC, Langerhans cells and macrophages). Furthermore IL-10 inhibits the full maturation of DC and down-regulates MHC class II

expression and the capacity to produce IL-12.[66] Finally IL-10 treated DC with/without TGF-β can become regulatory DC, which consequently are able to induce Tr1 cells.[66]

2.2.2.2. Th3 cells

Th3 cells are another adaptive Treg sub-set, defined by their high production of TGF-β. In this way Th3 cells are able to directly regulate T cell function by inhibiting T cell proliferation and indirectly by induction of $Foxp3^+$ Tregs in the periphery. Like the naturally occurring $CD4^+CD25^+$ Treg, Th3 cells express the transcription factor Foxp3. Unlike natural Treg, IL-2 is dispensable for induction and Th3 cells can differentiate from the same precursor as pathogenic T cells.[67]

Th3 can affect DCs, since DC that encounter TGF-β1 down-regulate expression of CD80, CD86, TLR4[68] and, importantly, CCR7[69]. Low expression of CCR7 inhibits MIP-3β-induced migration to lymph nodes. Therefore, T lymphocyte stimulation is inhibited. The inability to recognize pro-inflammatory microbial products combined with decreased signaling capacity likely renders TGF-β1-treated dendritic cells ineffective in driving T lymphocyte immunity. TGF-β is also important for the maintenance of peripheral Tregs. Several groups showed that *in vitro* sorted $CD4^+CD25^-$ T cells stimulated with TGF-β, resulted in the generation of $CD25^+Foxp3^+$ comprising 10-50% of the proliferating cells.[70,71,72] In addition it was shown that IL-2 was a prerequisite for the TGF-β mediated induction of Tregs.[73,74]

*2.2.3. Activation induced T lymphocyte death (AITCD)*

Apart from maintenance of naturally occurring Treg cells and the induction of adaptive Treg cells AITCD plays an important role in keeping autoreactive T cells under control. After an effective immune response the activated T cells have to be removed, since they may be dangerous due to their secretion of inflammatory molecules and their cytotoxic activity. Further full TCR stimulation of already activated and expanded T cells can result in the efficient induction of AITCD, an apoptotic process.[74] AITCD occurs via stimulation through CD95[76], TNFR1[77] and TRAILR[78] and is induced by fully mature DC.

## 3. SOME FREQUENTLY USED ANIMAL MODELS OF SPONTANEOUSLY DVELOPING AUTOIMMUNE DISEASE

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

Of the spontaneous animal models the Bio-Breeding Diabetes-Prone (BB-DP) rat and the Non Obese Diabetes (NOD) mouse are the best studied models, and in this thesis we will concentrate on the BB-DP rat.

### 3.1. The BB-DP rat

The BB-DP rat is primarily used as a model for autoimmune diabetes.[79] In fact, this animal model is a model for autoimmune polyglandular syndrome (APS) type 3a, since the animal also has autoimmune thyroiditis.

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 spontaneously develop in a couple of days a T cell dependent insulitis, leading to a ketosis-prone diabetes, which is clinically very similar to T1D in humans. In generating the BB-DP line the animals were subject to inbreeding of different lines with a 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 onwards due to a mutation in one of the Immune-associated nucleotide (Ian) genes on rat chromosome 4.[80,81] 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 sub-lines of the BB-DP rat, that are not lymphopenic and do not develop diabetes. These lines are referred to as Diabetes Resistant or BB-DR rats. The peripheral lymphopenia of the BB-DP rat is primarily due to a lack of $ART2^+$ T cells.[82] ART2 is a marker for a sub-set of regulatory T cells (see before). Transfers of $ART2^+$ T cells from BB-DR rats to BB-DP rats prevent disease.[83] Although diabetes-resistant BB-DR rats are sufficient in $ART2^+$ T cells, they are still prone to diabetes: infection with Kilham Rat Virus (KRV) is a known inducer of autoimmune diabetes in these rats.[84] 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 Th2 to Th1 mechanisms.[85] Also treatment with poly I:C induces diabetes in these rats. A standardized approach is to treat the BB-DR rats with monoclonal anti-ART2 antibodies (depleting $ART2^+$ T cells) and poly I:C (stimulating TLR3), 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.[86]

BB-DP rats also suffer from a form of focal lymphocytic infiltrations that under normal conditions do not lead to hypothyroidism.[87] 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.[88,89] The thyroiditis is genetically linked to the MHC class II $RT1^u$ haplotype rather than to the *Ian5* gene mutation.[90]

### 3.2. The NOD mouse

The NOD mouse is predominantly studied for its autoimmune diabetes and autoimmune sialo-adenitis. 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 accumulation of DC, accessory macrophages, T cells and B cells (peri-insulitis) 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.

In general the incidence of thyroiditis is very low in the NOD mouse (unlike in the BB rat), but it varies from colony to colony.[91] Certain dietary iodine regimens, however, have

a triggering effect on thyroiditis development. In mice with a pre-existing iodine-deficient goiter, a single administration of a high dose of iodine has a necrotic effect on hyperplastic iodine-deficient glands, but such dietary manipulation does not lead to thyroid autoimmunity in normal, non-autoimmune strains. In contrast in NOD mice it does lead to autoimmune thyroiditis following the initial phase of thyrocyte necrosis.[91] This shows the importance of a local triggering factor (high antigen release, necrosis) acting upon a dysregulated immune system (the NOD mouse background) in the development of at least this endocrine organ-specific autoimmune disease.[91,92] Like the BB rat, this model is in fact also a model for APS type 3a.

There does, however, exist a sub-line 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.[93] This sub-line is characterized by an alternative MHC haplotype, viz. the $IA^{k}$ allele instead of the regular $IA^{g7}$ on the NOD background, and the mice are called NOD-H-$2^{h4}$ mice.

### 3.3. The multigenic, multi-environmental nature of autoimmune thyroiditis and autoimmune insulitis in the BB-DP rat and the NOD mouse

The studies in the two above-described animal models have shown that the pathogenesis of an autoimmune failure of the thyroid or the islets of Langerhans are multistep processes, requiring several genetic and environmental abnormalities (or variants) to converge before full-blown disease develops. Hence, autoimmune thyroiditis and autoimmune insulitis are the outcome of an unfortunate combination of various genetic traits and environmental circumstance that by themselves do not need to be harmful, and may even be advantageous.

Studies of the initial etio-pathogenic phases of autoimmune thyroiditis and autoimmune insulitis are 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. In this thesis the focus is on the BB-DP rat model for type-1 diabetes, specifically focusing on DC abnormalities present in the initial phases of the disease.

## 4. BB RAT GENES

Four diabetes susceptibility genes (*iddm*) have been localized in the BB-DP rat genome and are commonly referred to as *iddm1*, *iddm2*, *iddm3* and *iddm4*. Table II lists the rat diabetes susceptibility genes in relation to the genes important in the human and the NOD mouse.

### 4.1. *Iddm1*

The most important diabetes susceptibility gene *iddm1* is the MHC class II complex locus with the (diabetes and thyroiditis) susceptible haplotype $RT1^{u}$ on chromosome 20.[94] This $RT1^{u}$ haplotype is an orthologue to the human HLA-DQ. Also in the human the genes showing the strongest linkage to susceptibility/resistance to diabetes are located in the MHC/HLA region on human chromosome 6p21 (mouse: chromosome 17). In humans, a positive association (susceptibility) with the disease is certain for HLA-DR3, DR4 and DQ8, while a negative association (resistance) is apparent for HLA-DR2. The peptide binding pockets P1, P4 and P9

**Table II. An overview of diabetes susceptibility genes involved in spontaneous autoimmune diabetes in the BB rat, NOD mouse and human T1D.**

| Rat/Mouse/Human | BB rat | NOD mouse | Human T1D |
|---|---|---|---|
| *Iddm1*/*idd1*/*IDDM1* | $RT1^{u}$ (+) | $IA^{g7}$ (+) | HLA-DQ8 (+)<br>HLA-DR3 (+)<br>HLA-DR4 (+)<br>HLA-DR2 (-)<br>HLA-DQ6 (-) |
| *Iddm2*/*idd2*/*IDDM2* | *Lyp*/*Ian5*/*GIMAP5* | undefined | *INS-IGF2* VNTR |
| *Iddm3*/*idd3*/*IDDM3* | Body Weight gene | il2 | IGF-1R# |
| *Iddm4*/*idd4*/*IDDM4* | TCRVβ4# | Lmp2(PSMB6/β1i)/ ALOX15/ STAT5# | Fgf3# |
| *Iddm5*/*idd5*/*IDDM5* | - | ctla4 | SUMO4 # |
| IDDM12 | - | - | CTLA-4 |

(+) susceptibility gene
(-) resistance gene
# candidate genes

of the MHC class II molecules are the main determinants in susceptibility versus protection in type-1 diabetes.[95]

NOD mice have the susceptible MHC class II haplotype $IA^{g7}$ (murine orthologue for HLA-DQ). $IA^{g7}$ is special as it has a proline and serine at positions 56 and 57, instead of the histidine and aspartic acid found in most murine IAβ chains. The human DQ and $IA^{g7}$ show striking similarities. Both have the same preference for amino acids binding in the pockets, because the pockets (except for P1) are quite similar.

### 4.2. *Iddm2*

*Iddm2* is a very selective and important gene for the diabetes of the BB-DP rat. Selective breeding of an outbred colony from which the BB strain was derived led to the development of two sub-lines, the diabetes prone (DP) rat and the diabetes resistant (DR) rat (see before).[96] The major genetic difference between the BB-DP rat and the BB-DR rat is at the level of the so-called *lyp* gene being a major diabetes susceptibility gene *(iddm2*) located on chromosome 4.

Inheritance of *iddm2*/*lyp* occurs in a Mendelian fashion.[97] Homozygosity for the *lyp* gene, as is the case in the BB-DP rat, leads to the severe T lymphocytopenia and the lack in a specific sub-set of regulatory T cells, i.e. the $ART2^{+}$ T cells (see before).[96,97,98,99,100]

The T lymphocytopenia is only detectable in the peripheral blood. Numbers of lymphocytes are virtually normal in the thymus, but after export from the thymus the T cells dramatically decrease in numbers in the BB-DP rat. The reduction of T cells in the peripheral pool is as much as 80%.

Recently the *lyp/iddm2* gene was found, by positional cloning, to represent a frameshift mutation in the *Ian5* (immune-associated nucleotide 5) gene.[80,81] Due to the point mutation in the *lyp* gene the *Ian5* protein is severely truncated. *Ian5* belongs to an uncharacterized gene family of GTP-binding proteins. Defective *Ian5*/*lyp* causes loss of mitochondrial integrity, increased mitochondrial stress-inducible chaperonins and induces specific apoptosis of recent thymic emigrating T cells.[101] Recently an association of functional *Ian5* with the anti-

apoptotic proteins Bcl-2 and Bcl-xL was shown.[102] The truncated mutated *Ian5* of the BB-DP rat is thought to loose its mitochondrial-binding domain, resulting in an increased apoptosis of T cells. These findings suggest *Ian5* to be, under physiological conditions, an anti-apoptotic protein. A recent report using EGFP-Ian5, however suggest that the Ian5 protein is located at ER.[103] Another recent report using antiserum against Ian5, disputes the localization of the Ian5 protein at mitochondria and ER or Golgi complex, their data support a sub-cellular localization outside these organelles, however the data also shows that Ian5 is still necessary for maintaining mitochondrial membrane integrity (Ian5 protein is thought to act upstream of mitochondria in this report).[104]

### 4.3. *Iddm3* and *iddm4*

*Iddm3*, a gene stretch related to the development of autoimmune diabetes in rats, was found to co-localize with body weight regulating genes on chromosome 2.[105] *Iddm4* on chromosome 4 *w*as found in close proximity to the *lyp* region, although it is distinct from the *lyp* region.[106] A recent study suggests TCRVβ4 as a candidate gene for *iddm4*.[107]

## 5. CELLULAR AND MOLECULAR ABERRANCIES IN CENTRAL TOLERANCE MECHANISMS OF THE BB-DP RAT

### 5.1. Thymus morphology

The BB rat thymus shows several morphological abnormalities. In BB-DP and BB-DR rats so-called 'empty spaces' exist in thymus: thymus epithelium is absent from these areas, as are MHC class II expressing cells (DC/macrophages).[108] This phenomenon develops after and is present in four-week old BB rats. These 'empty spaces' cover 3-5% of total thymus volume in BB rats[109] and are thought to contribute to the development of autoreactive T cells, since the absence of TEC and APC in the medulla theoretically may lead to a failure to delete (potentially) autoreactive T effector cells. Likewise the empty spaces may also be involved in the failure to positively select regulatory T cells.

Interestingly, NOD mice show abnormalities in thymus morphology as well, as they contain large peri-vascular spaces (PVS). These PVS contain a dense network of the ECM proteins laminin, fibronectin and type IV collagen around the lumen of vessels.[110] The cells found inside PVS mainly consist of mature T cells and some B cells.[111] Another defect includes a decrease in thymus medullary epithelial cells and a preliminary involution of NOD thymus at an age of 5 weeks (normal mice 8-10 months) accompanied by a disorganization of thymic reticulum.[110] This premature involution in NOD mice is accompanied by the presence of large cystic cavities across the thymus reticulum from 5 weeks onwards.[112]

### 5.2. Apoptosis of recent thymus emigrants

BB-DP rats (*lyp*/*lyp*) are deficient in $TCR\alpha\beta^{high}CD4^{-}CD8^{+}$ thymocytes.[113,114,115] These recent thymus emigrants (RTE: $Thy1^{-}ART2^{-}$) disappear within 7 days of export in spleen and lymph nodes due to apoptosis.[115,116] Only very few cells finally express the mature T cell phenotype ($Thy1^{-}ART2^{+}$).[117] The life span of RTE in BB-DP rats is extremely shortened due to a mutation in the *lyp* gene.

### 5.3. Reductions in peripheral Treg cells ($CD4^+CD25^+$, NKT cells, $ART2^+$ T cells)

The *lyp* gene has a strong impact on T cell longevity in BB-DP rats. The $CD4^+CD25^+Foxp3^+$ thymocytes were investigated, and normal numbers were found.[118] However these cells were incapable of homeostatic expansion and survival upon transfer to nude BB rats, while *Foxp3* expression levels were reduced in mature $CD4^+CD25^+$ T cells, thus suggesting that they are mostly activated T cells. This notion was supported by the finding that these T cells were incapable of suppressing $CD4^+CD25^-$ T cells. The *lyp* gene leads to the altered survival and function of Tregs, which results in the clonal expansion of diabetogenic autoreactive T cells among the $CD4^+CD25^+$ T cells.

In the NOD mouse a reduction in number of $CD4^+CD25^+$ Tregs have been found at 3, 8 and 15 weeks in spleen (and thymus) compared to Balb/c mice.[119] Contrary result have also been found when analyzing the percentage $CD25^+$ among $CD4^+$ cells and significant differences where not found in the Tregs of 6-week-old NOD mice.[120] While a reduction of the percentage/number of Treg is under debate in the NOD mouse, there is consensus about the disturbed function of Tregs. During ageing (8 vs. 16 weeks) NOD Tregs loose the ability to suppress diabetogenic T cells (and prevent diabetes) upon transfer into NOD scid mice.[121] This loss-of-function is, at least in part, caused by loss of membrane-bound TGF-β from the surface of $CD4^+CD25^+$ T cells.[122]

Similar findings, as in the NOD mouse, have been observed in Tregs from diabetic patients, where the function is impaired. Co-cultures of Tregs with $CD4^+CD25^-$ T cells showed that diabetic Tregs where 2-fold less able to inhibit proliferation of $CD4^+CD25^-$ T cells as compared to Tregs from HLA-identical controls. The cytokine profile in the co-cultures of T1D patients showed a pro-inflammatory profile (more IFN-gamma, less IL-10).[123]

## 6. THE HISTOPATHOLOGY OF ISLET AUTOSENSITIZATION IN THE BB-DP RAT: A PRIME ROLE FOR AN EARLY LOCAL APC ACCUMULATION

Increased numbers of APC have been found in the very early stages of the autoimmune insulitis, autoimmune thyroiditis and autoimmune sialo-adenitis in the BB-DP rat and the NOD mouse prior to large lymphocytic infiltration. Similar observations have been done in the other animal model of spontaneous endocrine autoimmunity (the OS chicken),[124,125] in the thymectomy mouse model,[1] the virus- and chemically-induced models,[126,127] and the RIP-LCMV mouse model.[128] Also in the glands of patients with Graves' disease, Hashimoto goiter[129] autoimmune insulitis[130] and sialo-adenitis[131] MHC class II-positive DC and macrophages are present inside and outside the lymphocytic accumulations, but here only later stages of the diseases have been studied.

DC are antigen-presenting cells (APC) *par excellence*, and well equipped for the stimulation of naïve T cells.[124,132] Macrophages have various functions, ranging from the production of factors for wound healing and remodelling of tissues,[133] via the phagocytosis and degradation of unwanted material, the regulation of immune responses to a superb capability to stimulate T cells. It must be noted that DC have also been implicated in the remodelling of tissues.[134,135]

The (local) presence of APC has been shown to be indispensable for the development of autoimmune insulitis, since prevention of their accumulation in the pancreas of NOD mice,[136,137] in the pancreas of BB-DP rats results in a prevention of insulitis/diabetes.[138] Also splenic lymphocytes from macrophage-depleted NOD mice fail to transfer diabetes to recipients.[139]

The destiny of the majority of APC accumulated in tissues is to enter the lymphatics[126,134] and to travel to the draining lymph nodes while transporting antigens to these nodes and, under steady state, i.e. non-inflammatory, non-danger conditions, inducing tolerance induction primarily by supporting autoantigen specific naturally occurring Treg cells (Figure 3). In the spontaneous animal models, however, a sensitization reaction is induced in the local draining lymph nodes after the very early accumulation of APC in the thyroid or around the islets.[140,141] 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 autoantibodies 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[140,141] and the earlier mentioned peri-insular lymphoid tissue in the NOD mouse.[142] 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.[140,141] 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.[140] 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.[143]

## 7. AIMS OF THIS THESIS

### 7.1. AIM I: To find abnormalities in the function of bone-marrow derived DC and thymus DC of the BB-DP rat

In previous studies of our group relatively mild defects in the function of spleen DC of the BB-DP rat were found. In a first study by Delemarre et al. spleen low-density non-adherent DC ingested more bacteria (which points to a more macrophage-like phenotype) and the spleen DC had a reduced MHC class II expression. Functionally the abnormal spleen DC of BB-DP rats had a decreased capability to stimulate T cells to proliferate and more importantly the cells showed a defective capability to support the expansion of the regulatory $ART2^+$ T cell population of the rat.[144] In a later study of Delemarre et al. spleen DC of the BB-DP rat showed a lower homotypic clustering capability, a close correlate of maturation, as compared to control Wistar rats.[145]

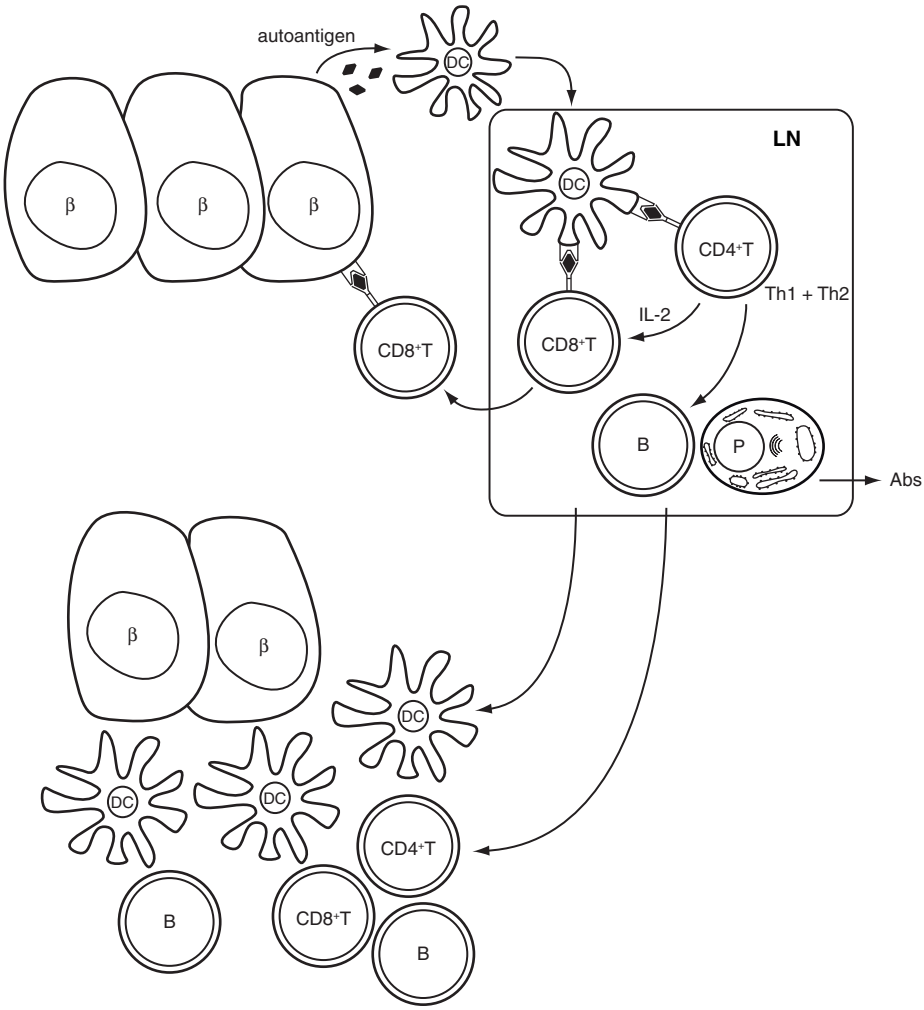


**Figure 3. The initial phase in type 1 diabetes.**
In the initial phase DCs are the very first immune cells to accumulate around β cells, where they take up autoantigen(s). Thereafter DC migrate with these autoantigens to the draining pancreatic lymph node (LN) where they present the autoantigen(s) to $CD4^+$ T cells, $CD8^+$ T cells and B cells. Part of the B cells differentiate into antibody-producing plasma cells (P) (top of the Figure).
In the expansion phase of type 1 diabetes DCs, $CD4^+$ T cells, $CD8^+$ T cells and B cells may form aggregates around the β cells in sort of local lymphoid tissue (bottom of the Figure).

In another study Simons et al. isolated non-adherent low-density APC from the thyroids of BB-DP rats; it appeared that fewer prototypical MHC class $II^+$ DC were found in these isolates (5-10%) as compared to non-adherent low-density cells from thyroids of Wistar rats control rats (15-20%). At the same time more $ED1^+$ (monocyte/macrophage-like) cells (70%) were in

the isolates as compared to Wistar rat thyroids (30-35%).[134]

In sum, our reports previous to this thesis work, provide evidence that spleen and thyroid DC of the BB-DP rat are abnormal, more macrophage-like with a poor APC function and particularly less capable of inducing $ART2^+$ regulatory T cells. However abnormalities found were not outspoken and many animals were needed to obtain significant differences.

Investigations regarding bone-marrow precursor derived DC and studies on thymus DC had not been performed in these previous studies in the BB-DP rat model. Furthermore linkage studies between DC aberrancies and gene abnormalities in the BB rat had not been performed. We hoped that these bone-marrow derived DC would show a clearer and more solid picture of aberrancies and therefore embarked on such studies.

*This thesis therefore addressed the following questions:*

- Are bone-marrow derived DC of the BB-DP rat defective in differentiation and function (Chapter 2)?
- Do bone-marrow derived DC of the F344.*lyp*/*lyp* rat show similar DC defects as bone-marrow derived DC of the BB-DP rat and are we able to make a detailed linkage of the found DC defects with the genetic background (Chapter 3)?
- Are thymus DC of the BB-DP rat defective in function (Chapter 4)?

### 7.2. AIM II: Targeting antigens to endocytical proteases in human monocyte-derived DC

The internalization and the subsequent processing of autoantigens leading to the generation of autoantigenic peptides presented on the MHC class II molecule have hardly been studied in type-1 diabetic DC. To approach this field I started to study the internalization and processing of antigen in human DC.

We used human monocyte-derived DC (MO-DC), since MO-DC provide attractive cellular tools in human autoimmune disorders. Myeloid DC internalize exogenous autoantigen and deliver it to the MHC class II-associated proteolytic machinery to generate antigenic peptides for the MHC class II-mediated activation of T cells. The generation of antigenic peptides is governed by endocytic proteases (cathepsins). The main endocytic proteases present in APC belong to the family of papain-like cysteine proteases. The pattern of endocytic proteases that is encountered by antigen internalized by intact human MO-DC is unknown. Understanding the rules that govern the transport of antigen to endocytic proteases and the successive processing pathway in MO-DC might help us to better exploit the therapeutic potential of these cells by maximizing the access of antigen to the MHC class II processing compartment, by inhibiting endocytic proteases responsible for the generation of the major immunogenic epitope in immunogenic DC or by facilitating the generation of the major immunogenic epitope in tolerogenic DC.

In previous studies MO-DC are usually exposed to soluble antigen or antigenic peptide, which is internalized by non-specific means, processed by endocytic proteases and loaded on MHC class II so that ultimately, antigen-specific T cells can be triggered. The phagocytosis of internalized material in DC has been difficult to study. Previous studies have focused on isolating lysosomes, late endosomes and early endosomes and showed that they differ with respect to the pattern of active proteases in all major types of APC. However such approaches have disrupted major determinants important in the regulation of protease activity like

endocytic pH, redox potential, protease inhibitors, thus being of little value in the study of the rendezvous between antigen and active proteases.

The use of chemical tools has recently overcome this limitation recently with the introduction of activity-base probes (ABP) facilitating the study of antigen delivery to the endolysosomal compartment.

The delivery of the endocytosed antigens (mimicked by the ABP) to the endocytic proteases (cathepsins) can be studied in intact viable DC. ABP specifically and irreversibly binds to the active centre of papain-like cysteine proteases and this enables the visualization of the targeted proteases via a detection tag.

The visualization of the active proteases occurs in a Western blot-like approach. While in Western blot both the inactive and the active form of the protease are visible and indistinguishable from each other, the activity-based probe approach solely allows the active form of the protease to be detected, since only the active form of the protease has an active center allowing irreversible binding and visualization with the ABP.

*This thesis therefore also addressed a 4$^{th}$ experimental question related to antigen processing by DC:*

- Which cathepsins are targeted by the endocytosis of antigen (ABP) and is this pattern altered when the antigen (ABP) is coupled to a cell-penetrating peptide (Chapter 5)?

## REFERENCES


1. Alderuccio F, Sentry JW, Marshall AC, Biondo M, Toh BH. Animal models of human disease: experimental autoimmune gastritis-a model for autoimmune gastritis and pernicious anemia. *Clin. Immunol.* 2002;102: 48-58.
2. Cheta D. Animal models of type I (insulin-dependent) diabetes mellitus. *J. Pediatr. Endocrinol. Metab.* 1998;11:11-19.
3. Todd JA. From genome to aetiology in a multifactorial disease, type 1 diabetes. *Bioessays*. 1999;21:164-174.
4. Mc Duffie M. Genetics of autoimmune diabetes in animal models. *Curr. Opin. Immunol.* 1998;10:704-709.
5. Kotsa K, Watson PF, Weetman AP. A CTLA-4 gene polymorphism is associated with both Graves disease and autoimmune hypothyroidism. *Clin. Endocrinol.* (Oxford) 1997;46:551-554.
6. Ueda H, Howson JM, Esposito L, Heward J, Snook H, Chamberlain G, Rainbow DB, Hunter KM, Smith AN, Di Genova G, Herr MH, Dahlman I, Payne F, Smyth D, Lowe C, Twells RC, Howlett S, Healy B, Nutland S, Rance HE, Everett V, Smink LJ, Lam AC, Cordell HJ, Walker NM, Bordin C, Hulme J, Motzo C, Cucca F, Hess JF, Metker ML, Rogers J, Gregory S, Allahabadia A, Nithiyananthan R, Tuomilehto-Wolf E, Tuomilehto J, Bingley P, Gillespie KM, Undlien DE, Rønningen KS, Guja C, Ionescu-Tîrgovişte C, Savage DA, Maxwell AP, Carson DJ, Patterson CC, Franklyn JA, Clayton DG, Peterson LB, Wicker LS, Todd JA, Gough SC. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature* 2003;423:506-511.
7. Bottini N, Vang T, Cucca F, Mustelin T. Role of PTPN22 in type 1 diabetes and other autoimmune diseases. *Semin. Immunol*. 2006;18:207-213.
8. Lee YH, Rho YH, Choi SJ, Ji JD, Song GG, Nath SK, Harley JB.The PTPN22 C1858T functional polymorphism and autoimmune diseases-a meta-analysis. Rheumatology (Oxford) 2007;46:49-56.
9. Gregersen PK, Lee HS, Batliwalla F, Begovich AB.PTPN22: setting thresholds for autoimmunity. *Semin. Immunol*. 2006;18:214-223.
10. Bennett ST, Wilson AJ, Esposito L, Bouzekri N, Undlien DE, Cucca F, Nisticò L, Buzzetti R, Bosi E, Pociot F, Nerup J, Cambon-Thomsen A, Pugliese A, Shield JP, McKinney PA, Bain SC, Polychronakos C, Todd JA. Insulin VNTR allele-specific effect in type 1 diabetes depends on identity of untransmitted paternal allele. The IMDIAB Group. *Nat. Genet.*1997;17:350-352.

11. Anderson MS, Venanzi ES, Klein L, Chen Z, Berzins SP, Turley SJ, von Boehmer H, Bronson R, Dierich A, Benoist C, Mathis D. Projection of an immunological self shadow within the thymus by the Aire protein. *Science* 2002;298:1395-1401.
12. Brix TH, Christensen K, Holm NV, Harvald B, Hegedüs L. A population-based study of Graves' disease in Danish twins. *Clin. Endocrinol.* 1998;48:397-400.
13. Dahlquist G. The aetiology of type 1 diabetes: an epidemiological perspective. *Acta Paediatr. Suppl.* 1998;425: 5-10.
14. Brix TH, Kyvik KO, Hegedüs L. A population-based study of chronic autoimmune hypothyroidism in Danish twins. *J. Clin. Endocrinol. Metab.* 2000;85:536-539.
15. Ciofani M, Zuniga-Pflucker JC. Notch promotes survival of pre-T cells at the beta-selection checkpoint by regulating cellular metabolism. *Nat. Immunol*. 2005;6:881-888.
16. Kyewski B, Klein L. A central role for central tolerance. *Annu. Rev. Immunol*. 2006;24:571-606.
17. Matzinger P, Guerder S. Does T-cell tolerance require a dedicated antigen-presenting cell? *Nature* 1989;338: 74-76.
18. Brocker T. The role of dendritic cells in T cell selection and survival. *J Leukoc. Biol.* 1999;66:331-335.
19. Donskoy E, Goldschneider I. Two developmentally distinct populations of dendritic cells inhabit the adult mouse thymus: demonstration by differential importation of hematogenous precursors under steady state conditions. *J. Immunol.* 2003;170:3514-3521.
20. Wu L, Shortman K. Heterogeneity of thymic dendritic cells. *Seminars in Immunology* 2005;17:304-312.
21. Blom B, Ligthart SJ, Schotte R, Spits H. Developmental origin of pre-DC2. *Hum. Immunol.* 2002;63: 1072-1080.
22. Cheng MH, Shum AK, Anderson MS. What's new in the Aire? *Trends Immunol.* 2007;28:321-327.
23. Ericsson A, Geenen V, Robert F, Legros JJ, Vrindts-Gevaert Y, Franchimont P, Brene S, Persson H. Expression of preprotachykinin-A and neuropeptide-Y messenger RNA in the thymus. *Mol. Endocr.* 1990;4:1211-1219.
24. Geenen V, Achour I, Robert F, Vandersmissen E, Sodoyez JC, Defresne MP, Boniver J, Lefebvre PJ, Franchimont P. Evidence that insulin-like growth factor 2 (IGF-2) is the dominant member of the insulin superfamily. *Thymus* 1993;21:115-127.
25. Jolicoeur C, Hanahan D, Smith KM. T-cell tolerance toward a transgenic beta-cell antigen and transcription of endogenous pancreatic genes in thymus. *Proc. Natl. Acad. Sci*. 1994;91:6707-6711.
26. Kecha O, Martens H, Franchimont N, Achour I, Hazée-Hagelstein MT, Charlet-Renard C, Geenen V, Winkler R. Characterization of the insulin-like growth factor axis in the human thymus. *J. Neuroendocrinol.* 1999; 11:435-440.
27. Sebzda E, Mariathasan S, Ohteki T, Jones R, Bachmann MF, Ohashi PS. Selection of the T cell repertoire. *Annu. Rev. Immunol.* 1999;17:829-874.
28. Smith H, Sakamoto Y, Kasai K, Tung KSK. Effector and regulatory cells in autoimmune oophoritis elicited by neonatal thymectomy. *J. Immunol.* 1991;147:2928-2933.
29. Tung KS, Smith S, Teuscher C, Cook C, Anderson RE. Murine autoimmune oophoritis, epididymo-orchitis, and gastritis induced by day 3 thymectomy. *Am. J. Pathol.* 1987;126:293-302.
30. Chatenoud L, Salomon B, Bluestone JA. Suppressor T cells-they're back and critical for regulation of autoimmunity! *Immunol. Rev*. 2001;182:149-163.
31. McHugh RS, Shevach EM, Thornton AM. Control of organ-specific autoimmunity by immunoregulatory CD4(+)CD25(+) T cells. *Microbes Infect*. 2001;3:919-927.
32. Piccirillo CA, Shevach EM. Naturally-occurring CD4+CD25+ immunoregulatory T cells: central players in the arena of peripheral tolerance. *Semin. Immunol*. 2004;16:81-88.
33. Miyara M, Sakaguchi S. Natural regulatory T cells: mechanisms of suppression. *Trends Mol. Med.* 2007;13: 108-116.
34. Cupedo T, Nagasawa M, Weijer K, Blom B, Spits H. Development and activation of regulatory T cells in the human fetus. *Eur. J. Immunol.* 2005;35:383-390.
35. Apostolou I, Sarukhan A, Klein L, von Boehmer H. Origin of regulatory T cells with known specificity for antigen. *Nat. Immunol.* 2002;3:756-763.
36. Hori S, Nomura T, Sakaguchi S: Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003;299:1057-1061.

37. Fontenot JD, Rudensky AY. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat. Immunol.* 2005;6:331-337.
38. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat. Immunol.* 2003;4:337-342.
39. Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat. Immunol*. 2007;8:277-284.
40. Liston A, Rudensky AY. Thymic development and peripheral homeostasis of regulatory T cells. *Curr. Opin. Immuno*l. 2007;19:176-185.
41. Darrasse-Jeze G, Marodon G, Salomon BL, Catala M, Klatzmann D. Ontogeny of CD4+CD25+ regulatory/ suppressor T cells in human fetuses. *Blood* 2005;105:4715-4721.
42. Watanabe N, Wang YH, Lee HK, Ito T, Wang YH, Cao W, Liu YJ. Hassall's corpuscles instruct dendritic cells to induce CD4+CD25+ regulatory T cells in human thymus. *Nature* 2005;436:1181-1185.
43. Jiang Q, Coffield VM, Kondo M, Su L. TSLP is involved in expansion of early thymocyte progenitors. *BMC Immunol*. 2007;8:11.
44. Hillebrands JL, Whalen B, Visser JT, Koning J, Bishop KD, Leif J, Rozing J, Mordes JP, Greiner DL, Rossini AA. Regulatory CD4+ T cell subset in the BB rat model of autoimmune diabetes expresses neither CD25 nor Foxp3. *J. Immunol.* 2006;177:7820-7832.
45. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu. Rev. Immunol.* 2007;25:297-336.
46. Chen YG, Choisy-Rossi CM, Holl TM, Chapman HD, Besra GS, Porcelli SA, Shaffer DJ, Roopenian D, Wilson SB, Serreze DV. Activated NKT cells inhibit autoimmune diabetes through tolerogenic recruitment of dendritic cells to pancreatic lymph nodes. *J. Immunol.* 2005;174:1196-1204.
47. Sharif S, Arreaza GA, Zucker P, Mi QS, Sondhi J, Naidenko OV, Kronenberg M, Koezuka Y, Delovitch TL, Gombert JM, Leite-De-Moraes M, Gouarin C, Zhu R, Hameg A, Nakayama T, Taniguchi M, Lepault F, Lehuen A, Bach JF, Herbelin A. Activation of natural killer T cells by alpha-galactosylceramide treatment prevents the onset and recurrence of autoimmune type 1 diabetes. *Nat. Med.* 2001;7:1057-1062.
48. Carnaud C, Gombert J, Donnars O, Garchon H, Herbelin A. Protection against diabetes and improved NK/NKT cell performance in NOD.NK1.1 mice congenic at the NK complex. *J. Immunol.* 2001;166:2404-2411.
49. Baxter AG, Kinder SJ, Hammond KJ, Scollay R, Godfrey DI. Association between alphabetaTCR+CD4-CD8- T-cell deficiency and IDDM in NOD/Lt mice. *Diabetes* 1997;46:572-582.
50. Iwakoshi NN, Greiner DL, Rossini AA, Mordes JP. Diabetes prone BB rats are severely deficient in natural killer T cells. *Autoimmunity* 1999;31:1-14.
51. Hermans IF, Silk JD, Gileadi U, Masri SH, Shepherd D, Farrand KJ, Salio M, Cerundolo V. Dendritic cell function can be modulated through cooperative actions of TLR ligands and invariant NKT cells. *J. Immunol*. 2007;178:2721-2729.
52. Ruprecht CR, Gattorno M, Ferlito F, Gregorio A, Martini A, Lanzavecchia A, Sallusto F. Coexpression of CD25 and CD27 identifies FoxP3+ regulatory T cells in inflamed synovia. *J. Exp. Med*. 2005;201:1793-1803.
53. Colovai AI, Mirza M, Vlad G, Wang S, Ho E, Cortesini R, Suciu-Foca N. Regulatory CD8+CD28- T cells in heart transplant recipients. *Hum. Immunol.* 2003;64:31-37.
54. Najafian N, Chitnis T, Salama AD, Zhu B, Benou C, Yuan X, Clarkson MR, Sayegh MH, Khoury SJ. Regulatory functions of CD8+CD28- T cells in an autoimmune disease model. *J. Clin. Invest.* 2003;112: 1037-1048.
55. Ménager-Marcq I, Pomié C, Romagnoli P, van Meerwijk JP. CD8+CD28- regulatory T lymphocytes prevent experimental inflammatory bowel disease in mice. *Gastroenterology* 2006;131:1775-1785.
56. Yamazaki S, Inaba K, Tarbell KV, Steinman RM. Dendritic cells expand antigen-specific Foxp3+ CD25+ CD4+ regulatory T cells including suppressors of alloreactivity. *Immunol. Rev.* 2006;212:314-329.
57. Tarbell KV, Petit L, Zuo X, Toy P, Luo X, Mqadmi A, Yang H, Suthanthiran M, Mojsov S, Steinman RM. Dendritic cell-expanded, islet-specific CD4+ CD25+ CD62L+ regulatory T cells restore normoglycemia in diabetic NOD mice. *J. Exp. Med.* 2007;204:191-201.
58. Fehérvári Z, Sakaguchi S. Control of Foxp3+ CD25+CD4+ regulatory cell activation and function by dendritic cells. *Int. Immunol.* 2004;16:1769-1780.
59. Voigtlander C, Rossner S, Cierpka E, Theiner G, Wiethe C, Menges M, Schuler G, Lutz MB. Dendritic cells matured with TNF can be further activated *in vitro* and after subcutaneous injection *in vivo* which converts their tolerogenicity into immunogenicity. *J. Immunother*. 2006;29:407-415.

60. Lutz MB, Schuler G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol.* 2002;23:445-449.
61. Morelli AE, Thomson AW. Dendritic cells: regulators of alloimmunity and opportunities for tolerance induction. *Immunol. Rev*. 2003;196:125-146.
62. Ohl L, Mohaupt M, Czeloth N, Hintzen G, Kiafard Z, Zwirner J, Blankenstein T, Henning G, Förster R. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity* 2004;21:279-288.
63. Menges M, Rossner S, Voigtlander C, Schindler H, Kukutsch NA, Bogdan C, Erb K, Schuler G, Lutz MB. Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity. *J. Exp. Med.* 2002;195:15-21.
64. Kool M, Lambrecht BN. Dendritic cells in asthma and COPD: opportunities for drug development. *Curr. Opin. Immunol.* 2007.
65. Levings MK, Sangregorio R, Galbiati F, Squadrone S, de Waal Malefyt R, Roncarolo MG.IFN-alpha and IL-10 induce the differentiation of human type 1 T regulatory cells. *J. Immunol.* 2001;166:5530-5539.
66. Steinbrink K, Graulich E, Kubsch S, Knop J, Enk AH. CD4(+) and CD8(+) anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. *Blood* 2002;99: 2468-2476.
67. Carrier Y, Yuan J, Kuchroo VK, Weiner HL. Th3 Cells in peripheral tolerance. II. TGF-β-transgenic Th3 cells rescue IL-2-deficient mice from autoimmunity. *J. Immunol.* 2007;178:172-178.
68. Mou HB, Lin MF, Cen H, Yu J, Meng XJ. TGF-beta1 treated murine dendritic cells are maturation resistant and down-regulate Toll-like receptor 4 expression. *J. Zhejiang Univ. Sci.* 2004;5:1239-1244.
69. Zhang Y, Zhang YY, Ogata M, Chen P, Harada A, Hashimoto S, Matsushima K. Transforming growth factor-beta1 polarizes murine hematopoietic progenitor cells to generate Langerhans cell-like dendritic cells through a monocyte/macrophage differentiation pathway. *Blood* 1999;93:1208-1220.
70. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J. Exp. Med.* 2003;198:1875-1886.
71. Fu S, Zhang N, Yopp AC, Chen D, Mao M, Chen D, Zhang H, Ding Y, Bromberg JS. TGF-beta induces Foxp3 + T-regulatory cells from CD4+ CD25- precursors. *Am. J. Transplant.* 2004;4:1614-1627.
72. Park HB, Paik DJ, Jang E, Hong S, Youn J. Acquisition of anergic and suppressive activities in transforming growth factor-beta-costimulated CD4+CD25- T cells. *Int. Immunol*. 2004;16:1203-1213.
73. Davidson TS, DiPaolo RJ, Andersson J, Shevach EM. Cutting Edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells. *J. Immunol.* 2007;178:4022-4026.
74. Zheng SG, Wang J, Wang P, Gray JD, Horwitz DA. IL-2 is essential for TGF-beta to convert naive CD4+CD25- cells to CD25+Foxp3+ regulatory T cells and for expansion of these cells. *J. Immunol.* 2007;178:2018-2027.
75. Roberts AI, Devadas S, Zhang X, Zhang L, Keegan A, Greeneltch K, Solomon J, Wei L, Das J, Sun E, Liu C, Yuan Z, Zhou JN, Shi Y. The role of activation-induced cell death in the differentiation of T-helper-cell subsets. *Immunol. Res.* 2003;28:285-293.
76. Fas SC, Fritzsching B, Suri-Payer E, Krammer PH. Death receptor signaling and its function in the immune system. *Curr. Dir. Autoimmun*. 2006;9:1-17.
77. Sytwu HK, Liblau RS, McDevitt HO.The roles of Fas/APO-1 (CD95) and TNF in antigen-induced programmed cell death in T cell receptor transgenic mice. *Immunity* 1996;5:17-30.
78. Janssen EM, Droin NM, Lemmens EE, Pinkoski MJ, Bensinger SJ, Ehst BD, Griffith TS, Green DR, Schoenberger SP. CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature* 2005;434:88-93.
79. Greiner DL, Rossini AA, Mordes JP. Translating data from animal models into methods for preventing human autoimmune diabetes mellitus: caveat emptor and primum non nocere. *Clin. Immunol.* 2001;100:134-143.
80. MacMurray AJ, Moralejo DH, Kwitek AE, Rutledge EA, Van Yserloo B, Gohlke P, Speros SJ, Snyder B, Schaefer J, Bieg S, Jiang J, Ettinger RA, Fuller J, Daniels TL, Pettersson A, Orlebeke K, Birren B, Jacob HJ, Lander ES, Lernmark A. Lymphopenia in the BB rat model of type 1 diabetes is due to a mutation in a novel immune-associated nucleotide (Ian)-related gene. *Genome Res.* 2002;12:1029-1039.
81. Hornum L, Romer J, Markholst H. The diabetes-prone BB rat carries a frameshift mutation in Ian4, a positional candidate of Iddm1. *Diabetes* 2002;51:1972-1979.

82. Greiner DL, Handler ES, Nakano K, Mordes JP, Rossini AA. Absence of the RT6+ T cells subset in diabetes-prone BB/W rats. *J. Immunol.* 1986;136:148-151.
83. Burstein D, Mordes JP, Greiner DL, Stein D, Nakamura N, Handler ES, Rossini AA. Prevention of diabetes in BB/Wor rat by singletransfusion of spleen cells: parameters that affect degree of protection. *Diabetes* 1989;38: 24-30.
84. Chung YH, Jun HS, Son M, Bao M, Bae HY, Kang Y, Yoon JW. Cellular and molecular mechanism for Kilham rat virus-induced autoimmune diabetes in DR-BB rats. *J. Immunol*. 2000;165:2866-2876.
85. Chung YH, Jun HS, Kang Y, Hirasawa K, Lee BR, Van Rooijen N, Yoon JW. Role of macrophages and macrophage-derived cytokines in the pathogenesis of Kilham rat virus-induced autoimmune diabetes in diabetes-resistant BioBreeding rats. *J. Immunol.* 1997;159:466-471.
86. Bieg S. Differential expression of p95vav in primary lymphoid tissue of BB rats congenic for the lymphopenia gene. *Autoimmunity* 1999;30:37-42.
87. Sternthal E, Like AA, Sarantis K, Braverman LE. Lymphocytic thyroiditis and diabetes in the BB/W rat (a new model of autoimmune endocrinopathy). *Diabetes* 1981;30:1058-1061.
88. Allen EM, Appel MC, Braverman LE; The effect of iodine ingestion on the development of spontaneous lymphocytic thyroiditis in the diabetes-prone BB/W rat. *Endocrinology* 1986;118:1977-1981.
89. Mooij P, de Wit HJ, Drexhage HA. An excess of dietary iodine accelerates the development of a thyroid-associated lymphoid tissue in autoimmune prone BB rats. *Clin. Immunol. Immunopathol.* 1993;69:189-198.
90. Pettersson A, Wilson D, Daniels T, Tobin S, Jacob HJ, Lander ES, Lernmark A. Thyroiditis in the BB rat is associated with lymphopenia but occurs independently of diabetes. *J. Autoimmun.* 1995;8:493-505.
91. Many MC, Maniratunga S, Denef JF. The non-obese diabetic (NOD) mouse: an animal model for autoimmune thyroiditis. *Exp. Clin. Endocrinol. Diabetes* 1996;104 Suppl 3:17-20.
92. Ruwhof C, Drexhage HA. Iodine and thyroid autoimmune disease in animal models. *Thyroid* 2001;11: 427-436.
93. Rasooly L, Burek CL, Rose NR. Iodine-induced autoimmune thyroiditis in NOD-H2h4 mice. *Clin. Immunol. Immunopathol.* 1996;81:287-292.
94. Colle E, Guttmann RD, Fuks A. Insulin-dependent diabetes mellitus is associated with genes that map to the right of the class I RT1. A locus of the major histocompatibility complex of the rat. *Diabetes* 1986;35: 454-458.
95. Cucca F, Lampis R, Congia M, Angius E, Nutland S, Bain SC, Barnett AH, Todd JA. A correlation between the relative predisposition of MHC class II alleles to type 1 diabetes and the structure of their proteins. *Hum. Mol. Genet.* 2001;10:2025-2037.
96. Markholst H, Eastman S, Wilson D, Andreasen BE, Lernmark Å. Diabetes segregates as a single locus in crosses between inbred BB rats prone or resistant to diabetes. *J. Exp. Med.* 1991;174:297-300.
97. Jacob HJ, Pettersson A, Wilson D, Mao Y, Lernmark A, Lander ES. Genetic dissection of autoimmune type I diabetes in the BB rat. *Nat. Genet.*1992;2:56-60.
98. Jackson R, Rassi N, Crump T, Haynes T, Eisenbarth GS. The BB diabetic rat. Profound T-cell lymphopenia. *Diabetes* 1981;30:887-889.
99. Guttmann RD, Colle E, Michel F, Seemayer T. Spontaneous diabetes mellitus syndrome in the rat. II. T lymphopenia and its association with clinical disease and pancreatic lymphocytic infiltration. *J. Immunol.* 1983;130:1732-1735.
100. Greiner DL, Handler ES, Nakano V, Mordes JP, Rossini AA. Absence of the RT-6 cell subset in diabetes-prone BB/W rats. *J. Immunol.* 1986;136:148-151.
101. Pandarpurkar M, Wilson-Fritch L, Corvera S, Markholst H, Hornum L, Greiner DL, Mordes JP, Rossini AA, Bortell R. Ian4 is required for mitochondrial integrity and T cell survival. *Proc. Natl. Acad. Sci.* 2003;100: 10382-10387.
102. Dalberg U, Markholst H, Hornum L. Both Gimap5 and the diabetogenic BBDP allele of Gimap5 induce apoptosis in T cells. *Int. Immunol.* 2007;19:447-453.
103. Nitta T, Nasreen M, Seike T, Goji A, Ohigashi I, Miyazaki T, Ohta T, Kanno M, Takahama Y. IAN family critically regulates survival and development of T lymphocytes. *PLoS Biol.* 2006;4:e103.
104. Keita M, Leblanc C, Andrews D, Ramanathan S. GIMAP5 regulates mitochondrial integrity from a distinct subcellular compartment. *Biochem. Biophys. Res. Commun.* 2007;361:481-486.

105. Klaff LS, Koike G, Jiang J, Wang Y, Bieg S, Pettersson A, Lander E, Jacob H, Lernmark A. BB rat diabetes susceptibility and body weight regulation genes colocalize on chromosome 2. *Mamm. Genome* 1999;10: 883-887.
106. Mordes JP, Leif J, Novak S, DeScipio C, Greiner DL, Blankenhorn EP. The iddm4 locus segregates with diabetes susceptibility in congenic WF.iddm4 rats. *Diabetes* 2002;51:3254-3262.
107. Blankenhorn EP, Descipio C, Rodemich L, Cort L, Leif JH, Greiner DL, Mordes JP. Refinement of the Iddm4 diabetes susceptibility locus reveals TCRVbeta4 as a candidate gene. *Ann. N. Y. Acad. Sci.* 2007;1103: 128-131.
108. Doukas J, Mordes JP, Swymer C, Niedzwiecki D, Mason R, Rozing J, Rossini AA, Greiner DL. Thymic epithelial defects and predisposition to autoimmune disease in BB rats. *Am. J. Pathol*. 1994;145: 1517-1525.
109. Rozing J, Coolen C, Tielen FJ, Weegenaar J, Schuurman HJ, Greiner DL, Rossini AA. Defects in the thymic epithelial stroma of diabetes prone BB rats. *Thymus* 1989;14:125-135.
110. Savino W, Boitard C, Bach JF, Dardenne M. Studies on the thymus in nonobese diabetic mouse. I. Changes in the microenvironmental compartments. *Lab. Invest.* 1991;64:405-417.
111. Savino W, Carnaud C, Luan JJ, Bach JF, Dardenne M. Characterization of the extracellular matrix-containing giant perivascular spaces in the NOD mouse thymus. *Diabetes* 1993;42:134-140.
112. Nabarra B, Andrianarison I. Thymus reticulum of autoimmune mice. 3. Ultrastructural study of NOD (non-obese diabetic) mouse thymus. *Int. J. Exp. Pathol*. 1991;72:275-287.
113. Groen H, Klatter FA, Brons NH, Mesander G, Nieuwenhuis P, Kampinga J. Abnormal thymocyte subset distribution and differential reduction of CD4+ and CD8+ T cell subsets during peripheral maturation in diabetes-prone BioBreeding rats. *J. Immunol*. 1996;156:1269-1275.
114. Plamondon C, Kottis V, Brideau C, Métroz-Dayer MD, Poussier P. Abnormal thymocyte maturation in spontaneously diabetic BB rats involves the deletion of CD4-8+ cells. *J. Immunol.* 1990;144:923-928.
115. Zadeh HN, Greiner DL, Wu DY, Tausche F, Goldschneider I. Abnormalities in the export and fate of recent thymic emigrant in diabetes-prone BB/W rats. *Autoimmunity* 1996;24:35-46.
116. Groen H, Klatter FA, Brons NH, Wubbena AS, Nieuwenhuis P, Kampinga J. High-frequency, but reduced absolute numbers of recent thymic migrants among peripheral blood T lymphocytes in diabetes-prone BB rats. *Cell. Immunol.* 1995;163:113-119.
117. Hosseinzadeh H, Goldschneider I. Recent thymic emigrants in the rat express a unique antigenic phenotype and undergo post-thymic maturation in peripheral lymphoid tissues. *J. Immunol*. 1993;150:1670-1679.
118. Poussier P, Ning T, Murphy T, Dabrowski D, Ramanathan S. Impaired post-thymic development of regulatory CD4+25+ T cells contributes to diabetes pathogenesis in BB rats. *J. Immunol.* 2005;174:4081-4089.
119. Wu AJ, Hua H, Munson SH, McDevitt HO.Tumor necrosis factor-alpha regulation of CD4+CD25+ T cell levels in NOD mice. *Proc. Natl. Acad. Sci.* 2002;99:12287-12292.
120. Berzins SP, Venanzi ES, Benoist C, Mathis D. T-cell compartments of prediabetic NOD mice. *Diabetes* 2003;52:327-334.
121. Gregori S, Giarratana N, Smiroldo S, Adorini L. Dynamics of pathogenic and suppressor T cells in autoimmune diabetes development. *J. Immunol.* 2003;171:4040-4047.
122. Gregg RK, Jain R, Schoenleber SJ, Divekar R, Bell JJ, Lee HH, Yu P, Zaghouani H. A sudden decline in active membrane-bound TGF-beta impairs both T regulatory cell function and protection against autoimmune diabetes. *J. Immunol*. 2004;173:7308-7316.
123. Lindley S, Dayan CM, Bishop A, Roep BO, Peakman M, Tree TIM. Defective suppressor function in CD4+CD25+ T-cells from patients with type 1 diabetes. *Diabetes* 2005;54:92-99.
124. Drexhage HA, Delemarre FGA, Radoševic K, Leenen PJM. 1999. Dendritic cells in autoimmunity. In: Lotze MT, Thomson AW (eds) Dendritic cells. Biology and Clinical Applications.
125. Hala K, Malin G, Dietrich H, Loesch U, Boeck G, Wolf H, Kaspers B, Geryk J, Falk, M, Boyd RL. Analysis of the initiation period of spontaneous autoimmune thyroiditis (SLT) in obese strain (OS) of chicken. *J. Autoimmun.* 1996;9:129-138.
126. Mooij P, de Wit HJ, Drexhage HA. An excess of dietary iodine accelerates the development of a thyroid-associated lymphoid tissue in autoimmune prone BB rats. *Clin. Immunol. Immunopathol.* 1993;69:189-198.
127. Yoon JW, Jun HS. Cellular and molecular pathogenic mechanisms of insulin-dependent diabetes mellitus. *Ann. N. Y. Acad. Sci.* 2001;928:200-211.

128. Von Herrath M, Holz A. Pathological changes in the islet milieu precede infiltration of islets and destruction of beta-cells by autoreactive lymphocytes in a transgenic model of virus-induced IDDM. *J. Autoimmun.* 1997;10:231-238.
129. Kabel PJ, Voorbij HAM, de Haan M, van der Gaag RD, Drexhage HA. Intrathyroidal dendritic cells. *J. Clin. Endocrinol. Metab.* 1988;66:199-207.
130. Jansen A, Voorbij HA, Jeucken PH, Bruining GJ, Hooijkaas H, Drexhage HA. An immunohistochemical study on organized lymphoid cell infiltrates in fetal and neonatal pancreases. A comparison with similar infiltrates found in the pancreas of a diabetic infant. *Autoimmunity* 1993;15:31-38.
131. Van Blokland SC, Wierenga-Wolf AF, van Helden-Meeuwsen CG, Drexhage HA, Hooijkaas H, van de Merwe JP, Versnel MA. Professional antigen presenting cells in minor salivary glands in Sjogren's syndrome: potential contribution to the histopathological diagnosis? *Lab. Invest.* 2000;80:1935-1941.
132. Steinman RM, Nussenzweig MC. Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. *Proc. Natl. Acad. Sci.* 2002;99:351-358.
133. Leenen PJM, and Campbell PA. 1993. Heterogeneity of mononuclear phagocytes. An interpretive review. In: Horon MH (ed) Blood Cell Biochemistry 5. New York, Plenum Press, pp.29-84.
134. Simons PJ, Delemarre FG, Drexhage HA. A functional and phenotypic study on immune accessory cells isolated from the thyroids of Wistar and autoimmune-prone BB-DP rats. *J. Autoimmun.* 2000;15:417-424.
135. Hoek A, Allaerts W, Leenen PJ, Schoemaker J, Drexhage HA. Dendritic cells and macrophages in the pituitary and the gonads. Evidence for their role in the fine regulation of the reproductive endocrine response. *Eur. J. Endocrinol*. 1997;136:8-24.
136. Hutchings P, Rosen H, O'Reilly L, Simpson E, Gordon S, Cooke A. Transfer of diabetes in mice prevented by blockade of adhesion-promoting receptor on macrophages. *Nature* 1990, 348:639-642.
137. Lee KU, Amano K, Yoon JW. Evidence for initial involvement of macrophage in development of insulitis in NOD mice. *Diabetes* 1988;37:989-991.
138. Hanenberg H, Kolb-Bachofen V, Kantwerk-Funke G, Kolb H. Macrophage infiltration precedes and is a prerequisite for lymphocytic insulitis in pancreatic islets of pre-diabetic BB rats. *Diabetologia* 1989;32: 126-134.
139. Jun HS, Yoon CS, Zbytnuik L, van Rooijen N, Yoon JW. The role of macrophages in T cell-mediated autoimmune diabetes in nonobese diabetic mice. *J. Exp. Med*. 1999;189:347-358.
140. Kabel PJ, Voorbij HA, de Haan-Meulman M, Pals ST, Drexhage HA. High endothelial venules present in lymphoid cell accumulations in thyroids affected by autoimmune disease: a study in men and BB rats of functional activity and development. *J. Clin. Endocrinol. Metab*. 1989;68:744-751.
141. Voorbij HAM, Kabel PJ, de Haan M, Jeucken PHM, van der Gaag RD, de Baets MH, Drexhage HA. Dendritic cells and class II MHC expression on thyrocytes during the autoimmune thyroid disease of the BB rat. *Clin. Immunol. Immunopathol*. 1990;55:9-22.
142. Rosmalen JG, Martin T, Dobbs C, Voerman JS, Drexhage HA, Haskins K, Leenen PJ. Subsets of macrophages and dendritic cells in nonobese diabetic mouse pancreatic inflammatory infiltrates: correlation with the development of diabetes. *Lab. Invest.* 2000;80:23-30.
143. Wong FS, Janeway CA Jr. Insulin-dependent diabetes mellitus and its animal models. *Curr. Opin. Immunol*. 1999;11:643-647.
144. Delemarre FG, Simons PJ, de Heer HJ, Drexhage HA. Signs of immaturity of splenic dendritic cells from the autoimmune prone biobreeding rat: consequences for the *in vitro* expansion of regulator and effector T cells. *J. Immunol.* 1999;162:1795-1801.
145. Delemarre FG, Hoogeveen PG, De Haan-Meulman M, Simons PJ, Drexhage HA. Homotypic cluster formation of dendritic cells, a close correlate of their state of maturation. Defects in the biobreeding diabetes-prone rat. *J. Leukoc. Biol.* 2001;69:373-380.

# II

## Defects in differentiation of bone-marrow derived dendritic cells of the BB rat are partly associated with *iddm2* (the *lyp* gene) and partly associated with other genes in the BB rat background

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## ABSTRACT

BB rats develop various organ-specific autoimmune diseases, e.g. autoimmune diabetes and thyroiditis and have proven important to dissect genetic factors that govern autoimmune disease development. The *lymphopenia (lyp)* gene *(iddm2)* is linked to autoimmune disease development and is a major genetic difference between diabetes-resistant (DR) and diabetes-prone (DP) BB rats. To study the effects of the *lyp* gene and other genes on dendritic cell (DC) differentiation from bone-marrow precursors, such differentiation was studied in BB-DP, BB-DR, Wistar and F344 control rats.

DC of BB-DP rats showed a lower MHC class II expression as compared to BB-DR, Wistar and F344 rats. LPS maturation did not restore this low MHC class II expression. DC of BB-DP rats also showed a poor capability to terminally differentiate into mature T cell stimulatory DC under the influence of LPS and produced significantly lower quantities of IL-10, yet these aberrancies were also found in BB-DR rats but did not occur in control rats.

This study thus shows that various aberrancies exist in the differentiation of myeloid DC from bone-marrow precursors in the BB rat model of organ-specific autoimmunity. These aberrancies are multigenically determined and partly associated with *iddm2 (lyp* gene) and partly associated with other genes in the BB rat.

# INTRODUCTION

Dendritic cells (DC), the antigen-presenting cells *par excellence*, play a pivotal role in the pathogenesis of organ-specific autoimmune diseases. In animal models that spontaneously develop such diseases, such as the BioBreeding-Diabetes Prone (BB-DP) rat and the Non-Obese Diabetic (NOD) mouse, MHC class $II^+$ DC accumulate in the thyroid gland, the pancreas and the salivary glands from the earliest phases of the disease onwards.[1-7] These DC take up relevant autoantigens and travel with these to the draining lymph nodes, where the specific autoimmune response is initiated. Indeed, the very early accumulation of DC in the target glands of the animal models is followed by an enlargement of the lymph nodes draining the glands and the production of autoantibodies by plasma cells located in these lymph nodes.[1,3] This lymph node reaction is followed later in time by a further infiltration of the target gland by large numbers of lymphocytes. It is likely that also in patients with type-1 diabetes mellitus, autoimmune thyroid disease and Sjögren's syndrome DC play a pivotal role as antigen-presenting cells (APC), since the cells are seen in large numbers in the target glands.[1,8-11]

With regard to the BB rat model we previously reported that *ex vivo* preparations of spleen DC and of thyroid DC displayed abnormalities. The spleen DC of the BB-DP rat showed a lower MHC class II expression, had a lower capability to form homotypic cell aggregations and had a reduced capability to stimulate T cells in syngeneic (syn)-MLR.[12,13] The DC preparation of the thyroid gland of the BB-DP rat contained more monocyte-like precursors and fewer cells with the characteristics of typical MHC class $II^+$ DC as compared to cell preparations of thyroids of control Wistar rats.[14] We took these abnormalities in both the DC populations as signs of defects in the differentiation/maturation of BB-DP DC.[12-14]

*In vitro* studies on the differentiation of DC from bone-marrow precursors have not been previously performed in BB-DP rats and this report describes such studies. To study the *in vitro* differentiation of DC from GM-CSF/IL-4 stimulated bone-marrow precursors, we used a culture procedure initially described for the Wistar rat and sampled the non-adherent cells, which are typical DC showing crucial markers and functional characteristics of this sub-set of APC. To further terminally differentiate these immature DC to mature DC we additionally stimulated the cells with LPS.

One of the advantages of studying the BB-DP rat model is the presence in this model of a well-defined regulatory T cell population. These regulatory T cells are characterized by ART2 expression and play a crucial role in the pathogenesis of the organ-specific autoimmune diseases in the BB-DP rat.[15] BB-DP rats are severely lymphopenic and lack in particular the $ART2^+$ regulatory T cells. There exists a sub-line of BB rats, the so-called BB-Diabetes Resistant (DR) rats, that are not lymphopenic, have $ART2^+$ regulatory T cells and do not develop organ-specific autoimmunity. Depletion of $ART2^+$ cells in the BB-DR rat induces organ-specific autoimmunity,[16] while transfer of these cells from BB-DR rats to BB-DP rats prevents organ-specific autoimmunity.[17] The major genetic difference between the BB-DP and BB-DR rats is the *lymphopenia* (*lyp*) gene, which is a major diabetes susceptibility gene for the rat (*iddm2*) located on chromosome 4. Homozygosity for the *lyp* gene, as is the case in the BB-DP rat, leads to the severe T lymphocytopenia and in particular a lack in $ART2^+$ regulatory T cells.[18,19] Recently, the *lyp* gene was found to carry a frameshift mutation in the gene for

*Ian5*. Due to the mutation in the *lyp* gene the predicted Ian5 protein is severely truncated.[20,21]

In this report we describe various abnormalities in the *in vitro* differentiation of DC from bone-marrow precursors in the BB-DP rat model as compared to BB-DR rats and non-autoimmune Wistar and F344 control rats. We used 3 different BB-DP rat sub-lines, i.e. the NB sub-line, the Groningen sub-line and the Seattle sub-line. To study the DC abnormalities in relation to the expression of the *lyp* gene we consider the Seattle sub-line the best, since the BB-DP/Seattle rat has been developed as a congenic strain on the BB-DR/Seattle rat background and only possesses a small *lyp* region containing part of chromosome 4 of the BB-DP rat (76.35-77.67 Mb). BB-DP/Seattle rats are lymphopenic and type-1 diabetes occurs in 100% of the Seattle BB-DP rats. We here also describe that we were able to link one of the found abnormalities in the bone-marrow derived DC, i.e. the lower MHC class II expression on DC, to the expression of the *lyp* gene containing part of chromosome 4 of the BB-DP rat. Other DC abnormalities, such as a poor capability of the DC to terminally differentiate into mature DC after LPS stimulation and to produce IL-10, appeared to be linked to non-*lyp* genes in the BB rat background.

## MATERIALS AND METHODS

### Animals

BB-DP rats from the NB sub-line were provided by BRM Inc. (Massachusetts, USA). BB Groningen rats (DP and DR) were provided by Dr. Jan Rozing (University of Groningen, The Netherlands). BB-DP/Seattle (BB-DP/S), BB-DR/Seattle (BB-DR/S) and Fischer/F344 control rats were developed and maintained at the Robert H. Williams Laboratory at the University of Washington, Seattle, U.S.A. Animals were housed under SPF conditions. All rats were kept under controlled light conditions (12/12 h light/dark cycle) throughout this study. A standard pelleted diet (0.35 mg iodine/kg; AM-II, Hope Farms BV, Woerden, The Netherlands) and tap water were provided *ad libitum*. For all experiments female animals were used and as controls age and sex matched F344 rats (R.H. Williams Laboratory) and/or Wistar rats (Harlan, Zeist, The Netherlands) were used. Use and care of laboratory animals was performed according to guidelines of the Dutch national law.

### Bone-marrow derived DC culture

Rat bone-marrow was obtained from tibia and femur. Erythrocytes were lysed by $NH_4Cl$ containing $KHCO_3$ and EDTA. After erythrocyte lysis cells were resuspended in RPMI 1640 containing 25mM HEPES buffer (Life Technologies), 10% FCSi, 50 μM β-mercapto-ethanol (Merck, Munich, Germany), and antibiotics. Cells were brought to a concentration of 10 x $10^6$/10 ml and cultured in 25 $cm^2$ culture bottles (Nunc, Roskilde, Denmark) for 9 days (37°C, 5% $C0_2$ incubator). To obtain DCs from bone-marrow precursor cells rat recombinant GM-CSF (17 μg/ml) and rat recombinant IL-4 (20 μg/ml) were added (Biosource, Camarillo, CA, USA) to the culture medium. Medium was refreshed twice at day 4 (with cytokines) and day 7 (without cytokines to enrich for DCs: deplete granulocytes). At day 7 the non-adherent cells were either resuspended in medium to obtain immature DC or in LPS (1 μg/ml) to obtain mature DC and were further cultured for 48 h. and were harvested by rinsing the bottles with medium twice.

**Methods for quantitative PCR**

Quantitative PCR was performed on an Mx4000 instrument (Stratagene, La Jolla, CA, USA) using DNAse treated RNA (100 ng of total RNA) in triplicate using the Brilliant Single-Step Quantitative RT-PCR Core Reagent Kit (Stratagene) along with serially diluted standard samples. The standards used were 1:4 serial dilutions of BB-DR/Seattle rat spleen poly A+ RNA.

Samples were multiplexed with a rat *Ian5* probe and a rat cyclophilin probe. The r*Ian5*-Q2 probe was designed to the 3´ end of exon 3, beyond the coding region.

Probes and primers were from Integrated DNA Technologies, Inc. (Coralville, IA, USA). The probe for rat *Ian5* was r*Ian5*-Q2: 5'-FAM-TTT CAC TAT CAT TTG ACT CCT GTG CA-BHQ-1-3'. The probe for rat cyclophilin was 5'-HEX-CTG CTT CGA GCT GTT TGC AGA C-BHQ-1-3'. These probes were carefully designed, assuring that they did not match any other gene in the rat database.

The primers for probe r*Ian5*-Q2 were r*Ian5*-Q2f: 5'-CAT GTT AGG GAA GCT CAG TC-3' and r*Ian5*-Q2r: 5'-GAA GGG TTC TAC TGT GTC TCA-3'. The primers for rat cyclophilin were rCyc-Qf: 5'-CAC CGT GTT CTT CGA CAT-3' and rCyc-Qr: 5'-TTT CTG CTG TCT TTG GAA CT-3'. Resulting C(t) values were converted to picograms and normalized to the values of cyclophilin from the same well and expressed as the average of triplicate samples G one standard deviation. Total RNA was isolated using Qiagen RNeasy (Qiagen, Chatsworth, CA, USA) or Stratagene Absolutely RNA RT-PCR Miniprep kits (Stratagene). To remove trace genomic DNA, Ambion DNA-free (Ambion, Austin, TX, USA) treatment was performed on samples extracted with Qiagen RNeasy kit.

**Antibodies**

The following mAbs were used: anti-MHC class II conjugated to phycoerythrin (PE) (1:400, MRC OX6; Serotec), anti-B7-1 (undiluted, CD80; BD Pharmingen, Flanders, NJ), anti-B7-2 (undiluted, CD86; BD Pharmingen), anti-CD40 (1:10, BD Pharmingen), anti-rat DC-FITC (undiluted, MRC OX62; Serotec), anti-rat CD11c-FITC (undiluted, Serotec), ED1 (1:10, DC, monocytes, MΦs; Serotec), ED2 (1:100, monocytes, MΦs; Serotec), ED3 (1:1000; Serotec), CD90 (undiluted, MRC OX7; Serotec).

**Flowcytometric analyses**

Cells were added in round-bottom 96-well plates (Nunc) at a concentration of ~$10^5$ cells/well and washed twice in PBS/0.5% BSA/20 mM sodium azide. Pelleted cells were resuspended in 20 µl solution with labelled primary Abs, incubated for 10 min., and followed by two washing steps. Using unconjugated Abs, a second step was incorporated with rabbit anti-mouse-FITC Abs (Dako, Glustrup, Denmark) with 1% normal rat serum or rabbit anti-mouse-PE Abs (CLB, Amsterdam) with 1% normal rat serum. For the visualizing of biotin-conjugated Abs, streptavidin-tricolor (Caltag Laboratories, San Francisco, CA, USA) was used. For cell analysis, 10,000 events were recorded with a FACS (FACSCalibur, Becton Dickinson, Sunnyvale, CA, USA). Dead cells, recognized by their uptake of 7-AAD and their specific forward- and side-scatter pattern, were excluded from analysis. For determination of background staining, cells were incubated with either labelled irrelevant Abs or with secondary Abs.

**MLR**

T cells from Wistar and Fischer rats were enriched using a nylon wool column. In short, spleens were minced and teased through a 105-µm filter, and the erythrocytes were removed by lysis. Cells were washed and loaded onto a nylon wool column (3 g; Polyscience, Eppelheim, Germany) packed into a 60 ml plastic syringe. After 1 h. in 5% $CO_2$ incubator, T cells (80-90% $CD3^+$ cells) were harvested by collecting the effluent. For the MLR, DCs were added at various ratios to T cells (fixed number of 150,000 T cells/well) in flat-bottom 96-well plates (Nunc). Subsequently, these cells were cultured for 3 days in RPMI 1640 containing 50 mM HEPES buffer (Life Technologies), 10% FCSi, 110 µg/ml sodium pyruvate (Merck), 0.5% (v/v) β-mercapto-ethanol (Merck), and antibiotics. In the MLR, T cell proliferation was measured via tritiated thymidine ($[^3H]$TdR) incorporation (0.5 µCi/well during the last 16 h. of total culture period). Finally, cells were harvested on filter papers, and radioactivity was counted in a liquid scintillation analyzer (LKB Betaplate, Wallac, Turun, Finland).

**IL-10, IL-12p40 and IL-12p70 production**

Supernatants were obtained from DCs by culturing them in 24-well plates (Nunc) for 24 h. Cells were resuspended in serum free medium (SF-1) at a concentration of 0.5 x $10^6$/ml. SF-1 medium was made by dissolving 1 ml SF-1 in 9 ml RPMI 1640 with antibiotics. Cells were either cultured in SF-1 medium alone for basal production, SAC (*Staphylococcus aureus* Cowan strain) for IL-10 production or SAC and IFN-γ for IL-12p40 and IL-12p70 production.[22] Rat IL-10, IL-12p40 and IL-12p70 ELISA kits were commercially available (Biosource).

**Statistical analyses**

Mean values ± SD are shown. Statistical analyses were performed using either Mann-Whitney test for two groups or Kruskal-Wallis test for multiple groups.

## RESULTS

**GM-CSF/IL-4 culture of bone-marrow precursors yields an adherent and a non-adherent DC population. The non-adherent population has all the characteristics of prototypic DC**

The culture of bone-marrow precursor cells in GM-CSF/IL-4 yields two populations: an adherent colony-forming cell population and a non-adherent floating single cell population (Figure 1A, B; this figure shows characteristics of the Wistar DC, but similar results were found for BB and Fischer rat DC (data not shown)).

Both populations expressed the rat DC-specific markers OX62 (MFI non-adherent: 3291 ± 1207; adherent: 3199 ± 844) and CD11c (MFI non-adherent: 3334 ± 1769; adherent: 3398 ± 1010) and both populations expressed MHC class II, the adherent population stronger than the non-adherent population (Figure 1C). Despite the higher MHC class II expression the adherent population appeared functionally considerably less able to stimulate T cell proliferation in MLR as compared to the non-adherent population (Figure 1D). The non-adherent population also contained many more cells, which were of veiled/dendritic shape (Figure 1B).

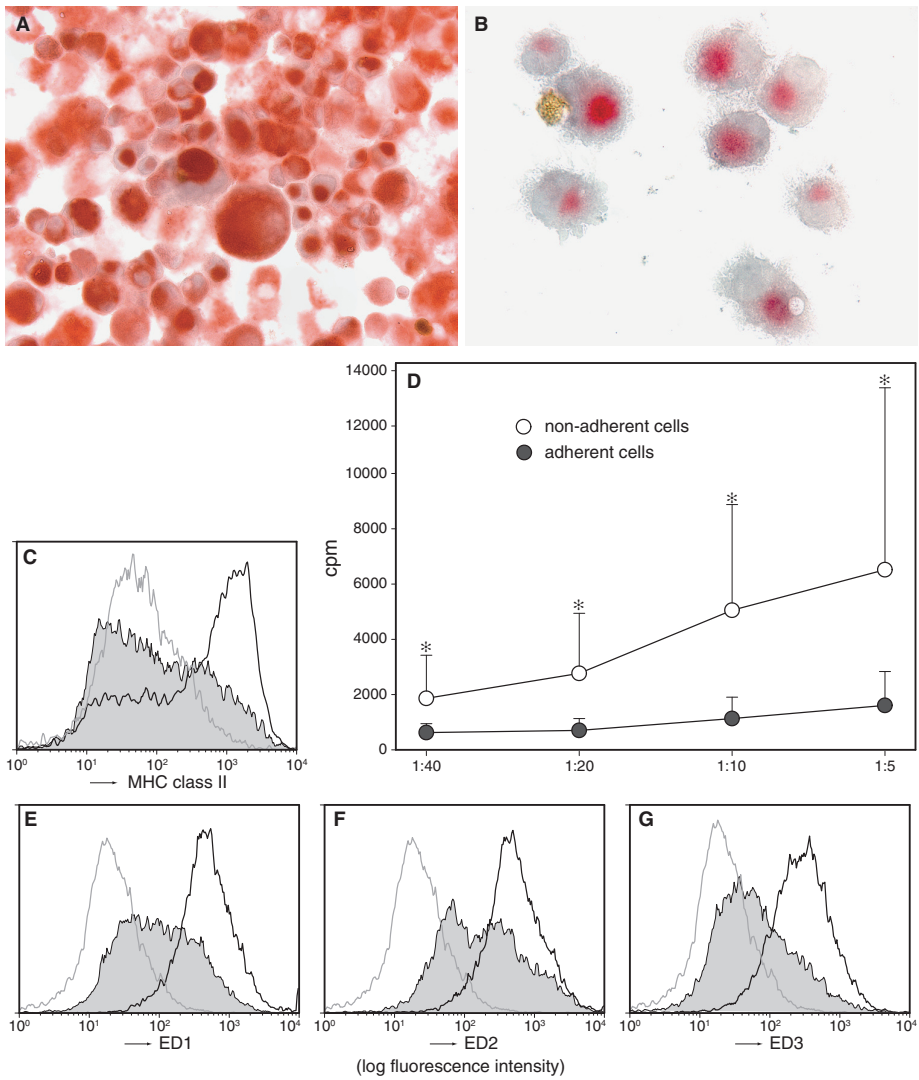
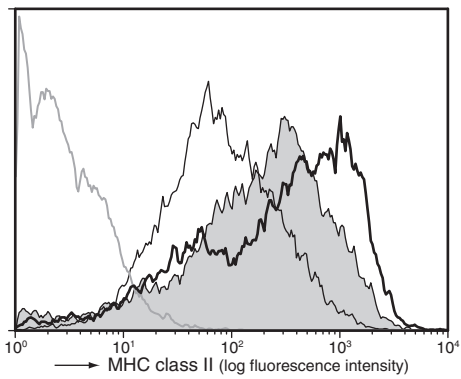



**Figure 1. Characteristics of rat bone-marrow derived DC.**
When bone-marrow precursors are cultured in GM-CSF/IL-4 two $OX62^{+}CD11c^{+}$ populations are obtained: (A) a population of adherent (colony-forming) cells and (B) a non-adherent population of floating single cells. Although the non-adherent cells show a definite, but lower expression of MHC class II (C) as compared to adherent cells, the cells have a much higher capability to stimulate T cells to proliferate (D, mean ± standard deviations are shown; *n*=10). The T cell stimulatory capacity was measured in syngeneic MLR and expressed as the incorporation of $^{3}$H-thymidine (c.p.m.) in the stimulated T cells. The adherent cells also have various macrophage characteristics like a higher ED1 (E), ED2 (F) and ED3 (G) expression and a stronger and ''spotty'' acid phosphatase activity (A). We therefore consider the non-adherent cells as the typical DC. The grey tone in (C, E, F and G) represents the non-adherent cells. The black line in (C, E, F and G) represents the adherent cells. The isotype control is depicted as a grey line. Representative figures are shown in (C, E, F and G) (*n*=18). Wistar rat DC are shown (similar results are found for BB and Fischer rats; data not shown). For colour figure see Appendix page 143.

To further underscore the non-dendritic cell character of the adherent population it had various characteristics reminiscent of macrophages, such as a stronger expression of acid phosphatase and the monocyte/macrophage markers ED1, ED2 and ED3 (Figure 1A, E-G). We therefore considered the non-adherent cell population as the more prototypic immature DC population and focused on this population in our subsequent experiments. We will refer to this population as immature DC throughout the further text. The adherent population probably represents precursors for the immature DC (also because one sees in culture the detachment of veiled cells from these colony-forming adherent cells).

**Marker expression: immature DC of BB-DP rats show a lower expression of surface MHC class II molecules as compared to BB-DR rats, irrespective of their state of maturation**

When studying the expression levels of the various markers in BB-DP rats we found that the most consistent abnormality was a lower expression of MHC class II on the GM-CSF/IL-4 generated immature DC (Figure 2). This lower expression was evident in all three BB-DP sub-lines and was statistically significant as compared to the expression level on the DC of the matching BB-DR rat strain tested in the same series of experiments. The lower expression was also statistically significant as compared to the control Wistar and F344 strains (apart from the immature DC of the Groningen sub-line rats) (Figure 2).




| Marker | Rat strain | NB immature DC | Groningen immature DC | Seattle immature DC | Seattle mature DC |
|---|---|---|---|---|---|
| MHC class II | BB-DP | 2388 ± 1412[a] | 2053 ± 948[c] | 1437 ± 254[b,c] | 2295 ± 575[c] |
| | BB-DR | n.d. | 3483 ± 1711 | 2285 ± 851 | 3251 ± 74 |
| | Wistar/F344 | 3258 ± 1138 | 2332 ± 425 | 2407 ± 1102 | 2178 ± 600 |

**Figure 2. Immature DC of BB-DP rats are consistently lower in surface MHC class II molecules as compared to immature DC of BB-DR (see table).**
Statistical analysis using the Kruskal-Wallis test for multiple groups showed the following statistically significant differences: (a) $p<0.05$ BB-DP vs. Wistar, (b) $p<0.05$ BB-DP vs. F344, (c) $p<0.05$ BB-DP vs. BB-DR. The figure shows a representative example of MHC class II expression, the immature DC of BB-DP (thin black line), the immature DC of BB-DR (shaded) and the immature DC of F344 (thick black line). The isotype control is depicted as a grey line.

With regard to the other tested molecules such as the rat DC-specific markers OX62 and CD11c, the monocyte/DC marker ED1 and the co-stimulatory molecules CD80, CD86 and CD40, consistent abnormalities could not be found in the expression levels on immature DC populations of the BB-DP vs. those of the BB-DR, Wistar or F344 control rats (there was only some lower expression of CD80, CD40 and ED1 on the immature DC of the BB/Seattle rats vs. its controls, data not shown).

To investigate whether the low expression of MHC class II on the immature DC of the BB-DP rat was still present after a final differentiation (maturation) of the cells, we first looked for a proper maturation factor for rat bone-marrow derived immature DC. In a preliminary set of experiments we used several maturation factors (LPS, TNF-α and *Mycobacterium tuberculosis* (MBT)) to find the optimal maturation factor for Wistar DC. TNF-α (Figure 3A) and MBT (data not shown) had no effect on MHC class II expression, while LPS slightly increased the MHC class II expression with an optimal concentration of 1 μg/ml (Figure 3B). In this optimal concentration LPS induced a strong up-regulation of the T cell stimulatory capacity of rat immature DC. TNF-α and MBT had no effects. We therefore used LPS in further experiments as a maturation factor.

When using LPS maturation in the BB rat model, there was a clear up-regulation of MHC class II expression in both the BB-DP/S and BB-DR/S rats due to the LPS exposure, but the lower expression level in the BB-DP/S rat vs. that of the BB-DR/S rat was not corrected (Figure 2).

**T cell stimulatory function: the inability of DC of both the BB-DP/S and BB-DR/S rats to terminally differentiate to strong T cell stimulators after LPS stimulation**

Figure 4 shows the T cell stimulatory capacities of the immature DC and mature DC of BB-DP/S and BB-DR/S rats as compared to those of Wistar and F344 rats. Although the

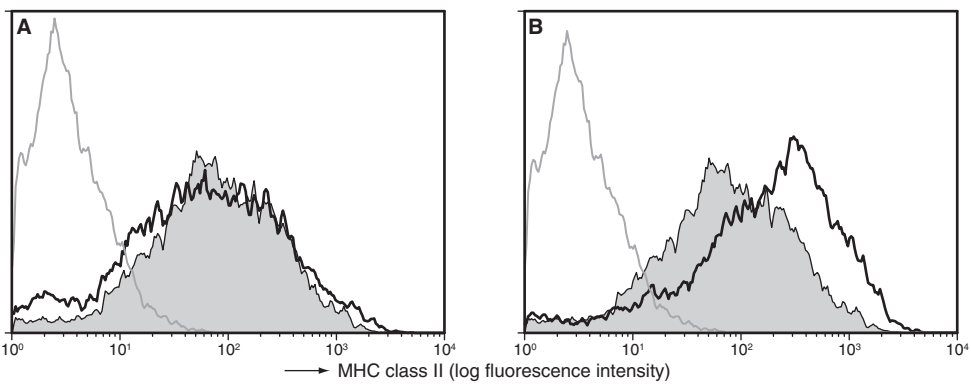



**Figure 3. Maturation with LPS was the most effective maturation factor for rat DC.**
(A) The non-stimulated DC (dark grey tone) vs. TNF-α-stimulated DC (black line) and (B) the non-stimulated DC (dark grey tone) vs. LPS-stimulated DC (black line). BB-DR rats were used in (A and B) (*n*=4-34). Similar effects are seen in Wistar (*n*=3-5) and Fischer rats (*n*=45) (data not shown). The figure illustrates that TNF-α-stimulated DC do not show an up-regulation of MHC class II (A), while LPS-stimulated DC do (B). The isotype control is depicted as a grey line.

MHC class II expression of immature DC of BB-DP/S rats was lower as compared to that of immature DC of BB-DR/S and of Wistar and F344 rats (see previous section), their T cell stimulatory capacity was not reduced (Figure 4A). As expected, after LPS maturation the T cell stimulatory capacities of mature DC of both the Wistar and F344 control strains were clearly higher as compared to T cell stimulatory capacity of the immature DC of the same strains (see above and Figure 4B). Remarkable was that this phenomenon, i.e. an increase in the T cell stimulatory capacity by maturation did not occur for the DC in both the BB-DP/S and DR/S sub-lines.

**IL-12 and IL-10 production: the IL-10 of immature DC of BB-DP/S and DR/S rats is lower as compared to that of F344 control rat DC. Only in BB-DR/S rats is the defective IL-10 production partially corrected after LPS maturation**

With regard to the IL-12p70 production capability of the rat DC populations, we found this production very low and values were not suitable for a reliable statistical evaluation and data are thus not shown. The IL-12p40 and IL-10 production were considerable and Table 1 shows this cytokine production of immature and mature DC of BB-DR/S, BB-DP/S and F344 control rats (Wistar rats were not used in this series of experiments) in the absence or presence of *Staphylococcus aureus* Cowan strain (SAC) and SAC plus IFN-γ, which are effective stimulators of IL-10 and IL-12 production, respectively.[22]

SAC-stimulated immature DC of BB-DP/S and BB-DR/S rats produced lower quantities of IL-10 (Table 1) as compared to such cells of F344 control rats, indicating a defect in IL-10 production that is not linked to the *lyp* gene but to other genes in the BB rat

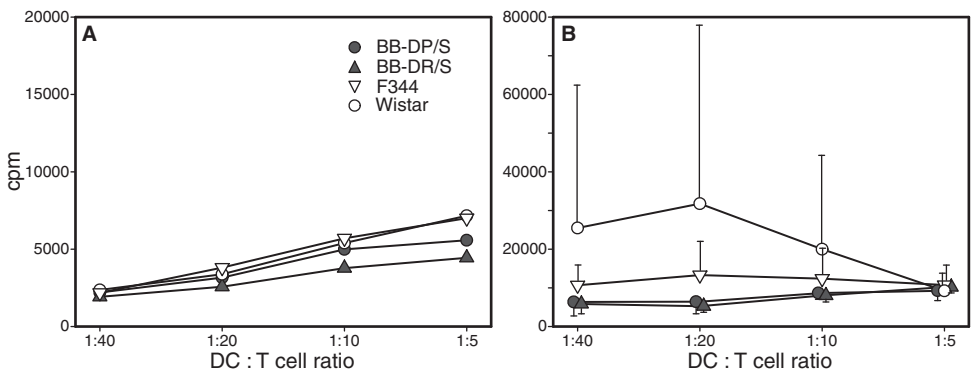



**Figure 4. Poor maturation of BB rat immature DC to T cell stimulatory mature DC.**
The T cell stimulatory capacity of immature DC (A) and mature DC (B) of BB-DP/S and BB-DR/S, Wistar and F344 rats. The T cell stimulatory capacity was measured in syngeneic MLR and expressed as the incorporation of $^{3}$H-thymidine (c.p.m.) in the stimulated T cells. Despite the low MHC class II expression of immature DC of the BB-DP/S rat, such immature DC do not show a lowered capability to stimulate syngeneic T cells in MLR (as compared to Wistar, F344 and even BB-DR/S rats; *n*=4-22). Immature DC of both BB-DP/S and BB-DR/S are incapable to mature to high T cell stimulating mature DC under the influence of LPS (B). Immature DC of Wistar and F344 rats do mature to such mature DC (mean ± standard deviations are shown; *n*=4-8). Note: Wistar and BB DC were stimulated with syngeneic Wistar T cells, since F344 rats have a different MHC haplotype, they were stimulated with syngeneic F344 T cells.

background. After terminal differentiation of the immature DC to mature DC with LPS the SAC-stimulated IL-10 production was not significantly altered for both the two BB/S strains and the F344 control rats (Table 1). It must be noted, however, that for the mature DC of the BB-DR/S rats there was a trend for a slightly higher production of IL-10 and the statistically significant difference with the IL-10 production of the mature DC of the F344 control rats was lost.

After a terminal differentiation to mature DC the spontaneous (i.e. not SAC and IFN-γ stimulated) IL-12p40 production was clearly raised for the mature DC of the F344 control rats in line with the view that mature DC are the better IL-12 producers as compared to immature DC (Table 1). With regard to the IL-12p40 production of the immature and mature DC of the BB-DP/S and BB-DR/S rats, this was in general not significantly different from that of the immature and mature DC of F344 rats (Table 1). However, in all instances there were clear trends for a reduced production capability of IL-12p40 by the immature and mature DC of both the BB rat strains (Table 1).

**Ian5 is not expressed in myeloid DCs**

We studied the expression of the *Ian5* transcripts in the immature DC, their $OX7^+$ bone-marrow precursors and in the non-fractioned bone-marrow population. We found that the non-fractioned bone-marrow population and the $OX7^+$ DC precursors expressed *Ian5* mRNA, but at an extremely low level as compared to T cells (Figure 5). The descending immature DC did not express *Ian5* mRNA (see Figure 5). Since the non-fractioned bone-marrow cell population had a higher *Ian5* mRNA expression as compared to $OX7^+$ DC precursors and since both populations are contaminated with some T cells (the non-fractioned bone-marrow cell population with around 10%, the $OX7^+$ bone-marrow DC precursors with around 5%), we consider the *Ian5* mRNA expression in the precursor cells as due to the T cell contamination.

**Table 1. The IL-10 and IL-12p40 cytokine production of bone-marrow derived immature DC (iDC) and mature DC (mDC) of BB-DP, BB-DR and F344 rats (*n*=3-7)**

| Cytokine (pg/ml) | Rat strain | iDC (unstimulated) | iDC (stimulated) | mDC (unstimulated) | mDC (stimulated) |
|---|---|---|---|---|---|
| IL-10 | BB-DP/S | 18 ± 8 | 479 ± 177 | 91 ± 37 | 471 ± 240 |
| | BB-DR/S | 18 ± 13 | 486 ± 127 | 98 ± 87 | 627 ± 376 |
| | F344 | 24 ± 14 | 856 ± 215[c] | 99 ± 39 | 934 ± 322[b] |
| IL-12p40 | BB-DP/S | 93 ± 86 | 1088 ± 729 | 131 ±92 | 577 ± 438 |
| | BB-DR/S | 39 ± 25 | 942 ± 483 | 54 ± 51 | 322 ± 149 |
| | F344 | 82 ± 24 | 1979 ± 117 | 714 ± 229[a] | 2151 ± 372 |

Statistical analysis using the Kruskal-Wallis test for multiple groups showed the following statistically significant differences: [a]$p<0.05$ F344 vs. BB-DR; [b]$p<0.05$ F344 vs. BB-DP; [c]$p<0.05$ F344 vs. BB-DP and BB-DR.

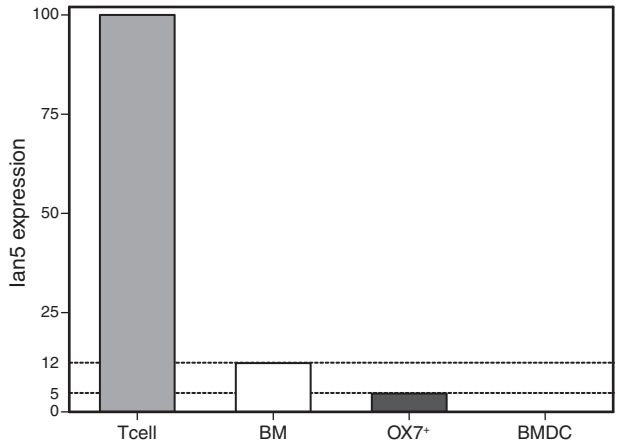



**Figure 5. Low *Ian5* mRNA expression in rat DC precursors and DC.**
The expression of *Ian5* mRNA in T cells, DC precursors (OX7$^{+}$), total BM (BM) and bone-marrow derived DC (BMDC). The *y*-axis represents the *Ian5* expression (relative to T cells, which were set to 100%).

## DISCUSSION

Here, we firstly show effects of the presence of the *lyp* region of BB-DP chromosome 4 on the differentiation of DC from bone-marrow precursors in the BB rat model of autoimmune thyroiditis/diabetes. In BB-DP rats a consistently lower expression of MHC class II on generated immature and mature DC was found as compared to such APC of BB-DR rats (and as compared to such cells of control rat strains).

Which mechanisms might be responsible for this lower expression of MHC class II associated with the *lyp* gene? Direct effects of the mutated *Ian5* gene can practically be ruled out, since it was not expressed in myeloid DC and their precursors (this report). Direct effects of the other *Ian* genes, such as *Ian1*, *Ian2*, *Ian3*, *Ian6* and *Ian7*, which are also expressed in the *lyp* gene region cannot be ruled out, since we did not investigate their expression in the DC. Indirect effects not related to gene expression are also possible. Here, it must be noted that the DP rat sub-lines from which the bone-marrow was collected all displayed a severe lymphopenia. Bone-marrow cultures normally contain approximately 10% of T cells, but the cultures of DP animals contained approximately 5% of T cells (data not shown) and a relative lack of T cell products in the culture might have accounted for the defective DC differentiation. That T cell factors are important for DC development has been shown in experiments with RAG2$^{-/-}$ T cell-deficient mice. These mice exhibit a deficiency in epidermal Langerhans cells and lymph node lymphoid- and myeloid-related DC. An adoptive transfer of normal T cells corrected these DC deficiencies.[23] Macrophage-like cells are also present in the bone-marrow cultures and it must be noted that macrophages of BB-DP rats are special in that they show

an excessive production of L-arginine, NO[24-26] and other reactive oxygen species.[27] All these factors are known to be involved in the differentiation and maturation of DC.[28,29] Clearly, the effects of and the production of T cell factors, such as IFN-γ, and macrophage factors (NO and other reactive oxygen species) need to be investigated in the GM-CSF/IL-4-induced bone-marrow cultures of the BB rats in future experiments to solve this item.

Secondly, we show here a set of abnormalities in bone-marrow precursor derived DC characteristic of both BB-DP and BB-DR rats and different from Wistar and F344 control rat DC. Such abnormalities must thus be due to genes in the BB gene background other than those associated with the *lyp* gene. The aberrancies include a low IL-10 production of the DC and a poor terminal differentiation of immature to mature T cell stimulatory (and fully IL-12 producing) DC under the influence of LPS (TLR4 stimulator). The poor terminal differentiation of BB rat DC could either be due to unresponsiveness of the mDC to LPS, or due to an intrinsic maturation defect of the cells. To distinguish between these two possibilities other maturation protocols need to be tested using alternative maturation factors like TLR2 stimulators (peptidoglycan or lipoteichoic acid), TLR3 stimulators (dsRNA or poly I:C) or a TLR9 stimulator (CpG).

What could be the genes responsible for these DC aberrancies characteristic of both the BB-DP and BB-DR rat? One or more of the major diabetes susceptibility (*iddm*) genes such as *iddm1* (the MHC class II ($RT1^u$)) on chromosome 20[30], *iddm3* (which co-localizes with body weight regulation genes) on chromosome 2[31] and *iddm4* located on chromosome 4 in close proximity to *iddm2*[32,33] are candidates to govern these aberrancies, since they are shared by BB-DP/S and BB-DR/S rats in comparison to control non-autoimmune strains. Clearly, to unravel whether these genes are involved, similar studies as reported here but with rats congenic for these *iddms* need to be performed (e.g. studies on the congenic WF.*iddm4* rats carrying the BB rat (''d'') allele, which become hyperglycemic in ~70% of animals after treatment to induce diabetes[33]).

Interestingly, the here found aberrancies in the differentiation and function of antigen-presenting cells are not specific for the BB rat model of autoimmune thyroiditis/diabetes. Similar aberrancies have been found in the other important rodent model of autoimmune diabetes, the NOD mouse, and in type-1 diabetes patients.

In the NOD mouse model studies have mainly concentrated on the GM-CSF induced development of DC from bone-marrow precursors. This development was found aberrant as compared to control animals in the majority of the studies, leading to the generation of immature DC with a macrophage-like phenotype and a low capability to stimulate T cells.[34-36] A further terminal differentiation of such NOD immature DC with LPS was hampered similar to the here reported defect in the BB rat strain. Contradictory findings have, however, also been made in the NOD mouse regarding the aberrant development of immature DC and mature DC from precursors: a hyperactive NF-κB in NOD bone-marrow derived DC (and macrophages) has also been found, which led to a strong T cell stimulatory capacity.[37-39] Although this is perhaps partially compatible with a more pro-inflammatory macrophage-like character of the generated NOD DC, we found it also dependent on the culture conditions used, e.g. seeding concentrations of the cells, application of additional growth factors (IL-4) and plastics used.[34] The hyperactive NF-κB in NOD MΦs and DC also led to an increased production of the pro-inflammatory cytokines IL-12p70, TNF-α and IL-1α.[40]

In type-1 diabetes patients and high-risk first-degree relatives aberrancies in the differentiation and function of monocyte-derived DC have been reported as well.[41] Also, the generation of another similar antigen-presenting cell (APC) population, i.e. of macrophage-like veiled cells, was found hampered in type-1 diabetic and autoimmune thyroiditis patients.[42] This hampered differentiation resulted in a low T cell stimulatory capacity of the APC. Other reports show a decreased *in vitro* IL-10 and IL-4 production by peripheral blood cells of first-degree relatives at a high risk of type-1 diabetes.[43] Additional evidence for an IL-10 production aberrancy comes from twin studies: type-1 diabetes-discordant twins with a raised risk (i.e. islet cell antibody-positive) show a reduced IL-10 response to hsp60 as compared to twins at low risk.[44] In addition there appears to be a defect in the production of IL-10 (and IL-4) in the pancreas of recently diagnosed type-1 diabetes mellitus patients.[45]

Collectively our rat data and those of others in mice and humans thus suggest that individuals prone to type-1 diabetes are characterized by an aberrant differentiation of antigen-presenting cells from precursors. How might such aberrancies in APC lead to proneness for auto-aggressive self-reactivity?

It is not difficult to envisage the reduced IL-10 production of the immature and mature DC of rats of the BB background (and those described in humans) as contributing to the autoimmune status, since IL-10 is an important immune suppressive molecule. However, the poor T cell stimulatory function and the relatively low IL-12 production of mature DC is more difficult to view as a factor promoting autoimmunity. There are, however, a few indications in the literature that poorly differentiated DC of the BB-DP rat and the NOD mouse contribute significantly to imbalances in the T cell system in such a way that autoimmunity prevails. First of all, transfers of properly terminally differentiated DC prevent diabetes development in the NOD mouse.[46] Dahlen et al. suggested that such transfers worked due to the correction of the low level of co-stimulation given by defective NOD DC: the transferred maturated DC would give a full activation of autoreactive T cells and would consequently induce Activation Induced T cell Death (AITCD) and/or the up-regulation of CTLA-4, an important switch-off signal for activated T cells.[35] However, other mechanisms might play a role as well: properly terminally differentiated mature DC are also better equipped to skew T cell responses towards Th2 immune responses[47] and to directly expand $CD4^+CD25^+$ regulatory T cells.[48,49] Indeed, in the BB-DP rat model the poorly differentiated lymph node and spleen DC of the BB-DP rat showed a reduced potential of expanding the important $ART2^+$ regulatory T cell population of the rat.[12] That indeed a full stimulation of T cells via the co-stimulatory pathways leads to a prevention of autoimmunity is further illustrated in experiments directly interfering in the CD28 stimulating pathway. When the BB-DP rat is treated with a stimulating anti-CD28 antibody (thus correcting the poor stimulating activity of the animals APC and activating the T cells), autoimmunity does not develop.[50] Also, in the NOD mouse interventions in the activation pathway between functionally active DC and T cells (by deleting CD80-CD28 interactions) disrupt existing faulty tolerance even further, thus accelerating diabetes development. Interestingly, these interventions in the NOD mouse led to a hampered generation of $CD4^+CD25^+$ regulatory T cells.[51]

In conclusion, the here-presented study provides evidence that the differentiation of immature and mature DC from bone-marrow precursors is hampered in the BB-DP rat model of autoimmune thyroiditis/diabetes. One of these disturbances is *lyp* (*iddm2*) gene

associated, i.e. the low MHC class II expression of DC arising from bone-marrow precursors. The others are governed by BB rat genes not associated with the *lyp* gene and also occurred in BB-DR/S rats, such as the low IL-10 production of the DC and a poor terminal differentiation of immature DC to mature T cell stimulatory DC under the influence of LPS.

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## REFERENCES

1. Kabel PJ, Voorbij HAM, van der Gaag RD, Wiersinga WM, de Haan M, Drexhage HA. Dendritic cells in autoimmune thyroiditis. *Acta Endocrinol. Suppl.* 1987;281:42-48.
2. Voorbij HAM, Jeucken PH, Kabel PJ, de Haan M, Drexhage HA. Dendritic cells and scavenger macrophages in pancreatic islets of prediabetic BB rats. *Diabetes* 1989;8:1623-1629.
3. Voorbij HAM, Kabel PJ, de Haan M, Jeucken PH, van der Gaag RD, de Baets MH, Drexhage HA. Dendritic cells and class II MHC expression on thyrocytes during the autoimmune thyroid disease of the BB rat. *Clin. Immunol. Immunopathol.* 1990;55:9-22.
4. Ziegler AG, Erhard J, Lampeter EF, Nagelkerken LM, Standl E. Involvement of dendritic cells in early insulitis of BB rats. *J. Autoimmun.* 1992;5:571-579.
5. Jansen A, Homo-Delarche F, Hooijkaas H, Leenen PJ, Dardenne M, Drexhage HA. Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of the insulitis and β-cell destruction in NOD mice. *Diabetes* 1994;43:667-675.
6. Many MC, Maniratunga S, Varis I, Dardenne M, Drexhage HA, Denef JF. Two-step development of Hashimoto-like thyroiditis in genetically autoimmune prone non-obese diabetic mice: effects of iodine-induced cell necrosis. *J. Endocrinol.* 1995;147:311-320.
7. Van Blokland SC, van Helden-Meeuwsen CG, Wierenga-Wolf AF, Drexhage HA, Hooijkaas H, van de Merwe JP, Homo-Delarche F, Versnel MA. Two different types of sialoadenitis in the NOD- and MRL/lpr mouse models for Sjögren's syndrome: a differential role for dendritic cells in the initiation of sialoadenitis? *Lab. Invest.* 2000;80:575-585.
8. Kabel PJ, Voorbij HAM, De Haan M, van der Gaag RD, Drexhage HA. Intrathyroidal dendritic cells. *J. Clin. Endocrinol. Metab.* 1988;66:199-207.
9. Jansen A, Voorbij HA, Jeucken PH, Bruining GJ, Hooijkaas H, Drexhage HA. An immunohistochemical study on organized lymphoid cell infiltrates in fetal and neonatal pancreases: a comparison with similar infiltrates found in the pancreas of a diabetic infant. *Autoimmunity* 1993;15:31-38.
10. Yamakawa M, Kato H, Takagi S, Karube Y, Seki K, Imai Y. Dendritic cells in various human thyroid diseases. *In Vivo* 1993;7:249-56.
11. Xanthou G, Tapinos NI, Polihronis M, Nezis IP, Margaritis LH, Moutsopoulos HM. CD4 cytotoxic and dendritic cells in the immunopathologic lesion of Sjögren's syndrome. *Clin. Exp. Immunol.* 1999;118:154-163.
12. Delemarre FG, Simons PJ, de Heer HJ, Drexhage HA. Signs of immaturity of splenic dendritic cells from the autoimmune prone biobreeding rat: consequences for the *in vitro* expansion of regulator and effector T cells. *J. Immunol.* 1999;162:1795-1801.
13. Delemarre FG, Hoogeveen PG, De Haan-Meulman M, Simons PJ, Drexhage HA. Homotypic cluster formation of dendritic cells, a close correlate of their state of maturation. Defects in the biobreeding diabetes-prone rat. *J. Leukoc. Biol.* 2001;69:373-380.
14. Simons PJ, Delemarre FG, Drexhage HA. A functional and phenotypic study on immune accessory cells isolated from the thyroids of Wistar and autoimmune-prone BB-DP rats. *J. Autoimmun.* 2000;15:417-424.

15. Greiner DL, Malkani S, Kanaitsuka T, Bortell R, Doukas J, Rigby M, Whalen B, Stevens LA, Moss J, Mordes JP, Rossini AA. The T cell marker RT6 in a rat model of autoimmune diabetes. *Adv. Exp. Med. Biol.* 1997;419:209-16.
16. Greiner DL, Mordes JP, Handler ES, Angelillo M, Nakamura N, Rossini AA. Depletion of RT6.1+ T lymphocytes induces diabetes in resistant biobreeding/Worcester (BB/W) rats. *J. Exp. Med.* 1987;166:461-475.
17. Burstein D, Mordes JP, Greiner DL, Stein D, Nakamura N, Handler ES, Rossini AA. Prevention of diabetes in BB/Wor rat by single transfusion of spleen cells: parameters that affect degree of protection. *Diabetes* 1989;38:24-30.
18. Jackson R, Rassi N, Crump T, Haynes T, Eisenbarth GS. The BB diabetic rat. Profound T-cell lymphopenia. *Diabetes* 1981;30:887-889.
19. Greiner DL, Handler ES, Nakano V, Mordes JP, Rossini AA. Absence of the RT-6 cell subset in diabetes-prone BB/W rats. *J. Immunol.* 1986;136:148-151.
20. MacMurray AJ, Moralejo DH, Kwitek AE, Rutledge EA, Van Yserloo B, Gohlke P, Speros SJ, Snyder B, Schaefer J, Bieg S, Jiang J, Ettinger RA, Fuller J, Daniels TL, Pettersson A, Orlebeke K, Birren B, Jacob HJ, Lander ES, Lernmark Å. Lymphopenia in the BB rat model of type 1 diabetes is due to a mutation in a novel immune-associated nucleotide (Ian)-related gene. *Genome Res.* 2002;12:1029-1039.
21. Hornum L, Romer J, Markholst H. The diabetes-prone BB rat carries a frameshift mutation in Ian4, a positional candidate of Iddm1. *Diabetes* 2002;51:1972-1979.
22. Ruwhof C, Canning MO, Grotenhuis K, de Wit HJ, Florencia ZZ, de Haan-Meulman M, Drexhage HA. Accessory cells with a veiled morphology and movement pattern generated from monocytes after avoidance of plastic adherence and of NADPH oxidase activation. A comparison with GM-CSF/IL-4-induced monocyte-derived dendritic cells. *Immunobiology* 2002;205:247-266.
23. Shreedhar V, Moodycliffe AM, Ullrich SE, Bucana C, Kripke ML, Flores-Romo L. Dendritic cells require T cells for functional maturation *in vivo*. *Immunity* 1999;11:625-636.
24. Lau A, Ramanathan S, Poussier P. Excessive production of nitric oxide by BB macrophages is secondary to the T lymphopenic state of these animals. *Diabetes* 1998;47:197-205.
25. Wu G, Flynn NE. The activation of arginine-citrulline cycle in macrophages from the spontaneously diabetic BB rat. *Biochem. J.* 1993;294:113-118.
26. Lee KU. Nitric oxide produced by macrophages mediates suppression of conA-induced proliferative responses of splenic leukocytes in the diabetes-prone BB rat. *Diabetes* 1994;43:1218-1220.
27. Corinti S, Pastore S, Mascia F, Girolomoni G. Regulatory role of nitric oxide on monocyte-derived dendritic cell functions. *J. Interferon Cytokine Res.* 2003;23:423-431.
28. Brenner HH, Burkart V, Rothe H, Kolb H. Oxygen radical production is increased in macrophages from diabetes prone BB rat. *Autoimmunity* 1993;15:93-98.
29. Kusmartsev S, Gabrilovich DI. Inhibition of myeloid cell differentiation in cancer: the role of reactive oxygen species. *J. Leukoc. Biol.* 2003;74:186-196.
30. Colle E, Guttmann RD, Seemayer T. Spontaneous diabetes mellitus syndrome in the rat. *J. Exp. Med.* 1981;154:1237-1242.
31. Klaff LS, Koike G, Jiang J, Wang Y, Bieg S, Pettersson A, Lander E, Jacob H, Lernmark Å. BB rat diabetes susceptibility and body weight regulation genes colocalize on chromosome 2. *Mamm. Genome* 1999;10: 883-887.
32. Martin AM, Maxson MN, Leif J, Mordes JP, Greiner DL, Blankenhorn EP. Diabetes-prone and diabetes-resistant BB rats share a common major diabetes susceptibility locus, iddm4: additional evidence for a ‘‘universal autoimmunity locus’’ on rat chromosome 4. *Diabetes* 1999;48:2138-2144.
33. Mordes JP, Leif J, Novak S, DeScipio C, Greiner DL, Blankenhorn EP. The iddm4 locus segregates with diabetes susceptibility in congenic WF.iddm4 rats. *Diabetes* 2002;51:3254-3262.
34. Nikolic T, Bunk M, Drexhage HA, Leenen PJ. Bone marrow precursors of nonobese diabetic mice develop into defective macrophage-like dendritic cells *in vitro*. *J. Immunol.* 2004;173:4342-4351.
35. Dahlen E, Hedlund G, Dawe K. Low CD86 expression in the nonobese diabetic mouse results in the impairment of both T cell activation and CTLA-4 up-regulation. *J. Immunol.* 2000;164:2444-2456.
36. Strid J, Lopes L, Marcinkiewicz J, Petrovska L, Nowak B, Chain BM, Lund T. A defect in bone marrow derived dendritic cell maturation in the nonobese diabetic mouse. *Clin. Exp. Immunol.* 2001;123:375-381.

37. Poligone B, Weaver Jr DJ, Sen P, Baldwin Jr AS, Tisch R. Elevated NF-κB activation in nonobese diabetic mouse dendritic cells results in enhanced APC function. *J. Immunol.* 2002;168:188-196.
38. Marleau A, Singh B. Myeloid dendritic cells in non-obese diabetic mice have elevated costimulatory and T helper-1-inducing abilities. *J. Autoimmun.* 2002;19:23-35.
39. Steptoe RJ, Ritchie JM, Harrison LC. Increased generation of dendritic cells from myeloid progenitors in autoimmune-prone nonobese diabetic mice. *J. Immunol.* 2002;168:5032-5041.
40. Sen P, Bhattacharyya S, Wallet M, Wong CP, Poligone B, Sen M, Baldwin AS, Tisch R. NF-κB hyperactivation has differential effects on the APC function of nonobese diabetic mouse macrophages. *J. Immunol.* 2003;170: 1770-1780.
41. Takahashi K, Honeyman MC, Harrison LC. Impaired yield, phenotype, and function of monocyte-derived dendritic cells in humans at risk for insulin-dependent diabetes. *J. Immunol.* 1998;161:2629-2635.
42. Jansen A, van Hagen M, Drexhage HA. Defective maturation and function of antigen- presenting cells in type 1 diabetes. *Lancet* 1995;345:491-492.
43. Szelachowska M, Kretowski A, Kinalska I. Decreased *in vitro* IL-4 [corrected] and IL-10 production by peripheral blood in first degree relatives at high risk of diabetes type-I. *Horm. Metab. Res.* 1998;30:526-530.
44. Kallmann BA, Lampeter EF, Hanifi-Moghaddam P, Hawa M, Leslie RD, Kolb H. Cytokine secretion patterns in twins discordant for type I diabetes. *Diabetologia* 1999;42:1080-1085.
45. Farilla L, Dotta F, Di Mario U, Rapoport B, McLachlan SM. Presence of interleukin 4 or interleukin 10, but not both cytokines, in pancreatic tissue of two patients with recently diagnosed diabetes mellitus type I. *Autoimmunity* 2000;32:161-166.
46. Feili-Hariri M, Dong Z, Alber SM, Watkins SC, Salter RD, Morel PA. Immunotherapy of NOD mice with bone marrow-derived dendritic cells. *Diabetes* 1999;48:2300-2308.
47. Feili-Hariri M, Falkner DH, Morel PA. Regulatory Th2 response induced following adoptive transfer of dendritic cells in prediabetic NOD mice. *Eur. J. Immunol.* 2002;32:2021-2030.
48. Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat. Immunol.* 2001;2:725-731.
49. Yamazaki S, Iyoda T, Tarbell K, Olson K, Velinzon K, Inaba K, Steinman RM. Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. *J. Exp. Med.* 2003;198:235-247.
50. Beaudette-Zlatanova BC, Whalen B, Zipris D, Yagita H, Rozing J, Benjamin CD, Hunig T, Drexhage HA, Amari MJ, Leif J, Mordes JP, Greiner DL, Sayegh MH, Rossini AA. Costimulation and autoimmune diabetes in BB rats. *Am. J. Transplant.* 2006;6:894-902.
51. Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, Bluestone JA. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 2000;12:431-440.

# III

# Aberrancies in the differentiation and maturation of dendritic cells from bone-marrow precursors are linked to various genes on chromosome 4 and other chromosomes of the BB-DP rat

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# ABSTRACT

BB-Diabetes Prone (BB-DP) rats, a model for endocrine autoimmune diseases, are severely lymphopenic, especially lacking ART2$^+$ regulatory T cells. BB-Diabetes Resistant (DR) rats are not lymphopenic and do not develop autoimmunity. BB-DP and BB-DR rats only differ at the *lymphopenia (lyp)* gene *(iddm2)* on chromosome 4. Since BB-DP rats also show aberrancies in the differentiation of dendritic cells (DC) from bone-marrow precursors, we tested the hypothesis that F344 rats congenic for a BB-DP chromosome 4 region (42.5-93.6 Mb; including the *lyp* gene, but also *iddm4)* display an *in vitro* DC differentiation different from normal F344 rats.

Here we show that the 42.5-93.6 Mb BB-DP chromosome 4 region is linked to an increased DC precursor apoptosis, a low MHC class II expression, a reduced IL-10 production and a reduced T cell stimulatory capacity of DC.

From our previous report on DC differentiation defects in BB rats (only differing in *iddm2)* and the present report, we deduce that the abnormal apoptosis and low MHC class II expression is linked to *iddm2*. The reduced T cell stimulatory capacity is linked to other genes on chromosome 4 (candidate gene: *iddm4)*. The reduced IL-10 production has a complex linkage pattern.

# INTRODUCTION

Dendritic cells (DC), the antigen-presenting cells *par excellence*, play a pivotal role in the pathogenesis of organ-specific autoimmune diseases. In animal models that spontaneously develop such diseases, such as the BioBreeding-Diabetes Prone (BB-DP) rat and the Non-Obese Diabetic (NOD) mouse, MHC class II$^+$ DC accumulate in the earliest phases of the disease in the thyroid gland, the pancreas and the salivary glands.[1-7] These DC take up and travel with relevant autoantigens to draining lymph nodes, where a specific autoimmune response is initiated by the stimulation of naïve autoreactive T cells. Indeed an early accumulation of DC in glands later targeted by the autoimmune response is followed by an enlargement of the lymph nodes draining the glands and the production of autoantibodies by plasma cells located in these lymph nodes.[1,3] This lymph node reaction is later followed by an infiltration of the target gland by large numbers of lymphocytes. In patients with type-1 diabetes mellitus, autoimmune thyroid disease and Sjögren's syndrome, DC are also seen in large numbers in the target glands.[1,8-11]

The advantage of studying the BB-DP rat model of organ-specific autoimmunity is the presence of a well-defined suppressor/regulatory T cell population expressing the Ag ART2 in the rat.[12] These T cells play a crucial regulatory role in the pathogenesis of organ-specific autoimmunity.[12] BB-DP rats are severely lymphopenic, in particular lacking ART2$^+$ T cells. There exists a sub-line of BB rats, the so-called BB-Diabetes Resistant (DR) rats, that are not lymphopenic and do not develop diabetes and thyroid autoimmunity because of the presence of ART2$^+$ T cells. Depletion of these cells in the BB-DR rat induces organ-specific autoimmunity,[13] while transfer of these cells obtained from BB-DR rats into BB-DP rats prevents organ-specific autoimmunity in the latter.[14] The genetic difference between the BB-DP rat and the BB-DR rat is at the level of the *lymphopenia* (*lyp*, *iddm2*) gene, a major diabetes susceptibility gene located on chromosome 4. Homozygosity for the defective *lyp* gene, as is the case in the BB-DP rat, leads to severe T lymphocytopenia and the lack of ART2$^+$ regulatory T cells.[15-19] Recently, the *lyp* gene (also called *Ian5*, *Gimap5*, *Ian4L1*) in BB-DP rats was found to carry a single nucleotide deletion causing a frameshift mutation and a severe truncation of the protein.[20,21]

In addition to T lymphocytopenia, DC are defective in the BB-DP rat. We previously reported abnormalities in *ex vivo* preparations of spleen DC and thyroid DC. The spleen DC of the BB-DP rat showed a lower MHC class II expression, had a lower capability to form homotypic cell aggregations and had a reduced capability to stimulate T cells in syngeneic MLR.[22,23] The DC preparation isolated from the thyroid gland of the BB-DP rat contained fewer cells with the characteristics of typical MHC class II$^+$ DC. Also more of monocyte-like precursors were present in the BB-DP thyroid isolates as compared to cell preparations of thyroids of control Wistar rats.[24] We viewed these abnormalities as signs of defects in the differentiation and maturation of DC from precursors in the BB-DP rat. We indeed later found such hampered differentiation and maturation from bone-marrow precursors in BB-DP/Seattle (S) rats. Cultures of bone-marrow precursors in GM-CSF/IL-4 yielded DC with a lower MHC class II expression not only as compared to Wistar and F344 rats, but also in comparison to BB-DR/S rats, that only differ from the BB-DP/S rats with regard to a 1.3-Mb region on chromosome 4 (76.35 Mb-77.67 Mb). This observation suggests the

involvement of the *lyp* gene (*iddm2*) in this MHC class II expression defect of the DC, since this BB-DP gene is located in the 1.3-Mb region on chromosome 4. The DC of BB-DP rats cultured from bone-marrow precursors also showed other abnormalities, i.e. a poor capability to terminally differentiate into DC with a strong T cell stimulatory capacity, and the cells also produced lower quantities of IL-10 and IL-12p40. These latter aberrancies, however, were also found in the BB-DR/S rat but did not occur in the control Wistar and F344 rats. We therefore concluded that there exist various aberrancies in the differentiation of myeloid DC from precursors in the BB rat model of organ-specific autoimmunity and that various genes, including the *lyp* gene (*iddm2*), control these aberrancies.

We decided to study the linkage of the BB-DP rat DC aberrancies to gene regions further by using F344 rats congenic for the region of the BB-DP rat chromosome 4, on which the *lyp* gene is located. Congenic F344 rats homozygous for this region of the BB-DP rat chromosome 4 (designated ''F344.*lyp*/*lyp* rats'') have a similar extent of T lymphocytopenia and other T cell abnormalities as the BB-DP rat (an increased number of $CD25^+$ and $Thy1^+$ T cells; nearly absent $ART2^+$ T cells and increased numbers of circulating granulocytes, large granular lymphocytes and natural killer cells).[25,26] The rats do not, however, develop organ-specific autoimmunity, since they apparently lack important *iddm* genes on other chromosomes for such development.

Recent studies indicate that the congenic region of F344.*lyp*/*lyp* rats is large (51.1 Mb) and covers a considerable part of the BB-DP rat chromosome 4, i.e. the region from 42.5 Mb to 93.6 Mb. This also implies that the congenic F344 rats share both the *iddm2* and the *iddm4* gene with the BB-DP rat. The latter is located on chromosome 4 at position 64.9-69.3 Mb.

Here we report the *in vitro* differentiation of DC from bone-marrow precursors and their further maturation after LPS stimulation in rats of this congenic F344 strain. We compared F344 rats homozygous and heterozogous for the *lyp*-containing region (designated ''F344.*lyp*/*lyp*'' and ''F344.*lyp*/+'' rats) versus wild-type non-congenic (''+/+'') F344 rats, enabling us to study the effects of genes within the 42.5 Mb-93.6 Mb region of the BB-DP rat chromosome 4 on the differentiation and maturation of rat DC.

We found that DC precursors of F344.*lyp*/*lyp* and *lyp*/+ rats showed a higher apoptosis during culture to DC, leading to a lower yield of DC after 7 days of culture. In addition, the generated DC had a low MHC class II expression, a low capability to stimulate T cells in allogeneic (allo)-MLR and, if ''*lyp*/*lyp*'', a reduced capability to produce IL-10. Their further final differentiation to mature DC by LPS was not, or hardly, hampered. In a comparison to our earlier reported findings on abnormalities in the differentiation and maturation of DC of BB-DP/S rats versus those of BB-DR/S rats (see above) we are able to construct a more solid and detailed linkage of *iddm2* and other genes to the DC aberrancies typical of the BB rat model of diabetes.

## MATERIALS AND METHODS

### Animals

Female Wistar rats were obtained from Harlan (Zeist, The Netherlands). F344.+/+, F344.*lyp*/+ and F344.*lyp/lyp* rats[26] were developed and maintained at the Robert H. Williams

Laboratory at the University of Washington, Seattle, USA. The animals were housed under SPF conditions. All rats were kept under controlled light conditions (12/12 h light/dark cycle) throughout this study. A standard pelleted diet (0.35 mg iodine/kg; AM-II, Hope Farms BV, Woerden, The Netherlands) and tap water were provided *ad libitum*. For all experiments, age matched F344 rats were used.

**Bone-marrow derived dendritic cell culture**

Rat bone-marrow was obtained from tibia and femur. Red blood cells were lysed by $NH_4Cl$ containing $KHC0_3$ and EDTA. After red blood cell lysis cells were resuspended in RPMI 1640 containing 25 mM HEPES buffer (Life Technologies), 10% FCSi, 50 μM β-mercapto-ethanol (Merck), penicillin (100 U/ml; Seromed, Biochrom, Berlin, Germany), and streptomycin (0.1 mg/ml; Seromed). Cells were brought to a concentration of 10 x $10^6$/10 ml and cultured in 25 $cm^2$ culture bottles (Nunc, Roskilde, Denmark) for 9 days (37°C, 5% CO2 incubator). To obtain dendritic cells from bone-marrow precursors rat recombinant GM-CSF (17 μg/ml) and rat recombinant IL-4 (20 μg/ml) was added (Biosource, Camarillo, CA, USA) to the culture medium. Medium was refreshed at day 4 (with cytokines) and day 7 (without cytokines to enrich for dendritic cells and to deplete granulocytes). At day 7 the non-adherent cells were further cultured for 48 h. (either resuspended in medium to obtain immature DC or in LPS (1 μg/ml) to obtain mature DC) and were harvested by rinsing the bottles with medium twice.

**Methods for quantitative PCR**

Quantitative PCR was performed on an Mx4000 instrument (Stratagene, La Jolla, CA, USA) using DNAse treated RNA (100 ng of total RNA) in triplicate using the Brilliant Single-Step Quantitative RT-PCR Core Reagent Kit (Stratagene) along with serially diluted Standard samples. The standards used were 1:4 serial dilutions of BB-DR/Seattle rat spleen poly AC RNA.

Samples were multiplexed with a rat *Ian5* probe and a rat cyclophilin probe. The *Ian5* probe was designed to the 3**'** end of exon 3, beyond the coding region.

Probes and primers were from Integrated DNA Technologies, Ine. (Coralville, IA, USA). The probe for rat *Ian5* was: 5'-FAM-TTT CAC TAT CAT TTG ACT CCT GTG CA-BHQ-1-3'. The probe for rat cyclophilin was 5'-HEX-CTG CTT CGA GCT GTT TGC AGA C-BHQ-1-3'. These probes were carefully designed, assuring that they did not match any other gene in the rat database. The primers for *Ian5* were f: 5'-CAT GTT AGG GAA GCT CAG TC-3' and r: 5'-GAA GGG TTC TAC TGT GTC TCA-3'. The primers for rat cyclophilin were f: 5'-CAC CGT GTT CTT CGA CAT-3' and: 5'-TTT CTG CTG TCT TTG GAA CT-3'. Resulting C(t) values were converted to picograms and normalized to the values of cyclophilin from the same well and expressed as the average of triplicate samples G one standard deviation. Total RNA was isolated using Qiagen RNeasy (Qiagen, Chatsworth, CA, USA) or Stratagene Absolutely RNA RT-PCR Miniprep kits (Stratagene, La Jolla, CA, USA). To remove trace genomic DNA, Ambion DNA-free (Ambion, Austin, TX, USA) treatment was performed on samples extracted with Qiagen RNeasy kit.

**Antibodies**

The following mAbs were used: anti-MHC class II conjugated to phycoerythrin (PE) (1:400, MRC OX6; Serotec), anti-B7-1 (undiluted, CD80; BD Pharmingen, Flanders, NJ), anti-B7-2 (undiluted, CD86; BD Phar-mingen), anti-CD40 (1:10; BD Pharmingen), anti-rat DC-FITC (undiluted, MRC OX62; Serotec), anti-rat CD11c-FITC (Serotec), ED1 (1:10, DC, monocytes, MΦ; Serotec), ED2 (1:100, monocytes, MΦ; Serotec), ED3 (1:1000; Serotec), CD90 (undiluted, MRC OX7; Serotec). Unconjugated antibodies were labelled with FITC via a second step using rabbit anti-mouse-FITC Abs.

**Flowcytometric analyses**

Cells were added in round-bottom 96-wells plates (Nunc) at a concentration of ~$10^5$ cells/well and washed twice in PBS/0.5% BSA/20 mM sodium azide. Pelleted cells were resuspended in 20 µl solution with labelled primary Abs, incubated for 10 min., and followed by two washing steps. Using unconjugated Abs, a second step was incorporated with rabbit anti-mouse-FITC Abs (Dako, Glustrup, Denmark) with 1% normal rat serum or rabbit anti-mouse-PE Abs (CLB, Amsterdam, The Netherlands) with 1% normal rat serum. For the visualizing of biotin-conjugated Abs, streptavidin-tri-color (Caltag Laboratories, San Francisco, CA, USA) was used. For cell analysis, 10,000 events were recorded with a FACS (FACSCalibur, Becton Dickinson, Sunnyvale, CA, USA). Dead cells, recognized by their uptake of 7-AAD and their specific forward- and side-scatter pattern, were excluded from analysis. For determination of background staining, cells were incubated with either labelled irrelevant Abs or with secondary Abs. Due to the considerable auto-fluorescence of the tested dendritic cell populations we used FACS instrument settings to correct for that (reduced voltage in the fluorescent channel) and to increase specificity of the marker signal. This, however, also reduced the magnitude of the specific marker signal and expression levels of 500 (Mean Fluorescence Intensity) are hence high and maximal. The expression level of the isotype control antibodies was 3 + 1 (for FITC) and 10 + 1 (for PE).

**DNA staining for cell cycle and cell apoptosis analysis**

Cells were added in round-bottom 96-wells plates (Nunc) at a concentration of ~$10^5$ cells/well and washed twice in PBS/0.5% BSA/20mM sodium azide. Pelleted cells were resuspended in 100 µl PBS with 100 µl 0.5% PFA to fixate the cell, incubated for 60 min. at 4°C followed by one washing step. After washing, cells were resuspended with 200 µl PBS/0.2% Tween20 and incubated for 15 min. at 37°C to permeabilize the cells followed by two washing steps. Cells were resuspended in 200 µl PBS/0.2% Tween20. 100 µl was taken out and 200 µl 7-AAD was added (30 µg/ml) for DNA staining. For cell analysis, 10,000 events were recorded with a FACS (FACSCalibur, Becton Dickinson, Sunnyvale, CA, USA).

**MLR**

T cells from F344 and Wistar rats were enriched using a nylon wool column. In short, spleens were minced and teased through a 105-µm filter, and the erythrocytes were removed by lysis. Cells were washed and loaded onto a nylon wool column (3 g; Polyscience, Eppelheim, Germany) packed into a 60-ml plastic syringe. After 1 h. in 5% $C0_2$ incubator, T cells (80-90% $CD3^+$ cells) were harvested by collecting the effluent.

For the MLR, BM derived DC were added at various ratios to T cells (fixed number of 150,000 T cells/well) in flat-bottom 96-wells plates (Nunc, Roskilde, Denmark). Subsequently, these cells were cultured for 3 days in RPMI 1640 containing 50 mM HEPES buffer (Life Technologies), 10% FCSi, 110 μg/ml sodium pyruvate (Merck, Munich, Germany), 0.5% (v/v) β-mercapto-ethanol (Merck), penicillin (100 U/ml; Seromed, Bio-chrom, Berlin, Germany), and streptomycin (0.1 mg/ml; Seromed). In the MLR, T cell proliferation was measured via tritiated thymidine ([$^3$H]TdR) incorporation (0.5 μCi/well during the last 16 h. of total culture period). Finally, cells were harvested on filter papers, and radioactivity was counted in a liquid scintillation analyzer (LKB Betaplate, Wallac, Turun, Finland).

**IL-10 and IL-12 production**

Supernatants were obtained from dendritic cells by culturing them in 24-well plates (Nunc, Roskilde, Denmark) for 24 h. Cells were resuspended in serum free medium (SF-1) at a concentration of 0.5 x $10^6$/ml. SF-1 medium was made by dissolving 1 ml SF-1 in 9 ml RPMI 1640 with antibiotics. Cells were either cultured in SF-1 medium alone for basal production, SAC (*Staphylococcus aureus* Cowan strain) for IL-10 production or SAC and IFN-γ for IL-12p40 and IL-12p70 production.[27] Rat IL-10, IL-12p40 and IL-12p70 ELISA kits were commercially available (Biosource, Camarillo, CA, USA).

**Statistical analyses**

Mean values ± SD are shown. Statistical analyses were performed using Mann-Whitney test.

## RESULTS

GM-CSF/IL-4 culture of bone-marrow precursors yields an adherent and a non-adherent DC population. The non-adherent population has all the characteristics of prototypic DC.

The culture of bone-marrow precursor cells in GM-CSF/IL-4 yields two populations: an adherent colony-forming cell population and a floating non-adherent single cell population (Figure 1A and B shows characteristics of the Wistar rat DC). Similar results were found for Fischer rat DC (data not shown).

Both populations expressed the rat DC-specific markers OX62 (mean fluorescence intensity, MFI, non-adherent cells: 3291 ± 1207; adherent cells: 3199 ± 844) and CD11c (MFI non-adherent cells: 3334 ± 1769; adherent cells: 3398 ± 1010) and both populations expressed MHC class II, the adherent population stronger than the non-adherent population (Figure 1C). Despite the higher MHC class II expression the adherent population appeared functionally considerably less able to stimulate T cell proliferation in MLR as compared to the non-adherent population (Figure 1D). The non-adherent population also contained many more cells, which had the typical veiled/dendritic shape known of DC (Figure 1B).

To underscore the non-dendritic cell character of the adherent population we found it to also have various characteristics reminiscent of macrophages, such as a stronger expression of acid phosphatase and of the monocyte/macrophage markers ED1, ED2 and ED3 (Figure 1A, E-G). We therefore considered the non-adherent cell population as the more

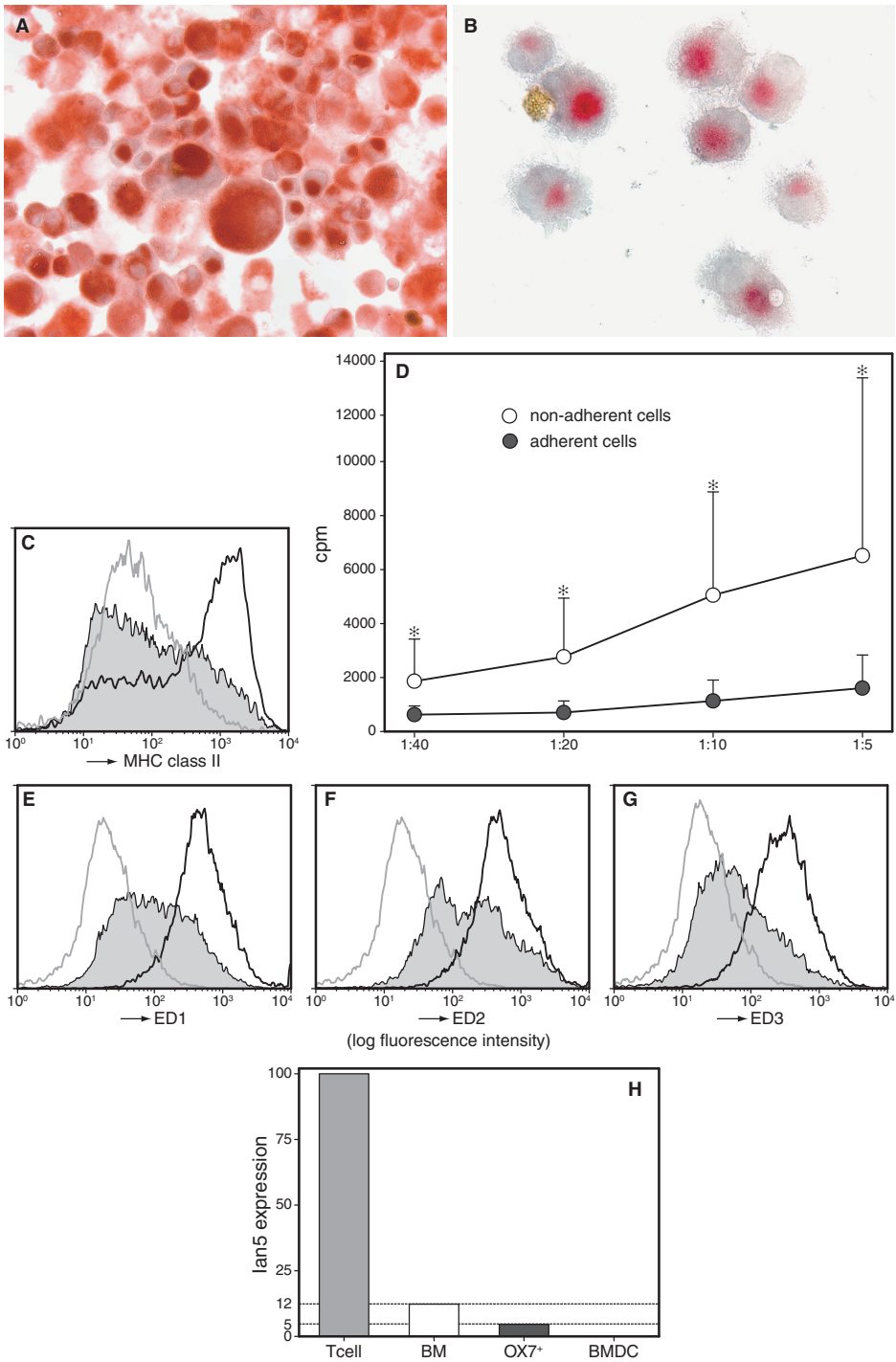
A
B
C
D
non-adherent cells
adherent cells
cpm
14000
12000
10000
8000
6000
4000
2000
0
1:40
1:20
1:10
1:5
MHC class II
E
F
G
ED1
ED2
ED3
(log fluorescence intensity)
H
Ian5 expression
100
75
50
25
12
5
0
Tcell
BM
OX7+
BMDC

**Figure 1. Characteristics of rat bone-marrow derived DC. When bone-marrow precursors are cultured in GM-CSF/IL-4 two $OX62^+CD11c^+$ populations are obtained: a population of adherent (colony-forming) cells and a non-adherent population of floating single cells.**
Although the non-adherent cells show a definite, but lower expression of MHC class II (C) as compared to adherent cells, the cells have a much higher capability to stimulate T cells to proliferate (D; *n*=10). The adherent cells also have more macrophage characteristics like a higher ED1 (E), ED2 (F) and ED3 (G) expression and a stronger and ''spotty'' acid phosphatase activity (A). We therefore consider the non-adherent cells as the typical DC. Interestingly, *Ian5* mRNA is not expressed in myeloid DC (H). The grey tone in (C), (E)-(G) represents the non-adherent cells. The dotted line represents isotype control. Representative figures are shown in (C), (E)-(G) (*n*=18). Experiments shown were performed in Wistar rats. The magnification for (A) and (B) is 64x and 96x, respectively. For colour figure see Appendix page 144.

prototypic immature DC population and focused on this population in our subsequent experiments. We will refer to this population as immature DC throughout the further text.

The adherent population probably represents precursors for the immature DC because one sees in culture the detachment of veiled/dendritic cells from these colony-forming adherent cells.

To be able to link putative aberrancies in DC and DC development in *lyp* gene carrying animals directly to gene influences, we also studied whether immature DC, their $OX7^+$ bone-marrow precursors and the non-fractioned bone-marrow population express the *Ian5* molecule. We found that the non-fractioned bone-marrow population and the $OX7^+$ DC precursors expressed *Ian5* mRNA, but at an extremely low level as compared to T cells (Figure 1H). The descending immature DC did not express *Ian5* mRNA (see Figure 1H). We therefore consider the *Ian5* mRNA expression in the precursor cells as due to T cell contamination of this population, because the non-fractioned bone-marrow cell population had a higher *Ian5* mRNA expression as compared to $OX7^+$ DC precursors, and because both populations are contaminated with some T cells (the non-fractioned bone-marrow cell population with around 10%, the $OX7^+$ bone-marrow DC precursors with around 5%).

DC bone-marrow precursors of congenic F344.*lyp*/*lyp* and *lyp*/+ rats show a higher apoptosis and the precursor derived DC show a lower expression of surface MHC class II molecules and a lower capability to stimulate T cells. These latter aberrancies are lost after a final differentiation of the cells with LPS.

Figure 2 shows that the F344.*lyp*/*lyp* and *lyp*/+ DC precursors exhibited a higher apoptosis as compared to +/+ cells in culture (Figure 2A). This resulted in a slightly lower (statistically not significant) yield of non-adherent floating DC (Figure 2B) after 7 days of culture (the yield of adherent cells was equal).

When studying the marker expression on bone-marrow precursor derived DC of the homozygous (*lyp*/*lyp*) and heterozygous (*lyp*/+) congenic F344 rats versus those of F344.+/+ animals, we found that the cells differed in their expression of MHC class II molecules on their surface: the *lyp* carrying animals had lower numbers of MHC class $II^{bright}$ cells, resulting in more MHC class $II^{dull}$ cells as compared to the +/+ animals (Table 1 and Figure 2C). With regard to the other tested important marker molecules such as OX62 and CD11c (the rat DC-specific markers) CD80, CD86, and CD40 (the co-stimulatory molecules) and ED1 (a monocyte/DC marker): neither the expression levels of these molecules, nor the percentages of positive cells for these markers differed between cells from *lyp*/*lyp*, *lyp*/+ and +/+ rats (Table 1).

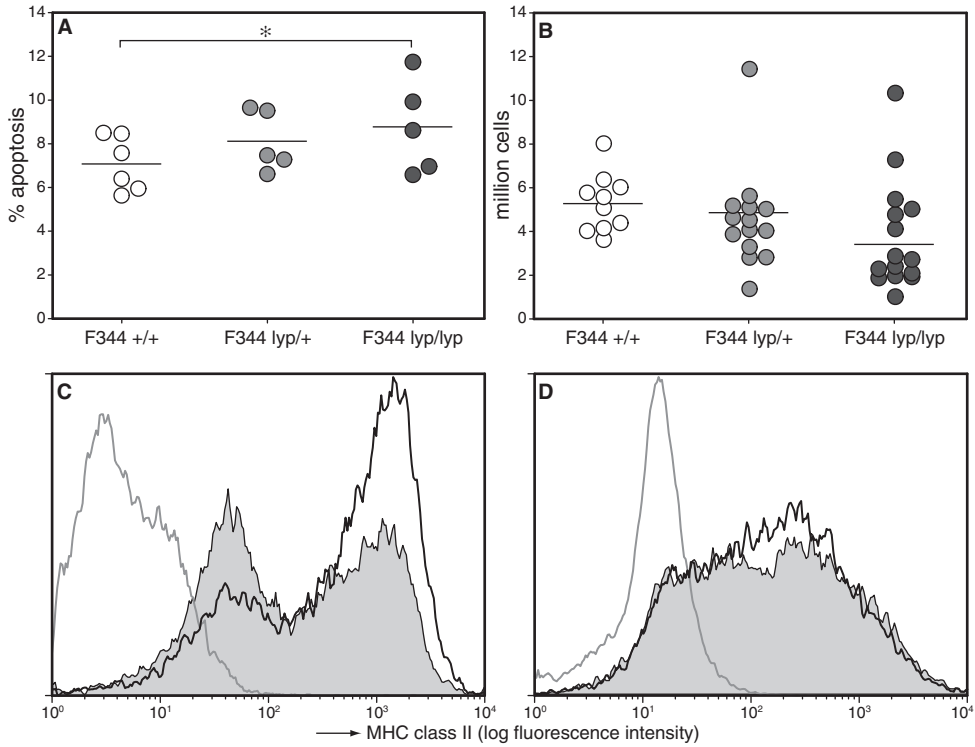


**Figure 2. Apoptosis and cell cycle in DC precursors and MHC class II expression of DC.**
The apoptosis (A) of precursor DC at day 4 of culture in F344.*lyp*/*lyp* rats as compared to F344.*lyp*/+ and F344.+/+ rats and measured by 7-AAD staining (*n*=5-6). There is a higher apoptosis of precursor DC of F344.*lyp*/*lyp* rats. The yield of cells (B) after 7 days of culture of bone-marrow precursors in GM-CSF/IL-4 in F344.*lyp*/*lyp* rats as compared to F344.*lyp*/+ and F344.+/+ rats (*n*=10-15). The yield is lower in F344.*lyp*/*lyp* rats, though not statistically significant. MHC class II expression of F344.*lyp*/*lyp* (solid grey fill) versus F344.+/+ (black line) of iDC (C) and mDC (D). Dotted line represents isotype control. It is clear that the number of MHC class II$^{dull}$ cells is higher in the iDC preparation of *lyp*/*lyp* versus +/+ rats.

The T cell stimulatory capacities of immature DC of F344.*lyp*/*lyp*, *lyp*/+ and +/+ rats were tested in an (allo)-MLR (Figure 3A). As can be seen, the T cell stimulatory capacity of immature DC of *lyp*/*lyp* and *lyp*/+ rats was considerably lower than that of +/+ rats, particularly at DC to T cell ratios 1:5 and 1:10.

To see whether the reduced expression of MHC class II molecules and the reduced T cell stimulatory capacity for T cells was still present for the F344.*lyp*/*lyp* and *lyp*/+ rats after a final differentiation (maturation) of the cells, we further differentiated the DC using LPS. In a preliminary small set of experiments, we had used several maturation factors (LPS, IFN-γ, TNF-α, and *Mycobacterium tuberculosis* (MBT)) to find the optimal maturation factor for rat DC (see Table 2). We had found 1 μg/ml LPS to be the best maturation factor to induce mature DC with a higher surface expression of MHC class II molecules and co-stimulatory

**Table 1. The Mean Fluorescence Intensity (MFI) of important marker molecules on the cell surface of bone marrow derived DC (*n*=5-12).**

| Marker | F344.*lyp/lyp* | F344.*lyp*/+ | F344.+/+ |
|---|---|---|---|
| OX62 | 339 ± 133 | 292 ± 66 | 379 ± 153 |
| CD11c | 58 ± 19 | 23 ± 4 | 66 ± 48 |
| MHC class II | **133 ± 63*** | **144 ± 25*** | 241 ± 110 |
| CD80 | 17 ± 9 | 12 ± 4 | 20 ± 7 |
| CD86 | 22 ± 11 | 19 ± 12 | 30 ± 13 |
| CD40 | 24 ± 18 | 16 ± 6 | 37 ± 21 |
| ED1 | 31 ± 29 | 11 ± 10 | 45 ± 10 |

* represents *p* values of <0.05 as compared to the +/+ condition

molecules and a clearly raised potency to stimulate T cells (Table 2).

With regard to the LPS maturation of the immature DC of the F344.*lyp*/*lyp* and *lyp*/+ rats it appeared that after LPS maturation the MHC class II$^{dull}$ population of DC had disappeared in the *lyp*/*lyp* and *lyp*/+ condition; all DC now expressed higher levels of MHC class II in the *lyp*/*lyp*, *lyp*/+ and +/+ conditions (Figure 2D and Table 3). The expressions of CD80, CD86 and CD40 were also not different between the mature DC of *lyp*/*lyp*, *lyp*/+ and +/+ animals (Table 3). In accord with the restoration of the MHC class II expression due to LPS maturation, the T cell stimulatory capacity of the *lyp*/*lyp* and *lyp*/+ rats was also restored by the LPS maturation (Figure 3B).

Of note is that we isolated in a separate small series of experiments a crude DC fraction

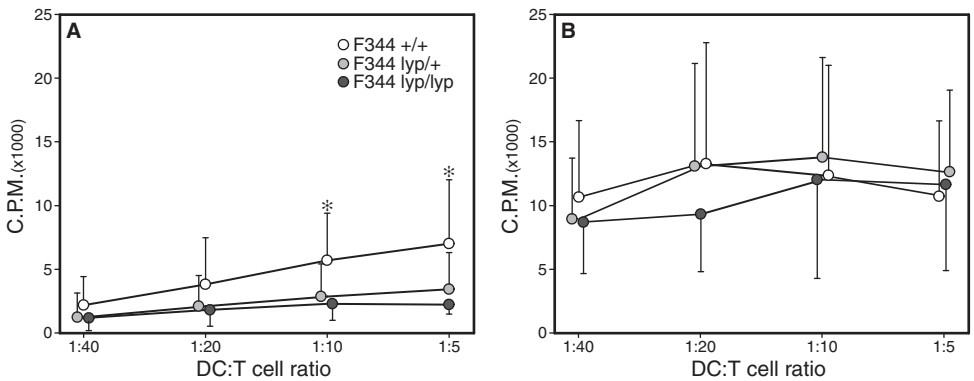


**Figure 3. The T cell stimulatory capacity of iDC and mDC.**
The T cell stimulatory capacity of iDC (A) and mDC (B) of F344.*lyp*/*lyp*, F344.*lyp*/+ and F344.+/+ rats. The T cell stimulatory capacity was measured in allogeneic MLR and expressed as the incorporation of $^{3}$H-thymidine (c.p.m.) in the stimulated T cells. iDC of *lyp*/*lyp* animals have a poor T cell stimulatory capacity as compared to the +/+ animals ($p$<0.05; $n$=4-10). The same holds true for *lyp*/+ animals, although this was not statistically significant. This defect is restored by maturation of the cells with LPS, while all mDC gain in T cell stimulatory capacity. Note: F344 DC were not stimulated with Wistar T cells (allogeneic MLR:MHC haplotypes are different between F344 and Wistar rats).

**Table 2. The Mean Fluorescence Intensity (MFI) of important marker molecules on the cell surface of bone marrow derived DC of Wistar rats after LPS, TNF-α and IFN-γ maturation (*n*=3) together with the T cell stimulatory capacity (MLR).**

| Marker | iDC | LPS-mDC | IFN-γ-mDC | TNF-α-mDC |
|---|---|---|---|---|
| MHC class II | 207 ± 35 | **317 ± 22*** | **462 ± 95*** | 365 ± 113 |
| CD80 | 27 ± 6 | **45 ± 1*** | **31 ± 2*** | 31 ± 11 |
| CD86 | 54 ± 33 | 84 ± 14 | 74 ± 10 | 68 ± 27 |
| CD40 | 45 ± 19 | 61 ± 27 | 57 ± 14 | 48 ± 14 |
| MLR | 3464 ± 2343 | 43,990 ± 36,867 | 1788 ± 941 | 2963 ± 746 |

* represents *p* values of <0.05 as compared to the iDC

(low-density cells) from the spleen of F344.*lyp*/*lyp* and +/+ rats (for isolation details, see ref. 22). Spleen DC can be considered as belonging to the series of mature DC[28], which have received their maturation signals *in vivo*. Figure 4 shows that the $OX62^{+}$ cells in the spleen low-density fraction of F344.*lyp*/*lyp* animals contained more MHC class $II^{negative}$ cells as compared to such fractions of F344.+/+ rats (thus showing a reduced expression of MHC class II on spleen DC), yet the T cell stimulatory capacity was equal between the spleen low-density cell fraction of F344.*lyp*/*lyp* versus +/+ rats (and similar to that of mature *in vitro* bone-marrow derived DC). Hence, we must conclude that LPS exposure *in vitro*, but only with regard to MHC class II expression, is more effective to induce a further differentiation of immature DC of the F344.*lyp*/*lyp* rats than the physiological maturation factors acting *in vivo* (fitting in with the idea that such cells are not fully mature, but in fact semi-mature[29]). Only DC of homozygous of F344.*lyp*/*lyp* rats show a lower capability to produce IL-10, the cells have a normal IL-12p40 production. The lower capability to produce IL-10 is partially restored after a final differentiation of the cells with LPS.

With regard to the cytokine production by the bone-marrow derived DC we tested the most relevant DC-related cytokines IL-12p70, IL-12p40 and IL-10 and used as stimulator *S. aureus* strain Cowan (in combination with IFN-γ for IL-12p70 and IL-12p40 production), since we had previously found that this was the most optimal stimulation for DC to produce

**Table 3. The Mean Fluorescence Intensity (MFI) of important marker molecules on the cell surface of bone-marrow derived DC of Wistar rats after LPS maturation (*n*=3-8) (statistically significant differences were not found between the three sub-lines).**

| Marker | F344.*lyp*/*lyp* | F344.*lyp*/+ | F344.+/+ |
|---|---|---|---|
| OX62 | 345 ± 276 | 474 ± 175 | 265 ± 139 |
| CD11c | 95 ± 11 | n.d. | 55 ± 12 |
| MHC class II | 203 ± 61 | 229 ± 58 | 218 ± 60 |
| CD80 | 22 ± 7 | 20 ± 3 | 35 ± 11 |
| CD86 | 26 ± 9 | 28 ± 13 | 36 ± 14 |
| CD40 | 32 ± 14 | 30 ± 6 | 33 ± 10 |
| ED1 | 14 ± 14 | 8 ± 3 | 10 ± 0 |

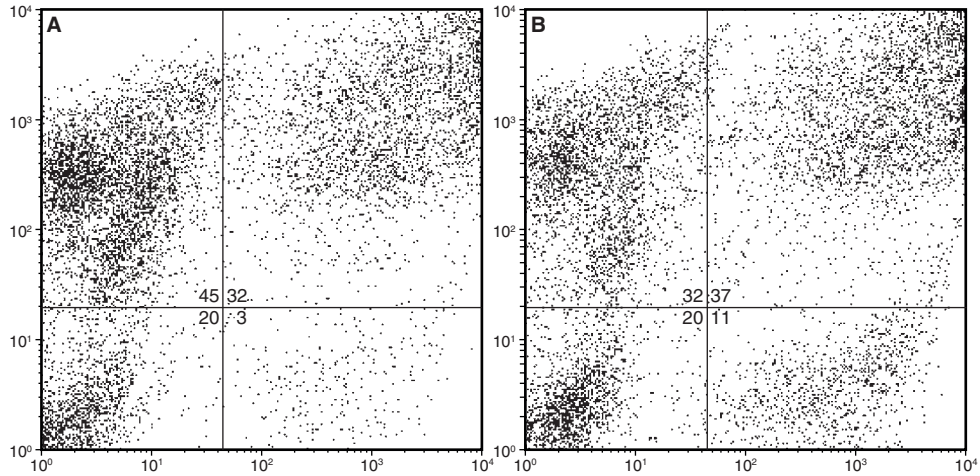


**Figure 4. The low-density cell fraction (a fraction enriched for DC) of the spleen of F344.*lyp/lyp* rats contains a higher number of OX62 cells (prototypic DC) with a reduced MHC class II expression as compared to such fractions of F344.+/+ rats.**

Dot plots of spleen low-density cells of F344.+/+ (A) and F344.*lyp*/*lyp* (B) rats with OX62 on the *x*-axis and MHC class II on the *y*-axis. The spleen low-density cells of the F344.*lyp*/*lyp* rat show a higher number of $OX62^{+}$ MHC class $II^{negative}$ cells as compared to such low-density cells of F344.+/+ rats. Percentages are indicated in the dot plots.

these cytokines.[27]

The IL-12p70 production by our DC populations was low, and there was no statistically significant difference between *lyp* carrying and +/+ bone-marrow derived DC, irrespective of their state of maturation (data not shown). The IL-12p40 production was, however, considerable, but did not differ between *lyp*/*lyp*, *lyp*/+ and +/+ conditions (Table 4), neither in the case of immature DC nor in the case of mature DC. It was evident that mature DC did produce more IL-12p40, as is expected from mature DC.

The IL-10 production of the rat DC populations was also considerable when stimulated with SAC. Interestingly, the IL-10 production capability of the bone-marrow derived DC of *lyp*/*lyp* animals was significantly lower compared to that of the DC of *lyp*/+ and +/+ animals

**Table 4. The IL-12p40 cytokine production of bone-marrow derived immature DC (iDC) and mature DC (mDC) of the various sub-lines of the F344 rats (statistically significant differences were not found between the three sub-lines (*n*=3)).**

| Cytokine (pg/ml) | Rat strain | IL-12 iDC (not stimulated) | IL-12 iDC (SAC/IFN-γ) | IL-12 mDC (not stimulated) | IL-12 mDC (SAC/IFN-γ) |
|---|---|---|---|---|---|
| IL-12p40 | F344.*lyp*/*lyp* | 57 ± 23 | 1646 ± 384 | 1294 ± 889 | 2254 ± 1492 |
| | F344.*lyp*/+ | 68 ± 9 | 1880 ± 203 | 624 ± 90 | 2121 ± 336 |
| | F344.+/+ | 82 ± 24 | 1979 ± 117 | 714 ± 229 | 2151 ± 372 |

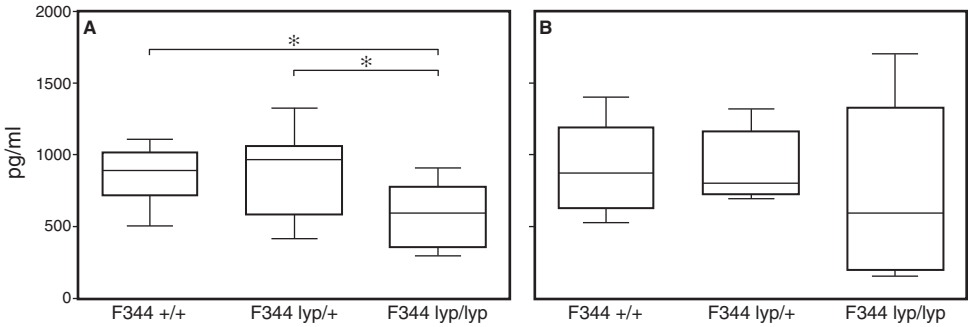


**Figure 5. The IL-10 production capacity of iDC and mDC. The IL-10 production capacity of iDC (A) and mDC (B) of F344.*lyp*/*lyp*, F344.*lyp*/+ and F344.+/+ rats.**
The IL-10 production capacity was measured after stimulation of the cells with SAC and measured in ELISA and expressed as pg/ml production over a 24-h period. iDC of *lyp*/*lyp* animals have a poor IL-10 production as compared to the *lyp*/+ and +/+ animals ($p<0.05$; $n=6$-8). This defect is partially restored by maturation of the cells with LPS.

(Figure 5A). A final differentiation with LPS corrected this low IL-10 production capability in DC populations of some of the animals, since there was an increase in the average IL-10 production, yet with a considerable standard deviation (Figure 5B), resulting in no significant difference between mature DC of *lyp*/*lyp* and +/+ animals.

## DISCUSSION

This study shows that the 42.5 Mb - 93.6 Mb region of chromosome 4 of the BB-DP rat, which contains both *iddm2* (the *lyp* gene) and *iddm4*, is not only responsible for the severe T lymphocytopenia of the rat[15-19], but is also linked to an aberrant differentiation of DC from bone-marrow precursors. While homozygosity for this region is essential for severe T lymphocytopenia, it turned out that heterozygosity for the genes on this region of chromosome 4, is sufficient to result in an enhanced apoptosis of DC precursors, a reduced MHC class II expression on precursor derived DC and a low capability of the DC to stimulate T cells in (allo)-MLR. A reduced IL-10 production capability of the cells was only found in congenic F344 homozygous for the 42.5 Mb to 93.6 Mb region of chromosome 4 of the BB-DP rat.

Previously we reported on effects on the DC differentiation and maturation in the presence of the *iddm2* (*lyp* gene) containing 1.3 Mb region of BB-DP chromosome 4 on the BB-DR rat background, thus representing the effects of only this small region on DC differentiation/maturation and thus likely closely linked to *iddm2*. Table 5 gives these data in relation to the effects of the larger 51.1 Mb region of BB-DP chromosome 4 (including *iddm2* and *iddm4*) on the wild-type F344 rat background here found.

As can be seen, the enhanced apoptosis of DC precursors and the reduced MHC class II expression on precursor derived DC can be linked to *iddm2*, since it occurs both in BB-DP/S and in congenic F344.*lyp*/*lyp* rats versus the BB-DR/S and F344.+/+, respectively.

Table 5 also shows that we are able to detect a set of abnormalities in the DC, which are

**Table 5. Linkage of DC phenotype with the genetic background of the various sub-lines of the F344 and BB rats.**

| Genes involved | | F344.+/+ | F344.*lyp/lyp* | BB.+/+ | BB.*lyp*/*lyp* |
|---|---|---|---|---|---|
| Genotype | | Normal genes | Normal genes plus BB genes on chromosome 4 (42.5-93.6 Mb) | BB genes minus *iddm2* | BB genes plus *iddm2* |
| *Iddm2 (lyp)* | Apoptosis precursors | normal | **increased** | normal | **increased** |
| | MHC class II expression | normal | **decreased** | normal | **decreased** |
| BB genes not on the 42.5 Mb-93.6 Mb region of chromosome 4 | Yield of DC | normal | normal | **increased** | **increased** |
| | *In vitro* maturation capacity after LPS | normal | normal | **decreased** | **decreased** |
| *iddm4* (candidate) | T cell stimulation | normal | **decreased** | normal | normal |
| Complex linkage | IL-10 production | normal | **decreased** | **decreased** | **decreased (but equal to BB.+/+)** |

characteristic of both BB-DP/S and BB-DR/S rats and are different as compared to control Wistar and F344 rats. Such abnormalities must thus be due to genes in the BB gene background other than those on the larger 51.1 Mb region of BB-DP chromosome 4 (including *iddm2* and *iddm4*). These aberrancies included a high yield of DC from precursors in the GM-CSF/IL-4 culture and a poor capacity of the generated immature DC to respond to LPS and to differentiate into strong T cell stimulatory mature DC (see Table 5).

There is also a DC abnormality, i.e. the poor T cell stimulatory capacity of DC, which was found in congenic F344.*lyp*/*lyp* rats but not in F344.+/+ rats. It is thus linked to the large 51.1 Mb region of BB-DP chromosome 4 containing both *iddm2* and *iddm4*. However, the T cell stimulatory capacity of DC is normal in BB-DP/S rats and not different from that of BB-DR/S rats. It is thus not linked to the small *iddm2* containing 1.3 Mb region of BB-DP chromosome 4. Hence, *iddm4* is a likely candidate gene for the poor capability of DC to stimulate T cells.

Finally, one of the DC abnormalities i.e. the reduced IL-10 production capacity, shows a complex linkage pattern. The IL-10 production was not different between BB-DP/S and BB-DR/S rats and is thus not linked to the small *iddm2* containing 1.3 Mb region of BB-DP chromosome 4. It is different in the F344 homozygous *lyp*/*lyp* rats versus the +/+ rats, hence *iddm4* or another gene on chromosome 4 is involved. However, it is also different between BB-DR/S and normal F344.+/+ rats and this observation indicates a linkage with additional genes in the BB-background (Table 5). Clearly to investigate whether these genes are really involved, similar studies as reported here but with rats transgenic for such *iddm*s need to be performed. A new and opposite approach introducing functional genes into rats could be another elegant research tool. Recently, Michalkiewicz and co-workers[30] were able to restore *Ian5* transcript and protein levels by creating a transgenic rat, completely rescuing the T cell lymphopenia in F344.*lyp*/*lyp* rats.

It is not difficult to envisage the reduced IL-10 production of DC of F344.*lyp*/*lyp* rats and rats of the BB-background as contributing to the autoimmune status of the animals, since IL-10 is an important immune suppressive molecule. Also in human diabetes, IL-10 production defects have been described. But how might a low MHC class II expression and a low T cell stimulatory capacity of DC contribute to proneness for the auto-aggressive self-reactivity of the BB-DP rat? There are a few indications in the literature that poorly differentiated DC contribute to imbalances in the T cell system in such a way that autoimmunity prevails. First of all, transfers of properly terminally differentiated DC prevent diabetes development in the NOD mouse. Dahlen et al.[31] suggested that such transfers worked due to the correction of the low level of co-stimulation given by defective NOD DC: the transferred mature DC would give a full activation of autoreactive T cells and would consequently induce Activation Induced T Cell Death (AITCD) and/or the up-regulation of CTLA-4, an important switch-off signal for activated T cells. However, other mechanisms might play a role as well: properly terminally differentiated mature DC are also better equipped to skew T cell responses towards Th2 immune responses[32] and to directly expand $CD4^+CD25^+$ regulatory T cells.[33,34] Indeed in the BB-DP rat model, the poorly differentiated lymph node and spleen DC of the BB-DP rat showed a reduced potential of expanding the important $ART2^+$ regulatory T cell population of the rat.[22] That indeed a full stimulation of T cells via the co-stimulatory pathways leads to a prevention of autoimmunity is further illustrated in experiments directly interfering in the CD28 stimulating pathway. When the BB-DP rat is treated with a stimulating anti-CD28 antibody (thus correcting the poor stimulating activity of the animals APC and activating the T cells), autoimmunity does not develop.[35]

Although the gene dose-effects (differences between *lyp/lyp* and *lyp/+*) of the DC aberrancies linked to *iddm2* suggest a direct effect of the gene on DC differentiation and maturation, it must be noted that we were not able to detect the expression of *Ian5* in the DC or its precursors (the mutated *Ian5* is considered the crucial abnormality determining the lymphopenia status). Which other indirect mechanisms could be responsible for the observed aberrancies in the differentiation of DC in the *lyp/lyp* and *lyp/+* animals? In the *lyp/lyp* and *lyp/+* animals, the lack of T cells or T cell products in the cultures to generate DC could have played a role. The bone-marrow cultures of the +/+ and *lyp/+* animals contained approximately 10% T cells, while the cultures of the *lyp/lyp* animals contained approximately 5%. That a deficiency in T cell factors in the culture is a possibility for an aberrant DC differentiation has been shown in experiments with $RAG2^{-/-}$ T cell-deficient mice that exhibit a striking deficiency in Langerhans cell numbers in the epidermis and significantly lower numbers and a deficient function of both lymphoid- and myeloid-related DC in the draining lymph node, all of which were corrected by an adoptive transfer of normal T cells.[36] A T cell factor that could account for an appropriate DC differentiation from precursors could be an essential interaction with the co-stimulatory CD40L on T cells present in the culture; CD40L is able to induce a final differentiation of DC.[37,38] Also T cell cytokines such as IL-4[39,40] and IFN-γ[41,42] might play a role. However, IL-4 was added to our cultures, but IFN-γ was not. This cytokine stimulates DC to up-regulate co-stimulatory/activation markers and the cell's capability to stimulate T cells.[41]

Macrophage-like cells are also present in our DC cultures and $OX7^+$ bone-marrow precursors can also give rise to macrophages, if stimulated appropriately with M-CSF. Some macrophage populations do express *Ian5* (our unpublished observations) and it must

be noted that macrophages of lymphopenic BB-DP rats are special in that they show an excessive production of L-arginine, NO[43-45] and other reactive oxygen species.[46] The TNF-α production by macrophages of the BB-DP rat is conjectural, a low[47] as well as a high TNF-α production[48] have been described. All these factors are known to be involved in the differentiation and maturation of DC. Moreover, bone-marrow derived DC, unlike spleen DC, are able to produce high levels of NO similar to peritoneal macrophages[49] and it has been shown that such oxidative radicals do suppress the differentiation of DC[50,51] and inhibited the T cell proliferation exerted by DC.[46] Clearly the production of IFN-γ, NO and other reactive oxygen species needs to be investigated in the GM-CSF/IL-4 induced bone-marrow cultures of the *lyp*/*lyp* and *lyp*/+ animals.

In conclusion we here show that the 42.5 Mb - 93.6 Mb region of chromosome 4 of the autoimmune prone BB-DP rat, which contains both the rat *iddm2* (the *lyp* gene) and *iddm4*, is linked to an increased apoptosis of DC bone-marrow precursor cells, a low MHC class II expression on DC differentiated from bone-marrow precursors, a reduced T cell stimulatory capacity, and a reduced IL-10 production of the DC.

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## REFERENCES

1. Kabel PJ, Voorbij HAM, van der Gaag RD, Wiersinga WM, de Haan M, Drexhage HA. Dendritic cells in autoimmune thyroiditis. *Acta Endocrinol. Suppl.* 1987;281:42-48.
2. Voorbij HAM, Jeucken PH, Kabel PJ, de Haan M, Drexhage HA. Dendritic cells and scavenger macrophages in pancreatic islets of prediabetic BB rats. *Diabetes* 1989;38:1623-1629.
3. Voorbij HAM, Kabel PJ, de Haan M, Jeucken PH, van der Gaag RD, de Baets MH, Drexhage HA. Dendritic cells and class II MHC expression on thyrocytes during the autoimmune thyroid disease of the BB rat. *Clin. Immunol. Immunopathol.* 1990;55:9-22.
4. Ziegler AG, Erhard J, Lampeter EF, Nagelkerken LM, Standl E. Involvement of dendritic cells in early insulitis of BB rats. *J. Autoimmun.* 1992;5:571-579.
5. Jansen A, Homo-Delarche F, Hooijkaas H, Leenen PJ, Dardenne M, Drexhage HA. Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of the insulitis and β-cell destruction in NOD mice. *Diabetes* 1994;43:667-675.
6. Many MC, Maniratunga S, Varis I, Dardenne M, Drexhage HA, Denef JF. Two-step development of Hashimoto-like thyroiditis in genetically autoimmune prone non-obese diabetic mice: effects of iodine-induced cell necrosis. *J. Endocrinol.* 1995;147:311-320.
7. Van Blokland SC, van Helden-Meeuwsen CG, Wierenga-Wolf AF, Drexhage HA, Hooijkaas H, van de Merwe JP, Versnel MA. Two different types of sialoadenitis in the NOD- and MRL/lpr mouse models for Sjögren's syndrome: a differential role for dendritic cells in the initiation of sialoadenitis? *Lab. Invest.* 2000;80:575-585.
8. Kabel PJ, Voorbij HAM, De Haan M, van der Gaag RD, Drexhage HA. Intrathyroidal dendritic cells. *J. Clin. Endocrinol. Metabol.* 1988;66:199-207.

9. Jansen A, Voorbij HA, Jeucken PH, Bruining GJ, Hooijkaas H, Drexhage HA. An immunohistochemical study on organized lymphoid cell infiltrates in fetal and neonatal pancreases: a comparison with similar infiltrates found in the pancreas of a diabetic infant. *Autoimmunity* 1993;15:31-38.
10. Yamakawa M, Kato H, Takagi S, Karube Y, Seki K, Imai Y. Dendritic cells in various human thyroid diseases. *In Vivo* 1993;7:249-256.
11. Xanthou G, Tapinos NI, Polihronis M, Nezis IP, Margaritis LH, Moutsopoulos HM. CD4 cytotoxic and dendritic cells in the immunopathologic lesion of Sjögren's syndrome. *Clin. Exp. Immunol.* 1999;118:154-163.
12. Greiner DL, Malkani S, Kanaitsuka T, Bortell R, Doukas J, Rigby M, Whalen B, Stevens LA, Moss J, Mordes JP, Rossini AA. The T cell marker RT6 in a rat model of autoimmune diabetes. *Adv. Exp. Med. Biol.* 1997;419: 209-216.
13. Greiner DL, Mordes JP, Handler ES, Angelillo M, Nakamura N, Rossini AA. Depletion of RT6.1+ T lymphocytes induces diabetes in resistant biobreeding/Worcester (BB/W) rats. *J. Exp. Med.* 1987;166:461-475.
14. Burstein D, Mordes JP, Greiner DL, Stein D, Nakamura N, Handler ES, Rossini AA. Prevention of diabetes in BB/Wor rat by single transfusion of spleen cells: parameters that affect degree of protection. *Diabetes* 1989;38:24-30.
15. Jackson R, Rassi N, Crump T, Haynes T, Eisenbarth GS. The BB diabetic rat. Profound T-cell lymphopenia. *Diabetes* 1981;30:887-889.
16. Guttmann RD, Colle E, Michel F, Seemayer T. Spontaneous diabetes mellitus syndrome in the rat. II. T lymphopenia and its association with clinical disease and pancreatic lymphocytic infiltration. *J. Immunol.* 1983;130:1732-1735.
17. Greiner DL, Handler ES, Nakano V, Mordes JP, Rossini AA. Absence of the RT-6 cell subset in diabetes-prone BB/W rats. *J. Immunol.* 1986;136:148-151.
18. Markholst H, Eastman S, Wilson D, Andreasen BE, Lernmark Å. Diabetes segregates as a single locus in crosses between inbred BB rats prone or resistant to diabetes. *J. Exp. Med.* 1991;174:297-300.
19. Jacob H, Pettersson A, Wilson D, Lernmark Å, Lander ES. Genetic dissection of autoimmune type 1 diabetes in the BB rat. *Nat. Genet.* 1992;2:56-60.
20. MacMurray AJ, Moralejo DH, Kwitek AE, Rutledge EA, Van Yserloo B, Gohlke P, Speros SJ, Snyder B, Schaefer J, Bieg S, Jiang J, Ettinger RA, Fuller J, Daniels TL, Pettersson A, Orlebeke K, Birren B, Jacob HJ, Lander ES, Lernmark Å. Lymphopenia in the BB rat model of type 1 diabetes is due to a mutation in a novel immune-associated nucleotide (Ian)-related gene. *Genome Res.* 2002;12:1029-1039.
21. Hornum L, Romer J, Markholst H. The diabetes-prone BB rat carries a frameshift mutation in Ian4, a positional candidate of Iddm1. *Diabetes* 2002;51:1972-1979.
22. Delemarre FG, Simons PJ, de Heer HJ, Drexhage HA. Signs of immaturity of splenic dendritic cells from the autoimmune prone biobreeding rat: consequences for the *in vitro* expansion of regulator and effector T cells. *J. Immunol.* 1999;162:1795-1801.
23. Delemarre FG, Hoogeveen PG, De Haan-Meulman M, Simons PJ, Drexhage HA. Homotypic cluster formation of dendritic cells, a close correlate of their state of maturation. Defects in the biobreeding diabetes-prone rat. *J. Leukoc. Biol.* 2001;69:373-380.
24. Simons PJ, Delemarre FG, Drexhage HA. A functional and phenotypic study on immune accessory cells isolated from the thyroids of Wistar and autoimmune-prone BB-DP rats. *J. Autoimmun.* 2000;15:417-424.
25. Hornum L, Lundsgaard D, Markholst H. An F344 rat congenic for BB/DP rat-derived diabetes susceptibility loci Iddm1 and Iddm2. *Mamm. Genome* 2001;12:867-868.
26. Moralejo DH, Park HA, Speros SJ, MacMurray AJ, Kwitek AE, Jacob HJ, Lander ES, Lernmark Å. Genetic dissection of lymhopenia from autoimmunity by introgression of mutated Ian5 gene onto the F344 rat. *J. Autoimmun.* 2003;21:315-324.
27. Ruwhof C, Canning MO, Grotenhuis K, de Wit HJ, Florencia ZZ, de Haan-Meulman M, Drexhage HA. Accessory cells with a veiled morphology and movement pattern generated from monocytes after avoidance of plastic adherence and of NADPH oxidase activation. A comparison with GM-CSF/IL-4-induced monocyte-derived dendritic cells. *Immunobiology* 2002;205:247-266.
28. Tan JK, O'Neill HC. Maturation requirements for dendritic cells in T cell stimulation leading to tolerance versus immunity. *J. Leukoc. Biol.* 2005;78:319-324.
29. Lutz MB, Schuler G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol.* 2002;23:445-449.

30. Michalkiewicz M, Michalkiewicz T, Ettinger RA, Rutledge EA, Fuller JM, Moralejo DH, Van Yserloo B, MacMurray AJ, Kwitek AE, Jacob HJ, Lander ES, Lernmark Å. Transgenic rescue demonstrates involvement of the Ian5 gene in T cell development in the rat. *Physiol. Genomics* 2004;19:228-232.
31. Dahlen E, Hedlund G, Dawe K. Low CD86 expression in the nonobese diabetic mouse results in the impairment of both T cell activation and CTLA-4 up-regulation. *J. Immunol.* 2000;164:2444-2456.
32. Feili-Hariri M, Falkner DH, Morel PA. Regulatory Th2 response induced following adoptive transfer of dendritic cells in prediabetic NOD mice. *Eur. J. Immunol.* 2002;32:2021-2030.
33. Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat. Immunol.* 2001;2:725-731.
34. Yamazaki S, Iyoda T, Tarbell K, Olson K, Velinzon K, Inaba K, et al. Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. *J. Exp. Med.* 2003;198:235-247.
35. Beaudette-Zlatanova BC, Whalen B, Zipris D, Yagita H, Rozing J, Benjamin CD, Hunig T, Drexhage HA, Amari MJ, Leif J, Mordes JP, Greiner DL, Sayegh MH, Rossini AA. Costimulation and autoimmune diabetes in BB rats. *Am. J. Transplant.* 2006;6:894-902.
36. Shreedhar V, Moodycliffe AM, Ullrich SE, Bucana C, Kripke ML, Flores-Romo L. Dendritic cells require T cells for functional maturation *in vivo*. *Immunity* 1999;11:625-636.
37. Caux C, Massacrier C, Vanbervliet B, Dubois B, Van Kooten C, Durand I, Banchereau J. Activation of human dendritic cells through CD40 cross-linking. *J. Exp. Med.* 1994;180:1263-1272.
38. Miga AJ, Masters SR, Durell BG, Gonzalez M, Jenkins MK, Maliszewski C, Kikutani H, Wade WF, Noelle RJ. Dendritic cell longevity and T cell persistence is controlled by CD154-CD40 interactions. *Eur. J. Immunol.* 2001;31:959-965.
39. Jackson SH, Yu CR, Mahdi RM, Ebong S, Egwuagu CE. Dendritic cell maturation requires STAT1 and is under feedback regulation by suppressors of cytokine signaling. *J. Immunol.* 2004;172:2307-2315.
40. Lutz MB, Schnare M, Menges M, Rossner S, Rollinghoff M, Schuler G, Gessner A. Differential functions of IL-4 receptor types I and II for dendritic cell maturation and IL-12 production and their dependency on GM-CSF. *J. Immunol.* 2002;169:3574-3580.
41. Banyer JL, Halliday DC, Thomson SA, Hamilton NH. Combinations of IFN-γ and IL-4 induce distinct profiles of dendritic cell-associated immunoregulatory properties. *Genes Immun.* 2003;4:427-440.
42. Rissoan M-C, Soumelis V, Kadowaki N, Grouard G, Briere F, de Waal Malefyt R, Liu YJ. Reciprocal control of T helper cell and dendritic cell differentiation. *Science* 1999;283:1183-1186.
43 Lau A, Ramanathan S, Poussier P. Excessive production of nitric oxide by macrophages from DP-BB is secondary to the T lymphopenic state of these animals. *Diabetes* 1998;47:197-205.
44. Wu G, Flynn NE. The activation of arginine-citrulline cycle in macrophages from the spontaneously diabetic BB rat. *Biochem. J.* 1993;294:113-118.
45. Lee KU. Nitric oxide produced by macrophages mediates suppression of conA-induced proliferative responses of splenic leukocytes in the diabetes-prone BB rat. *Diabetes* 1994;43:1218-1220.
46. Brenner HH, Burkart V, Rothe H, Kolb H. Oxygen radical production is increased in macrophages from diabetes prone BB rat. *Autoimmunity* 1993;15:93-98.
47. Lapchak PH, Guilbert LJ, Rabinovitch A. Tumor necrosis factor production is deficient in diabetes-prone BB rats and can be corrected by complete Freund's adjuvant: a possible immunoregulatory role of tumor necrosis factor in the prevention of diabetes. *Clin. Immunol. Immunopathol.* 1992;65:129-134.
48. Rothe H, Fehsel K, Kolb H. Tumor necrosis factor alpha production is upregulated in diabetes prone BB rats. *Diabetologia* 1990;33:573-575.
49. Powell TJ, Jenkins CD, Hattori R, MacPherson GG. Rat bone marrow-derived dendritic cells, but not *ex vivo* dendritic cells, secrete nitric oxide and can inhibit T-cell proliferation. *Immunology* 2003;109:197-208.
50. Corinti S, Pastore S, Mascia F, Girolomoni G. Regulatory role of nitric oxide on monocyte-derived dendritic cell functions. *J. Interferon Cytokine Res.* 2003;23:423-431.
51. Kusmartsev S, Gabrilovich DI. Inhibition of myeloid cell differentiation in cancer: the role of reactive oxygen species. *J. Leukoc. Biol.* 2003;74:186-196.

# IV

# Low-density cells isolated from the rat thymus resemble branched cortical macrophages and have a reduced capability of rescuing double-positive thymocytes from apoptosis in the BB-DP rat

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# ABSTRACT

BB-diabetes prone (DP) rats spontaneously develop organ-specific autoimmunity, are severely lymphopenic and particularly deficient in $ART2^+$ regulatory T cells. A special breed, the so-called BB-diabetes resistant (DR) rats, are not lymphopenic and do not develop organ-specific autoimmunity. The genetic difference between both strains is the *lymphopenia* (*lyp*) gene.

Intrathymic tolerance mechanisms are important to prevent autoimmunity and next to thymus epithelial cells, thymus antigen-presenting cells (APC) play a prominent part in this tolerance.

We here embarked on a study to detect defects in thymus APC of the BB-DP rat and isolated thymus APC using a protocol based on the low-density and non-adherent character of the cells. We used BB-DP, BB-DR, wild-type F344 and F344 rats congenic for the *lyp* gene containing region.

The isolated thymus non-adherent low-density cells (LDC) appeared to be predominantly $ED2^+$ branched cortical macrophages and not $OX62^+$ thymus medullary and cortico-medullary dendritic cells. Functionally these $ED2^+$ macrophages were excellent stimulators of T cell proliferation, but more importantly rescued double-positive thymocytes from apoptosis. The isolated thymus $ED2^+$ macrophages of the BB-DP and the F344.*lyp/lyp* rat exhibited a reduced T cell stimulatory capacity as compared with such cells of non-lymphopenic rats. They had a strongly diminished capability of rescuing thymocytes from apoptosis (also of $ART2^+$ T cells) and showed a reduced *Ian5* expression (as *lyp/lyp* thymocytes do).

Our experiments strongly suggest that branched cortical macrophages play a role in positive selection of T cells in the thymus and point to defects in these cells in BB-DP rats.

# INTRODUCTION

BB-diabetes prone (DP) rats spontaneously develop autoimmune insulitis, autoimmune thyroiditis and other organ-specific autoimmunity. The advantage of studying the BB rat model of organ-specific autoimmunity is the involvement of a well-defined, thymus derived regulatory T cell population. These regulatory T cells express the molecule ART2.[1] BB-DP rats are severely lymphopenic and particularly deficient in $ART2^+$ regulatory T cells.[2-6] A special breed of BB rats, the so-called BB-diabetes resistant (DR) rats, are not lymphopenic and do not develop organ-specific autoimmunity, because of the presence of $ART2^+$ regulatory T cells. Depletion of these cells in the BB-DR rat induces organ-specific autoimmunity,[7] while transfer of these cells obtained from BB-DR rats into BB-DP rats prevents organ-specific autoimmunity.[8] Recently this $ART2^+$ regulatory T cell population was investigated in more detail and it was shown that one sub-set was $CD4^+CD25^-Foxp3^-PD\text{-}1^+ART2^+$, and this population was as capable of preventing the development of diabetes in the BB-DP rat as the presently well established $CD4^+CD25^+Foxp3^+PD\text{-}1^+ART2^+$ regulatory T cell sub-set.[9]

The genetic difference between the BB-DP rat and the BB-DR rat is the *lymphopenia* (*lyp, iddm2*) gene, which is a major rat diabetes susceptibility gene located on chromosome 4. Homozygosity for the defective *lyp* gene, as is the case in the BB-DP rat, leads to the severe T lymphocytopenia and the lack of $ART2^+$ regulatory T cells.[10,11] Recently the *lyp* gene (also called *Ian5*, *Gimap5*, *Ian4L*) was found to carry a frameshift mutation and a severe truncation of the protein.[11,12] *Ian5* is thought to be involved in apoptosis, being associated with the anti-apoptotic proteins Bcl-2 and Bcl-xL.[12-14]

Intra-thymic tolerance mechanisms are important mechanisms to prevent organ-specific autoimmunity. Next to thymus epithelial cells, thymus antigen-presenting cells (APC), i.e. dendritic cells (DC) and macrophages, play a prominent role in these central tolerance mechanisms. Interestingly thymus transplantation experiments by Georgiou et al. showed a pivotal role of defects in antigen presentation by bone-marrow derived cells in the thymus of BB-DP rats.[15-17] These defects in antigen presentation were crucial for the T cell lymphopenia of the rat and the ultimate development of organ-specific autoimmunity.

A direct *in vitro* approach has not been used to study the defects of thymus bone-marrow derived APC in the BB-DP rat and we embarked on such a study. We isolated APC from the rat thymus using a protocol based on the low-density and non-adherent character of the cells, as this protocol is known to result in a population of APC, previously identified as thymus DC on the basis of their dendritic morphology, expression of MHC class II molecules in the absence of T and B cell markers, and their excellent T cell stimulatory capacity.[18,19] The low-density cell (LDC) isolation is a well-accepted method to isolate spleen DC and is in use since the early days of DC studies.[20] In our experiments BB-DP and BB-DR rats of the Seattle sub-line and wild-type F344 rats and F344 rats congenic for the region of the BB-DP rat chromosome 4, on which the *lyp* gene is located, were used. BB-DP/Seattle rats are lymphopenic, and autoimmune diabetes occurs in 100% of the Seattle BB-DP rats. Congenic F344 rats homozygous for the *lyp* region of the BB-DP rat chromosome 4 (designated "F344. *lyp/lyp* rats") have a similar extent of T cell lymphopenia and other cellular abnormalities as the BB-DP rat, including nearly absent $ART2^+$ T cells.[21,22] The rats do, however, not develop organ-specific autoimmunity, since they lack other important *iddm* genes for such development

on other chromosomes.

We here describe that thymus LDC are phenotypically rather reminiscent of branched cortical macrophages than of thymus medullary and cortico-medullary DC. The population of thymus LDC of the BB-DP and the F344.*lyp/lyp* rat had a strongly diminished capability of rescuing (double-positive) thymocytes from apoptosis and a reduced *Ian5* expression as compared with such LDC of non-lymphopenic rats.

# MATERIALS AND METHODS

**Animals**

Male and female BB-DP, BB-DR, F344.+/+ and F344.*lyp*/*lyp* rats were from the Seattle colony. Wistar rats were purchased from Harlan (Zeist, The Netherlands). All rats were kept under controlled light conditions (12/12 h light/dark cycle) throughout this study. A standard pelleted diet (AM-11, Hope Farms BV, Woerden, The Netherlands) and tap water were provided *ad libitum*. One hundred percent of the BB-DP rats became diabetic. BB-DP rats were daily tested for glucosuria (Gluketur test sticks; Boehringer Mannheim BV, Almere, The Netherlands). For all experiments, age matched rats were used.

**Cell preparations**

*Thymus LDC, spleen LDC, spleen macrophages and bone-marrow derived DC*

Thymus LDC and spleen LDC were enriched according to the method of Knight et al.[20] and Delemarre et al.,[23] with slight modifications. Briefly, thymi and spleens from BB-DP/BB-DR and F344 rats were minced and digested for 1 h. at 37°C in RPMI 1640 medium (Life Technologies, Breda, The Netherlands) with 25 mM glutamax-1 and 25 mM HEPES (hereafter referred to as $RPMI^+$) containing: 125 U/ml collagenase (type III; Worthington Biochemical, Freehold, NJ, USA) and 0.1 mg/ml DNase (Boehringer Mannheim BV).

The remaining tissue was teased through a 105-µm filter, and the erythrocytes were removed by lysis. Finally, the separated cells were washed and cultured in $RPMI^+$ supplemented with 10% inactivated FCS (FCSi), penicillin (100 U/ml; Seromed, Biochrom, Berlin, Germany), and streptomycin (0.1 mg/ml; Seromed). After an overnight culture period in culture flasks (Costar Europe, Badhoevedorp, The Netherlands; 37°C, 5% $C0_2$ incubator), the non-adherent cells and (in the case of the spleens) the adherent cells were harvested. The spleen adherent cells were for over 95% macrophages. LDC were isolated from the non-adherent cells by using a 14.5% (w/v) Nycodenz (Nycomed Pharma As, Oslo, Norway) density gradient (800 *g* for 20 min.). LDC were collected from the interphase and washed.

Bone-marrow precursor derived DC were obtained as described in detail previously.[24]

## Antibodies

In flowcytometric analysis we used: anti-MHC class II conjugated to phycoerythrin (PE) (1:400; MRC OX6, Serotec, UK), anti-B7-1 (undiluted, CD80; BD Pharmingen, Flanders, NJ, USA), anti-B7-2 (undiluted, CD86; BD Pharmingen), anti-CD40 (1:10, BD Pharmingen), anti-rat DC-FITC (undiluted, MRC OX62; Serotec), ED1 (1:10, DC, monocytes, МΦ; Serotec), ED2/anti-CD163 (1:100, МΦ; Serotec) and ED3/anti-CD169 (1:1000; Serotec).

In immunohistochemistry we used anti-MHC class II (1:5, Serotec), anti-rat DC/OX62 (1:10, Serotec) and ED2/anti-CD163 (1:20, Serotec). All were conjugated to biotin.

**Flowcytometric analysis**

Flowcytometric analysis was performed via standard procedures and as described previously.[24]

**Immunocytology**

Cytocentrifuge preparations were made of the isolated thymus LDC fractions and fixed in acetone (Fluka, St. Louis, MO, USA) for 10 min. (endogenous peroxidase was blocked by adding 0.05% $H_20_2$), and thereafter, incubated with normal rabbit serum (Dako, Glostrup, Denmark), 10% in PBS for 10 min. before incubating for 1 h. with the biotinylated anti-MHC class II antibody. After washing three times with PBS, the slides were incubated with streptavidin-biotin complex conjugated with alkaline phosphatase (Dako). The slides were rinsed three times in PBS and stained with Fast Blue BB Base (Sigma, Zwijndrecht, The Netherlands). Counterstaining was not performed. Finally, the slides were rinsed in water and mounted in Kaiser's gelatine (Merck, Rahway, NJ, USA).

For double-staining, fixation and subsequent incubation with the first mAb followed by a rabbit-anti mouse antiserum conjugated with horseradish peroxidase was performed. After blocking with normal mouse serum (10%), the slides were incubated with a directly biotinylated second mAb. Slides were rinsed three times in PBS and incubated with streptavidin-biotin complex conjugated with alkaline phosphatase (Dako). Slides were sequentially stained with Fast Blue BB Base (Sigma) and 3-amino-9-ethylcarbazole (AEC; Sigma). Finally, the slides were rinsed in water and mounted in Kaiser's gelatine (Merck).

**Immunohistology**

mAb against ED2 and OX62 were used to stain histological sections of the thymus. As a control, we used non-specific murine IgG1. Histological preparations were fixed in acetone (Fluka) for 10 min. (endogenous peroxidase was blocked by adding 0.05% $H_20_2$) and thereafter, incubated with normal rabbit serum (Dako), 10% in PBS for 10 min. before incubating for 1 h. with the appropriate mAb. Subsequently, the slides were incubated with rabbit-anti mouse antiserum conjugated with horseradish peroxidase for AEC staining. Finally, the slides were rinsed in water and mounted in Kaiser's gelatine (Merck).

**Methods for Quantitative PCR for *Ian5* expression**

Quantitative PCR for *Ian5* expression was performed as described in detail previously.[24]

**MLR**

T cells from Wistar rats were enriched using a nylon wool column as described in detail previously.[24] In short, spleens were minced and teased through a 105-µm filter, and the erythrocytes were removed by lysis. Cells were washed and loaded onto a nylon wool column (3g; Polyscience, Eppelheim, Germany). After 1h incubation, T cells (80–90% $CD3^+$ cells) were harvested by collecting the effluent. For the MLR, APC from the different rats were added at various ratios to T cells (fixed number of 150,000 T cells/well) in flat-bottom

96-wells plates (Nunc, Roskilde, Denmark). Subsequently, these cells were cultured for 3 days in RPMI$^+$ medium including $FCS_i$ and antibiotics. In the MLR, T cell proliferation was measured via tritiated thymidine ([$^3$H]TdR) incorporation. Radioactivity was counted in a liquid scintillation analyzer (LKB Betaplate, Wallac, Turun, Finland).

**Thymocyte apoptosis assay**

Thymocytes were isolated from the high-density fraction after overnight culture (macrophages and residual epithelial cells attach to the bottom of the flasks). These thymocytes were thereafter co-cultured with thymus LDC in flat-bottom 96-wells plates (Nunc) at a ratio of 2**:**1. After harvesting the cells the capacity of thymus LDC to rescue double-positive thymocytes from apoptosis was assessed by 7-amino-actinomycin D (7-AAD) staining using a flowcytometer (FACSCalibur, Becton Dickinson, Sunnyvale, CA, USA) gating on the $CD4^+CD8^+$ cells. For determination of background staining, cells were incubated with either labelled irrelevant Abs or with secondary Abs.

**Statistical analyses**

Statistical analyses were performed using the non-parametric Mann-Whitney test.

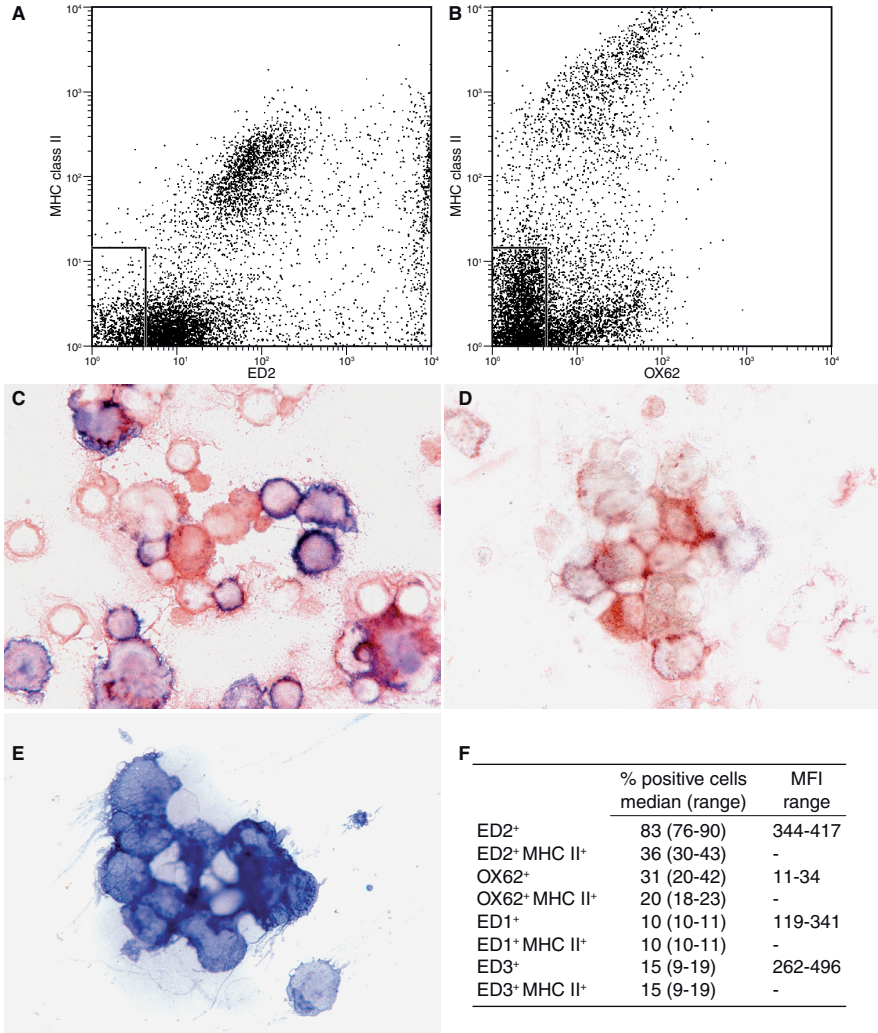
# RESULTS

**The phenotype of thymus low-density cells strongly suggests that the cells represent the thymus branched cortical macrophages**

Analysing the thymus LDC population with DC and macrophage specific markers we found that the thymus LDC were, in particular, positive for the macrophage marker ED2/CD163: 83% (median, range 76-90%, *n*=3) of the cells were positive in flowcytometric analysis in a dim-to-intermediate-staining manner (Figure 1A and F), and in immunocytology, approximately 80% of the LDC were clearly positive (Figure 1C and D). ED2/CD163 (a member of the scavenger receptor cysteine-rich group B family, functioning as a scavenger receptor for hemoglobin-haptoglobin complexes) identifies most sub-populations of mature tissue MΦ, including spleen red pulp MΦ, thymus cortical MΦ, Kupffer cells in the liver, resident bone-marrow MΦ and central nervous system perivascular and meningeal MΦ, but is absent on monocytes.[25]

Using the DC-specific marker OX62, only 31% (standard deviation 9%, *n*=7) of the thymus LDC were positive in flowcytometric analysis with a dim staining manner (Figure 1B and F), and in immunocytology OX62 was found to be stained weakly in 20-30% of the cells (Figure 1D). In double immunocytological staining it appeared that all $OX62^+$ cells were also positive for ED2, a marker that showed a much stronger staining pattern (Figure 1D and F).

About half (48% +/- 13, *n*=7) of the total thymus LDC was positive for MHC class II. This also applied to the $ED2^+$ LDC (Figure 1A, C and F). It must be noted that the MHC class $II^+ED2^+$ cells were the strongest positive for ED2 (Figure 1A). Of the $OX62^+$ cells, approximately two-thirds were positive for MHC class II (Figure 1B and F). In immunocytology, many of the MHC class $II^+$ cells showed strong branched/dendritic morphology (Figure 1E). Only small minorities of the thymus LDC were positive for the




F

| | % positive cells median (range) | MFI range |
|---|---|---|
| ED2$^+$ | 83 (76-90) | 344-417 |
| ED2$^+$ MHC II$^+$ | 36 (30-43) | - |
| OX62$^+$ | 31 (20-42) | 11-34 |
| OX62$^+$ MHC II$^+$ | 20 (18-23) | - |
| ED1$^+$ | 10 (10-11) | 119-341 |
| ED1$^+$ MHC II$^+$ | 10 (10-11) | - |
| ED3$^+$ | 15 (9-19) | 262-496 |
| ED3$^+$ MHC II$^+$ | 15 (9-19) | - |

**Figure 1.**
(A) Dot plot of thymus low-density cells (LDC) with ED2 expression on the *x*-axis and MHC class II on the *y*-axis. Negative cells are in the boxed area. Note that the majority of the LDC are ED2$^+$ and approximately half are MHC class II$^+$. The latter population is also the strongest positive for ED2 (for quantitative data, see Figure 1F). (B) Dot plot of thymus LDC with OX62 expression on the *x*-axis and MHC class II on the *y*-axis. Note that only a minority of LDC is intermediately positive for OX62 (for quantitative data see Figure 1F). (C) Immune cytology of thymus LDC with ED2 (red) and MHC class II (blue). Magnification X 1000; no counterstaining. It can clearly be seen that the majority of cells is ED2$^+$, and that about half of them stain double for MHC class II (particularly the large cells). (D) Immune cytology of thymus LDC with ED2 (red) and OX62 (blue). Magnification X 1000. A weaker staining was used for ED2 as compared with Figure 1C by shorter development of the staining step, since OX62 staining appeared rather faint; no counterstaining. It shows that part of the ED2$^+$ cells are also faintly OX62$^+$. (E) Immune cytology of thymus LDC with MHC class II (blue). Magnification X 1000. Overstaining was used by longer development of the staining step to clearly visualize the long, cellular protrusions (branches/dendrites). No counterstaining. (F) Quantification of marker positive LDC in flowcytometric analysis. For colour figure see Appendix page 146.

macrophage markers ED1 and ED3 (Figure 1F).
The CD80, CD86, and CD40 expression of the LDC was respectively 36 +/- 11, 41 +/- 13 and 42 +/- 7% (mean +/- standard deviations, *n*=7).

It is also important to note that the LDC fractions did not contain any cells with an epithelial morphology.

As the LDC were branched/dendritic cells and clearly positive for ED2/CD163 (and only in minority for OX62) we were of the opinion that the LDC better fit the phenotypic and morphological description of thymus branched cortical macrophages[26] than of medullary and cortico-medullary thymus DC, as described previously.[18,19] In addition, therefore, we stained thymus sections for ED2 and OX62 to verify the *in situ* phenotype of the cells. Indeed, a hallmark of the branched cortical macrophages was their strong ED2 positivity,

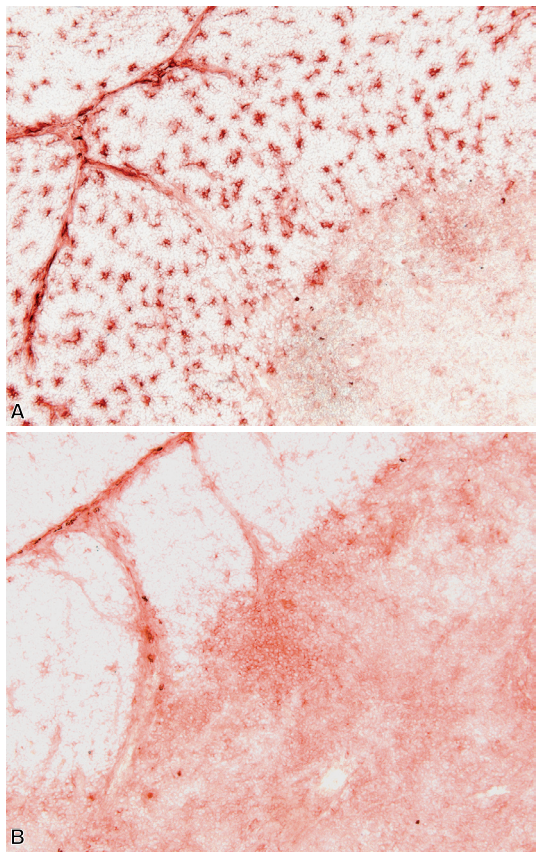



**Figure 2.**
(A) Immunohistochemistry of a F344.+/+ rat thymus stained for ED2 (magnification X 150). The ED2$^+$ cells in the cortex (i.e. the branched cortical macrophages) are clearly visible. (B) Immunohistochemistry of a F344.+/+ rat thymus stained for OX62 (magnification X 150). The positivity for OX62 of the cortico-medullary and medullary dendritic cells can clearly be seen. For colour figure see Appendix page 147.

and only few ED2$^+$ cells were found in the medulla of the thymus (Figure 2A). OX62$^+$ cells were found predominantly in the medulla and particular, at the cortico-medullary junction (Figure 2B), while there was some faint positivity of cells in the cortical area. Based on these phenotypic characteristics, we consider our isolated LDC as representing branched cortical macrophages.

In addition, when we compared the phenotype of the isolated thymus LDC with classical DC, i.e. spleen LDC and BM-precursor derived DC using flowcytometric analysis, it is clear that the latter two populations contained many more of classical DC than our thymus LDC population, i.e. of cells brightly and intermediately positive for OX62 and clearly positive for MHC class II (Figure 3).

**The T cell stimulatory capacity of thymus low-density cells**

With regard to the functional capability of the thymus LDC, we compared the T cell stimulatory capacity of thymus LDC to that of spleen LDC and BM-precursor derived DC. Figures 4A and B show that the thymus LDC were potent stimulators of allogeneic T cells, but reached half the potency of spleen LDC. Thymus LDC and spleen LDC were more potent than BM-precursor derived DC. The latter were in fact quite weak T cell stimulators. This is in accord with their relative immature character. We have reported previously that stimulating such BM-precursor derived DC with LPS to mature the cells further does up-regulate their MHC class II expression and their T cell stimulatory capacity to levels found with spleen LDC.[24]

**Thymus LDC of BB-DP and F344.*lyp/lyp* rats show a reduced capability to stimulate T cells, yet have a normal phenotype except for a lower expression of CD86**

T cell stimulatory capacities of thymus LDC of *lyp*/*lyp* and non-lymphopenic rats were tested in MLR using Wistar T cells as responder cells, representing an allogeneic MLR for F344 rats (Figure 4A) and a syngeneic MLR for BB rats (Figure 4B). As can be seen, the T cell stimulatory capacity of thymus LDC of *lyp*/*lyp* rats was significantly lower than that of non-lymphopenic rats, being it BB-DP or Fischer *lyp*/*lyp* rats (ratio APC/T cell: 1/5

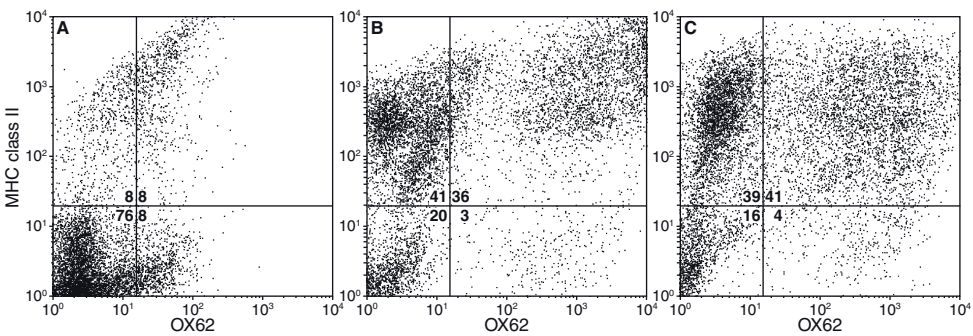



**Figure 3.**
Dot plots of thymus LDC (A), spleen LDC (B) and cultured bone-marrow precursor derived DC (C) with OX62 on the *x*-axis and MHC class II on the *y*-axis. Percentages of cells in each quadrant are indicated in the dot plots.

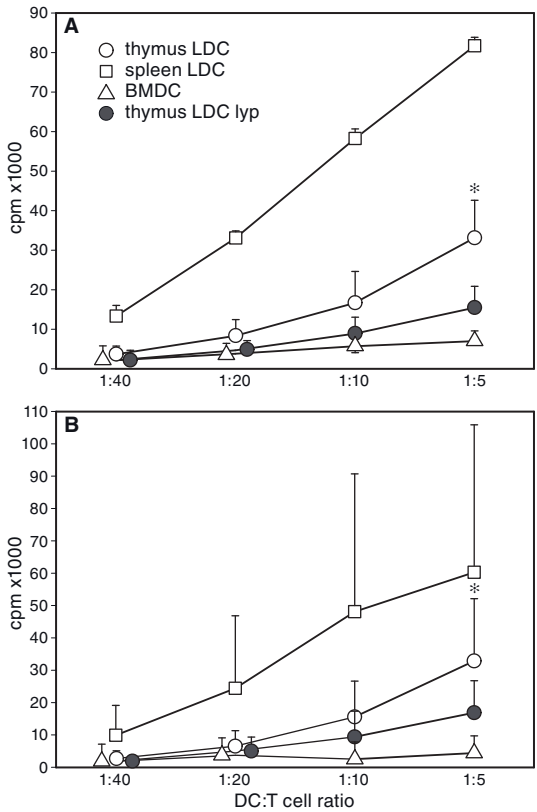



**Figure 4.**
The T cell stimulatory capacity of thymus LDC (circles), spleen LDC (squares) and bone-marrow derived DC (triangles). The black circles represent thymus LDC of lymphopenic rats. The T cell stimulatory capacity was measured using Wistar T cells as responder cells in allogeneic MLR for F344 rats (Figure 4A) and in syngeneic MLR for BB rats (Figure 4B) and expressed as $^{3}$H-Thymidine incorporation (c.p.m.) in stimulated T cells. Means plus/minus standard error of the mean are given ($n$=4). Significances ($p$<0.05) are given with the asterixes, meaning differences between thymus LDC of lymphopenic versus non-lymphopenic rats.

(c.p.m.); mean ± SEM; non-*lyp* BB: 32923 ± 19240, BB.*lyp/lyp*: 16915 ± 9905 ($n$=4; $p$<0.05); non-*lyp* F344: 33091 ± 9462, F344.*lyp/lyp*: 15432 ± 5374 ($n$=4; $p$<0.05), (Mann-Whitney paired analyses was performed, compare open and closed circles).

Despite this clear difference in T cell stimulatory capacity between thymus LDC of *lyp*/*lyp* and non-lymphopenic rats, the phenotype of the LDC fractions was not different, and equal numbers of ED2$^{+}$, OX62$^{+}$, and MHC class II$^{+}$ cells were found in the LDC fractions of *lyp/lyp* and non-lymphopenic rats. With regard to the co-stimulatory molecules there was a difference in the expression of CD86 between *lyp*/*lyp* and non-lymphopenic rats: 25 +/- 6% ($n$=7) thymus LDC of *lyp/lyp* rats were positive for CD86 against 33 +/- 9% ($n$=9) of LDC of non-lymphopenic rats ($p$<0.05). The expression of CD80 and CD40 was equal.

**Thymus LDC of *lyp/lyp* rats show a lower capability to rescue double-positive thymocytes from apoptosis as compared to non-lymphopenic rats**

Thymus LDC of *lyp*/*lyp* and non-lymphopenic BB and Fischer rats were co-cultured with double-positive thymocytes of *lyp*/*lyp* and non-lymphopenic animals. Thymus LDC of non-lymphopenic animals were able to rescue thymocytes from cell death (Figure 5A and D), further supporting the view that the thymus LDC represent branched cortical macrophages, since these cells are thought to be involved in positive selection.[26,27]

Thymus LDC of *lyp*/*lyp* animals hardly rescued thymocytes from apoptosis (Figure 5B and E), indicating that such LDC are functionally defective. This defect was independent of the thymocyte population used, being similar for thymocytes of *lyp*/*lyp* and non-lymphopenic animals (not shown). Also, these two thymocyte populations showed similar survival kinetics when cultured in isolation, i.e. both showed a high level of apoptosis (over 95%) in the absence of rescuing APC.

We also analysed by flowcytometry (in two experiments) the thymocytes exposed to the thymus LDC of lymphopenic and non-lymphopenic BB and Fischer rats and paid in particular attention to the rescue of the population of $CD4^+ART2^+$ T cells. The exposure of the thymocytes to thymus LDC of lymphopenic strains (BB-DP and Fischer.*lyp*/*lyp*) resulted in a lower percentage of $CD4^+ART2^+$ T cells in the stimulated thymocyte population as compared

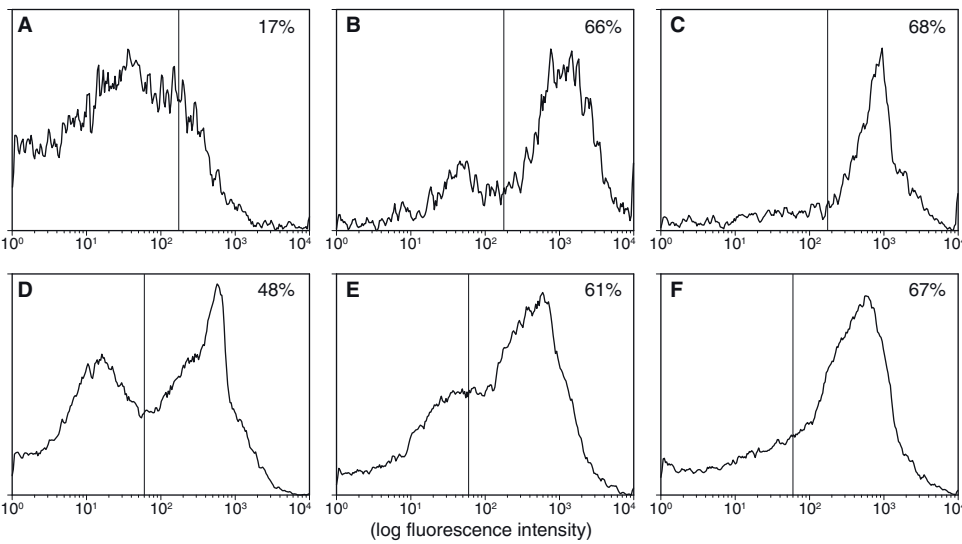



**Figure 5.**
The thymocyte rescuing capability of rat thymus LDC. After co-culture of thymus LDC with non-lymphopenic thymocytes, cell death of double-positive thymocytes was assessed via 7-AAD staining in $CD4^+CD8^+$ cells. Positivity for 7-AAD is given on the horizontal axis of the histograms and represents cell death. The upper rows depict experiments in F344 rats and the lower rows in BB rats. Figure 5C and F illustrate representative histograms of thymocytes cultured in the absence of thymus LDC. Figure 5A and D illustrate representative histograms of thymocytes cultured in the presence of thymus LDC of non-lymphopenic rats. Figure 5B and E illustrate representative histograms of thymocytes cultured in the presence of thymus LDC of lymphopenic rats.

with percentages found in thymocytes stimulated by thymus LDC of non-lymphopenic BB and Fischer rats (BB-DR and Fischer.+/+): i.e. 21% for BB-DP vs. 35% for BB-DR and 13% for Fischer.*lyp*/*lyp* vs. 23% for Fischer.+/+. This shows that the cortical branched macrophages of *lyp*/*lyp* rats are also less capable of rescuing T cell populations of the thymus, which contain an important fraction of the special regulatory T cells of the rat.

***Ian5/Gimap5/lyp* is highly expressed in thymus LDC and is decreased in thymus LDC of lymphophenic rats**

We studied the expression of *Ian5* transcripts in the thymus LDC, spleen LDC and bone-marrow precursor derived DC and spleen macrophages. We found that the thymus LDC expressed high levels of *Ian5* mRNA comparable with the expression levels in thymus T cells (Figure 6). Spleen macrophages also showed high expression levels of *Ian5* mRNA. Classical DC (spleen LDC and bone-marrow precursor derived DC) expressed low levels of *Ian5* mRNA (Figure 6). These data further support the idea that thymus LDC are macrophages rather than DC. Interestingly thymus LDC of lymphopenic (*lyp*/*lyp*) rats showed a decreased *Ian5* mRNA expression (45% as compared with thymus LDC of +/+ rats), while thymus LDC of *lyp*/+ rats showed an intermediate *Ian5* mRNA expression (74% as compared with thymus LDC of +/+ rats) (Figure 6). Similar observations of reduced *Ian5* mRNA have been made for *lyp*/*lyp* T cells.[10,11]

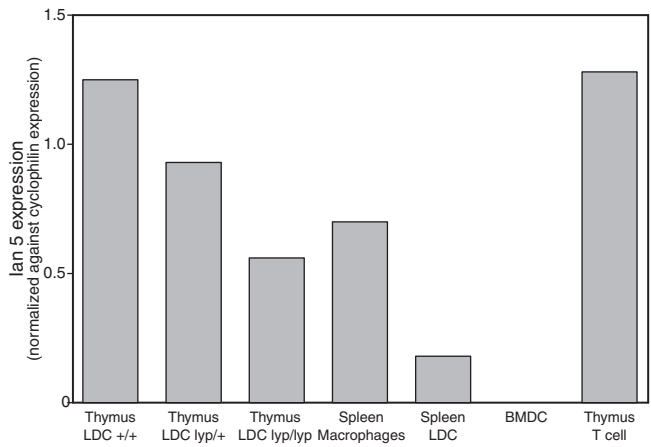



**Figure 6.**
The expression of *Ian5* mRNA in thymus LDC of F344.+/+, *lyp*/+ and *lyp*/*lyp* rats and in spleen macrophages, spleen LCD, bone-marrow precursor derived DC (BMDC), and thymocytes of F344.+/+ rats. The *Ian5* expression is normalized against cyclophilin expression.

## DISCUSSION

Within the thymus, non-lymphoid cells play an important role in the generation of thymocytes. Sessile epithelial and mobile accessory cells are the two prominent groups of cells within this non-lymphoid compartment. The latter appear as two distinct cell types, the thymus DC and the thymus macrophages.[28] The thymus DC are predominantly located in the medulla, express characteristic markers of DC (such as OX62 in the rat, see this report) and play a role in the negative selection of thymocytes. With regard to the thymus macrophages two different sub-populations can be identified: large rounded macrophages (in the rat positive for ED1) with a high phagocytic activity present in the cortex and medulla (and probably playing a role in the removal of apoptotic thymocytes) and non-phagocytic, large, branched cortical macrophages.[29] In rats these latter cells are characterized by their dendritic morphology and labelling with mAb to ED2/CD163.[25] The branched cortical macrophages have a slow turnover (in contrast to the other thymus macrophage population and the thymus DC) and probably originate from bone-marrow precursors (although an intra-thymic precursor has also been suggested for part of the cells).[30] Functionally, the cells have intimate contact with the cortical thymocytes and are capable of forming cell complexes with double-positive thymocytes *in vitro*. In addition the cells are largely positive for MHC class II molecules, and it has therefore been suggested that branched cortical macrophages play a role in thymocyte maturation and/or differentiation in the cortex.[30]

Our data show that the thymus LDC, which we isolated, fitted best the phenotype and suggested function of the rat branched cortical macrophages: the cells were predominantly positive for ED2 (and only for a proportion weakly positive for the DC marker OX62), had a branched/dendritic morphology, half the cells were positive for MHC class II, and the cells were excellent T cell stimulators and rescued double-positive thymocytes from apoptosis.

Thymus LDC have been isolated from the rat thymus before by the group of Ardavin and by Ilic et al.[18,19] In the reports of these investigators, the LDC were considered to be the thymus medullary DC on the basis of their branched morphology, their MHC class II positivity, and excellent T cell stimulatory capacity. We found these qualities as well. However, we also found the cells hardly positive for OX62, and OX62 proved to be a good marker for rat medullary thymus DC *in situ*. We are therefore of the opinion that thymus LDC fractions predominantly contain $ED2^+$ branched cortical macrophages and not classical DC (Banuls et al. also noted some ED2 positivity of the thymus LDC).[18] On the other hand we must note that Banuls et al.[18] and Ilic et al.[19] used slightly different isolation techniques to obtain thymus LDC, and this might have resulted in a predominant yield of medullary thymus DC. Banuls et al. predominantly isolated thymus DC from the thymus LDC by using two consecutive adherence steps[18], while Ilic et al. obtained mainly thymus DC by culturing thymus LDC for 3 days with conditioned medium derived from medullary thymic epithelial cells, which contained the cytokines IL-1, IL-6, TNF-α and IL-2.[19]

A second novel observation we made was that the thymus LDC populations of the BB-DP and F344.*lyp/lyp* rat were deficient in function as compared with the thymus LDC of non-lymphopenic BB-DR and F344 rats: the cells had a reduced T cell stimulatory capacity and, more importantly for branched cortical macrophages, a reduced capability to rescue double-positive thymocytes from apoptosis. This phenomenon of a reduced capability to rescue

double-positive thymocytes from apoptosis is of interest, as it could explain at least part of the severe lymphopenia characteristic of these rats. Classically, the lymphopenia is thought to be a result of a shortened life span of recently emigrated thymocytes because of a high "spontaneous" apoptosis of such cells. There are, however, previous reports that BB-DP thymocytes can at least be rescued partially from such a shortened life span. Ramanathan et al. showed that an early exposition to antigen could partially rescue recent thymic emigrants of the BB-DP rat from programmed cell death.[31] In particular, thymus branched cortical macrophages would be capable of delivering such early antigenic signals to developing thymocytes.

Is this inability of branched cortical macrophages in the BB-DP rat linked to the proneness for autoimmunity? Since our LDC fraction did not contain epithelial cells, we hold the APC as responsible for the rescue of the double-positive thymocytes. The mechanisms via which thymic APC induce tolerance are presently not known. Next to the MHC class $II^+$ thymus epithelial cells, the thymus APC shape the T cell repertoire via positive selection, including that of regulatory T cells, and via negative selection deleting strongly autoreactive T cells.

Several reports of Georgiou et al.[15-17] support the idea that appropriately functioning antigen-presenting cells in the thymus are able to prevent type-1 diabetes by partially restoring T cell abnormalities. These investigators showed that the transplantation of a non-lymphopenic thymus led to the restoration of T cell proliferative function in the BB-DP rat, significantly reduced the incidence of insulitis, and prevented the development of diabetes. It appeared from their experiments that a defect in bone-marrow derived thymus APC contributed to an abnormal maturation of BB-DP T lymphocytes, which in turn, predisposed the animals to autoimmune insulitis, since the transplantation of 'healthy' thymus APC restored the T cell population and prevented disease. In the NOD mouse Georgiou et al. showed that the neonatal transfer of 'healthy' thymus macrophages also prevented disease.[32,33] It is thus tempting to speculate, in view of our data, that the lymphopenia-restoring and diabetes-preventing APC of the transplant experiments of Georgiou et al. are able to act as the here-described thymus cortical branched macrophages rescuing double-positive thymocytes from apoptosis. Since these macrophages also rescue $ART2^+$ T cells (see this report), which harbour thymus-derived regulatory T cells, they might, in this way, contribute to prevention of disease.

With regard to the expression of relevant autoantigens in the thymus (e.g. the insulin peptide family) Kecha-Kamoun et al. reported that thymic macrophages express, in particular, insulin-like growth factor 1 (IGF-1), while IGF-2 is predominantly expressed by thymus epithelial cells and insulin by thymic DC and medullary epithelial cells.[34] The investigators showed that in the BB-DP rat model, IGF-2 expression was defective in 11 of 15 thymuses in close concordance with the diabetes incidence in their rat strain (while IGF-1 and insulin expression were intact). Hence, their experiments do not point to a defective function of thymus macrophages in the BB-DP rat, at least at the level of autoantigen expression.

Which mechanisms might be responsible for the decreased function of branched thymus cortical macrophages of BB-DP and the Fischer.*lyp*/*lyp* rats? Interestingly the thymus LDC of *lyp*/*lyp* rats showed a reduced expression of *Ian5* mRNA as compared with thymus LDC of +/+ rats with *lyp*/+ rats showing intermediate levels. A similar expression pattern exists in *lyp*/*lyp, lyp*/+ and +/+ thymocytes in the BB rat model, and the severely reduced levels of *Ian5* in *lyp*/*lyp* thymocytes lead to an enhanced apoptosis. We did not study whether the

*lyp*/*lyp* LDC had an increased apoptosis rate, but it is interesting that Rees and Dijkstra have described an *in situ* reduction in the number of thymus cortical ED2$^+$ macrophages in BB-DP rats.[35] Obviously, we need to test the exact yield and apoptosis rate of LDC isolated from BB-DP and F344.*lyp*/*lyp* rats in future experiments.

In conclusion, we found that non-adherent LDC isolated from the thymus represent ED2$^+$ branched cortical macrophages and that in the BB-DP and F344.*lyp/lyp* rat such cells had a reduced *Ian5* expression, T cell stimulatory capacity, and a strongly diminished capability to rescue thymocytes from apoptosis, including that of ART2$^+$ T cells. It is likely that these defects, in this important macrophage population of the thymus, are at least in part responsible for the severe T cell lymphocytopenia and the lack of regulatory T cells in the periphery of *lyp/lyp* gene-carrying rats.

## ACKNOWLEDGEMENTS

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## REFERENCES

1. Greiner DL, Malkani S, Kanaitsuka T, Bortell R, Doukas J, Rigby M, Whalen B, Stevens LA, Moss J, Mordes JP, Rossini AA. The T cell marker RT6 in a rat model of autoimmune diabetes. *Adv. Exp. Med. Biol.* 1997;419: 209-216.
2. Jackson R, Rassi N, Crump T, Haynes B, Eisenbarth GS. The BB diabetic rat. Profound T-cell lymphopenia. *Diabetes* 1981;30:887-889.
3. Guttmann RD, Colle E, Michel F, Seemayer T. Spontaneous diabetes mellitus syndrome in the rat. II. T lymphopenia and its association with clinical disease and pancreatic lymphocytic infiltration. *J. Immunol.* 1983;130**:**1732–1735.
4. Greiner DL, Handler ES, Nakano K, Mordes JP, Rossini AA. Absence of the RT-6 T cell subset in diabetes-prone BB/W rats. *J. Immunol.* 1986;136:148-151.
5. Markholst H, Eastman S, Wilson D, Andreasen BE, Lernmark Å. Diabetes segregates as a single locus in crosses between inbred BB rats prone or resistant to diabetes. *J. Exp. Med.* 1991;174:297-300.
6. Jacob HJ, Pettersson A, Wilson D, Mao Y, Lernmark Å, Lander ES. Genetic dissection of autoimmune type 1 diabetes in the BB rat. *Nature Genet.* 1992; 2:56–60.
7. Greiner DL, Mordes JP, Handler ES, Angelillo M, Nakamura N, Rossini AA. Depletion of RT6.1+ T lymphocytes induces diabetes in resistant biobreeding/Worcester (BB/W) rats. *J. Exp. Med.* 1987;166:461-475.
8. Burstein D, Mordes JP, Greiner DL, Stein D, Nakamura N, Handler ES, Rossini AA. Prevention of diabetes in BB/Wor rat by single transfusion of spleen cells: parameters that affect degree of protection. *Diabetes* 1989; 38:24-30.
9. Hillebrands JL, Whalen B, Visser JT, Koning J, Bishop KD, Leif J, Rozing J, Mordes JP, Greiner DL, Rossini AA. Regulatory CD4+ T cell subset in the BB rat model of autoimmune diabetes expresses neither CD25 nor Foxp3. *J. Immunol.* 2006;177:7820-7832.

10. MacMurray AJ, Moralejo DH, Kwitek AE, Rutledge EA, Van Yserloo B, Gohlke P, Speros SJ, Snyder B, Schaefer J, Bieg S, Jiang J, Ettinger RA, Fuller J, Daniels TL, Pettersson A, Orlebeke K, Birren B, Jacob HJ, Lander ES, Lernmark Å. Lymphopenia in the BB rat model of type 1 diabetes is due to a mutation in a novel immune-associated nucleotide (Ian)-related gene. *Genome Res.* 2002;12:1029-1039.
11. Hornum L, Romer J, Markholst H. The diabetes-prone BB rat carries a frameshift mutation in Ian4, a positional candidate of Iddm1. *Diabetes* 2002;51:1972-1979.
12. Pandarpurkar M, Wilson-Fritch L, Corvera S, Markholst H, Hornum L, Greiner DL, Mordes JP, Rossini AA, Bortell R. Ian4 is required for mitochondrial integrity and T cell survival. *PNAS* 2003;100:10382-10387.
13. Nitta T, Nasreen M, Seike T, Goji A, Ohigashi I, Miyazaki T, Ohta T, Kanno M, Takahama Y. IAN family critically regulates survival and development of T lymphocytes. *PLOS Biology* 2006;4:593-605.
14. Michalkiewicz M, Michalkiewicz T, Ettinger RA, Rutledge EA, Fuller JM, Moralejo DH, Van Yserloo B, MacMurray AJ, Kwitek AE, Jacob HJ, Lander ES, Lernmark Å. Transgenic rescue demonstrates involvement of the Ian5 gene in T cell development in the rat. *Physiol. Genomics* 2004;19:228-232.
15. Georgiou HM, Lagarde AC, Bellgrau D. T cell dysfunction in the diabetes-prone BB rat: a role for thymic migrants that are not T cell precursors. *J. Exp. Med.* 1988;167:132-148.
16. Georgiou HM, Bellgrau D. Thymus transplantation and disease prevention in the diabetes-prone Bio-Breeding rat. *J. Immunol.* 1989;142:3400-3405.
17. Georgiou HM, Bellgrau D. Modulation of insulitis following thymus transplantation. *Transplant. Proc.* 1989;21 (1 Pt 1):215-217.
18. Banuls MP, Alvarez A, Ferrero I, Zapata A, Ardavin C. Cell-surface marker analysis of rat thymic dendritic cells. *Immunology* 1993;79:298-304.
19. Ilić V, Colić M, Kosec D. Isolation, cultivation and phenotypic characterization of rat thymic dendritic cells. *Thymus* 1994-1995;24:9-28.
20. Knight SC, Farrant J, Bryant A, Edwards AJ, Burman S, Lever A, Clarke J, Webster AD. Non-adherent, low-density cells from human peripheral blood contain dendritic cells and monocytes, both with veiled morphology. *Immunology* 1986;57:595-603.
21. Hornum L, Lundsgaard D, Markholst H. An F344 rat congenic for BB/DP rat-derived diabetes susceptibility loci Iddm1 and Iddm2. *Mamm. Genome* 2001; 12:867-868.
22. Moralejo DH, Park HA, Speros SJ, MacMurray AJ, Kwitek AE, Jacob HJ, Lander ES, Lernmark Å. Genetic dissection of lymphopenia from autoimmunity by introgression of mutated Ian5 gene onto the F344 rat. *J. Autoimmun.* 2003;21:315-324.
23. Delemarre FG, Simons PJ, de Heer HJ, Drexhage HA. Signs of immaturity of splenic dendritic cells from the autoimmune prone biobreeding rat: consequences for the *in vitro* expansion of regulator and effector T cells. *J. Immunol.* 1999;162:1795-1801.
24. Sommandas V, Rutledge EA, Van Yserloo B, Fuller J, Drexhage HA, Lernmark Å. Defects in differentiation of bone-marrow derived dendritic cells of the BB rat are partly associated with iddm2 (the lyp gene) and partly associated with other genes in the BB rat background. *J. Autoimmun.* 2005; 25:46-56.
25. Polfliet MM, Fabriek BO, Daniels WP, Dijkstra CD, van den Berg TK. The rat macrophage scavenger receptor CD163: expression, regulation and role in inflammatory mediator production. *Immunobiology* 2006;211: 419-425.
26. Sminia T, van Asselt AA, van de Ende MB, Dijkstra CD. Rat thymus macrophages: an immunohistochemical study on fetal, neonatal and adult thymus. *Thymus* 1986;8:141-150.
27. Zepp F, Schulte-Wissermann H, Mannhardt W. Macrophage subpopulations regulate intrathymic T-cell development. I: Ia-positive macrophages augment thymocyte proliferation. *Thymus* 1984;6:279-293.
28. Durkin HG, Waksman BH. Thymus and tolerance. Is regulation the major function of the thymus? *Immunol. Rev.* 2001;182:33-57.
29. Vicente A, Varas A, Moreno J, Sacedon R, Jimenez E, Zapata AG. Ontogeny of rat thymic macrophages. Phenotypic characterization and possible relationships between different cell subsets. *Immunology* 1995;85: 99-105.
30. Murawska MB, Duijvestijn AM, Klatter FA, Ammerlaan W, Meedendorp B, Nieuwenhuis P. Differential kinetics of various subsets of thymic bone marrow-derived stromal cells in rat chimeras. *Scand. J. Immunol.* 1991; 33:473-484.

31. Ramanathan S, Norwich K, Poussier P. Antigen activation rescues recent thymic emigrants from programmed cell death in the BB Rat. *J. Immunol.* 1998;160:5757-5764.
32. Georgiou HM, Constantinou D, Mandel TE. Neonatal transfer of allogeneic thymic macrophages prevents autoimmunity in nonobese diabetic mice. *Transplant. Proc.* 1995;27:2168-2169.
33. Georgiou HM, Constantinou D, Mandel TE. Prevention of autoimmunity in nonobese diabetic (NOD) mice by neonatal transfer of allogeneic thymic macrophages. *Autoimmunity* 1995;21:89-97.
34. Kecha-Kamoun O, Achour I, Martens H, Collette J, Lefebvre PJ, Greiner DL, Geenen V. Thymic expression of insulin-related genes in an animal model of autoimmune type 1 diabetes. *Diabetes Metab. Res. Rev.* 2001;17: 146-152.
35. Van Rees EP, Dijkstra CD. Postnatal development of non-lymphoid and lymphoid cell populations in situ in diabetes-prone BB rats. *Adv. Exp. Med. Biol.* 1988;237:737-743.

# V

# Endocytosis targets exogenous material selectively to cathepsin S in live human dendritic cells, while cell-penetrating peptides mediate nonselective transport to cysteine cathepsins

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## ABSTRACT

The way the MHC II-associated proteolytic system of APC handles exogenous antigen is key to the stimulation of T cells in infections and immunotherapy settings. Using cell-impermeable, activity-based probe (ABP) for papain-cathepsins, the most abundant type of endocytic proteases, we have simulated the encounter between exogenous antigen and endocytic proteases in live human monocyte-derived dendritic cells (MO-DC). Although cathepsinS (CatS), -B, -H, and -X were active in DC-derived endocytic fractions *in vitro*, the peptide-size tracer was routed selectively to active CatS after internalization by macropinocytosis. Blocking of the vacuolar adenosine triphosphatase abolished this CatS-selective targeting, while LPS-induced maturation of DC resulted in degradation of active CatS. Conjugation of the ABP to a protein facilitated the delivery to endocytic proteases and resulted in labelling of sizable amounts of CatB and CatX, although CatS still remained the major protease reached by this construct. Conjugation of the probe to a cell-penetrating peptide (CPP) routed the tracer to the entire panel of intracellular cathepsins, independently from endocytosis or LPS-stimulation. Thus, different means of internalization result in differential targeting of active cathepsins in live MO-DC. CPP may serve as vehicles to target antigen more efficiently to protease-containing endocytic compartments.

# INTRODUCTION

Dendritic cells (DC) internalize exogenous antigen and deliver it to the MHC II-associated proteolytic machinery to generate antigenic peptides for the MHC II-mediated activation of T cells.[1-3] Human monocyte-derived DC (MO-DC) also provide attractive cellular tools for the manipulation of autoimmune disorders or the delivery of antigen-based cancer vaccines *in vivo*.[4] In such therapeutic vaccination protocols, MO-DC are usually exposed to soluble antigen or antigenic peptide, which is internalized by non-specific means, processed by endocytic proteases and loaded on MHC II so that ultimately, antigen-specific T cells are triggered. Understanding the rules that govern the transport of antigen to endocytic proteases and the successive processing pathway in MO-DC might help us to better exploit the therapeutic potential of these cells by maximizing the access of antigen to the MHC II processing compartment or by facilitating the generation of the major immunogenic epitope in such therapeutic vaccines.

Soluble peptide or protein antigen is internalized by MO-DC in an nonselective fashion via macropinocytosis and subsequently, reaches the endocytic compartment.[1] The majority of proteases in the endocytic compartment of DC belongs to the family of papain-like cysteine proteases [cathepsins (Cat) B, S, H, L, X], together with the aspartate proteases CatD and E, as well as the asparagine-specific endopeptidase AEP.[5-7] This proteolytic system is not redundant in general, as genetic elimination of individual cathepsins resulted in defects in the processing and presentation of selected antigens as well as invariant chain (Ii) *in vitro* and *in vivo*.[8-16] As CatS is expressed selectively in professional APC and controls the rate-limiting step in Ii degradation, it is considered the key endocytic protease for antigen presentation in DC.[5,17] The initial proteolytic attack on internalized antigen predetermines the subsequent processing pathway and can result in either destruction or generation of immunodominant epitopes.[18-20] Thus, identification of this dominant "unlocking" protease(s) is crucial for the understanding of the processing pathway of a given antigen.

Fractionation studies revealed that lysosomes, late endosomes and early endosomes differ with respect to the pattern of active proteases in all major types of APC.[9,21-23] However, these types of studies required the disruption of major regulators of protease activity such as the endocytic pH gradient and the redox potential, so that they are of limited value for an analysis of the encounter between antigen and active proteases in intact cells. The use of chemical tools has overcome this limitation recently and allowed us to monitor the delivery of internalized material directly to endocytic proteases in viable APC.[24] Activity-based probes (ABP) bind specifically and irreversibly to the active centre of papain-like cysteine proteases and enable the visualization of the targeted proteases via a detection tag.[25] ABP delivered to the endocytic compartment of live, murine bone-marrow derived APC via phagocytosis interacted progressively with different cathepsins, namely CatS, upon phagosome maturation in murine bone-marrow (BM) derived monocytes but not DC.[24] Human and murine APC and in particular, different types of DC vary significantly with respect to expression, activity, distribution and regulation of endocytic proteases.[6,8-10,21-23,26,27] The pattern of endocytic proteases that is encountered by antigen internalized by intact human MO-DC, is unknown.

Cell-penetrating peptides (CPP) serve as a carrier to cross the plasma membrane by direct permeation or endocytosis.[28] It has been suggested that CPP might also facilitate endocytosis,

so that they could represent a strategy to increase the transport of exogenous material to protease-containing MHC II peptide-loading compartments in vaccination approaches. Indeed, the efficiency of a cancer vaccine *in vivo* was increased substantially by attaching antigenic material to a CPP prior to vaccination.[29] However, neither the effect of CPP on the internalization of peptide-like antigen nor its influence on the pattern of proteases, which is targeted by such CPP-facilitated internalization, has been assessed directly in DC.

We here used ABP to address the delivery of exogenous material to active, papain-like cathepsins in human MO-DC. Internalization of ABP as soluble tracer, in conjugation with latex beads or protein, or facilitated by CPP modulated the patterns and efficiency of the delivery of exogenous material to endocytic proteases in intact, viable DC.

## MATERIALS AND METHODS

### Generation and characterization of dendritic cells

PBMC (peripheral blood mononuclear cells) were isolated by Ficoll/Paque (PAA Laboratories, Austria) density gradient centrifugation of heparinized blood obtained from buffy coats. Isolated PBMC were plated (1x $10^8$ cells/8 ml flask) into tissue-culture flasks (Cellstar 75 $cm^2$, Greiner Bio-One GmbH, Germany) in RPMI 1640 (Gibco, Grand Island, NY, USA) supplemented with 10% FCS, and antibiotics. After 1.5 h. of incubation at 37°C, nonadherent cells were removed, and the adherent cells were cultured in complete medium supplemented with GM-CSF (Leukomax, Sandoz, Hanover, NJ, USA) and IL-4 (R&D Systems, Minneapolis, MN, USA) for 6 days.[22] This resulted in a cell population consisting of approximately 70% DC, as judged by flowcytometry. For induction of DC maturation, medium was supplemented with LPS (Sigma Chemical Co., St. Louis, Mo, USA) at day 6 for an additional 24 h. Murine BM-derived DC were prepared according to the protocol of Lutz et al.[30] from bone-marrow cells of BALB/c mice cultured in the presence of 200 U of granulocyte-macrophage colony-stimulating factor (GM-CSF; produced by mouse myeloma strain P3X63)/ml for a total of 9 days. More than 90% of these cultures expressed CD11c as assessed by flowcytometric analysis, and characteristic clustering of DC was observed by light microscopy. Human monocytes were enriched from buffy coats using a percoll gradient as described, resulting in 60-80% pure preparations of $CD14^+$ cells.

Flowcytometry analysis was performed using a FACSCalibur. Antibodies for immunophenotyping (Becton Dickinson, San Diego, CA, USA), OVA-FITC (Sigma Chemical Co.) and fluorochrome-coupled streptavidin latex beads (YG, Polysciences Inc., Warrington, PA, USA) were obtained commercially.

### Affinity-labelling of active cysteine proteases

#### *General labelling procedure in cell lysates and endocytic fractions*

JPM-565 and DCG-0N, a derivative of DCG-04 with identical labelling characteristics, were synthesized and purified as previously described.[25,31] Crude endocytic extracts of at least 5x$10^7$ monocytes or human MO-DC were prepared according to the method described previously.[23] DC lysates were prepared from 5x$10^5$ immature DC in 2x lysis buffer (100 mM citrate/phosphate, 2 mM EDTA, 1% Nonidet P40 (NP-40), pH 5) and were lysed for

30 min. at 4°C, followed by removal of membrane fragments by centrifugation. Total endocytic protein (1.5 µg) of primary monocytes or 3 µg DC cell lysate protein was incubated with 10 µM DCG-0N, PS457 or DCG-0N preincubated in a ratio of 2:1 with streptavidin and 50 mM DTT at ambient temperature for 30 min. Reactions were terminated by addition of 6x SDS-reducing sample buffer and immediate boiling at 95°C for 10 min. Samples were resolved by 12.5% SDS-PAGE gel, and then blotted on a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Piscataway, NJ, USA). After blocking with PBS-0.2% Tween 20 and 10% Roti®-Block (Roth, Karlsruhe, Germany) and extensive washing with PBS-0.2% Tween 20, the membrane was probed with Vectastain® (Vector Laboratories, Burlingame, CA, USA) in PBS-0.2% Tween 20 for 60 min, followed by washes with PBS-0.2% Tween 20. The ECL detection kit (Amersham Biosciences) was used for visualization.

For the flowcytometric-based analysis of the internalization of the respective constructs, YG-coupled strepatividinlatex beads were purchased, which were otherwise identical to the particles used below (Polysciences Inc.), as well as FITC-coupled OVA (Sigma Chemical Co.). Con B was a kind gift from Hidde Ploegh (Department of Pathology, Harvard Medical School, Boston, MA, USA). N-morpholinurea-homophenyl-leucyl-vinylsulfone (LHVS) was synthesized essentially as described.[32] E-64 and CA-074 were obtained from Sigma Chemical Co.

**Labelling with DCG-0N coupled to streptavidin-coated latex beads**

Crude endocytic fractions were prepared as described above. Streptavidin-coated, carboxylated latex beads (1 µm diameter, Polysciences Inc.) were incubated with different concentrations of DCG-0N for 1 h. at room temperature. Beads were washed three times with PBS. The efficiency of the coupling and the washing steps were controlled by labelling cell lysates with beads pelleted after centrifugation or with the supernatant after washing, respectively, as described above. To label cysteine proteases in live cells with DCG-0N coupled to streptavidin-coated latex beads, essentially, the procedure published for murine APC was used.[24] Streptavidin-coated carboxylated latex beads were incubated with 100 µM DCG-0N for 1 h. at room temperature. Beads were washed three times with PBS and resuspended in complete medium. Cells were plated on 24-well plates ($1x10^7$ cells/ well) and pulsed for 1h at 37°C with 300 µl medium containing DCG-0N-coated beads. After the pulse, excess beads were removed by centrifuging them four times at 2000 rpm for 2 min. over an FCS cushion. Cells were washed with PBS and lysed with 100 µl of 2x hot, reducing SDS sample buffer supplemented with 100 µM-free JPM-565. Lysates were boiled, and the DNA was sheared with a syringe and sonification. Samples were analyzed by 12.5% SDS-PAGE and streptavidin blotting.

**Labelling of cysteine proteases in live cells with soluble DCG-0N and PS457**

At least $1x10^6$ DC per time point were pulsed in complete culture medium (300 µl per time point) for different times at 37°C with 25 µM DCG-0N or 10 µM PS457, if not mentioned otherwise. After the pulse, cells were washed to remove excess label at 4°C for four times in PBS. Where a chase was performed, cells were taken up in 37°C medium and then incubated for the indicated additional times. After labelling, medium was removed and cells were lysed with 50 µl 2x lysis buffer (100 mM citrate/phosphate, 2 mM EDTA, 1% NP-40,

pH 7), supplemented with 100 µM-free JPM-565. SDS reducing sample buffer (6x) was added to 50 µg total protein of each time-point and boiled immediately. Samples were resolved by 12.5 % SDS-PAGE gel and then blotted on a PVDF-membrane (Amersham Biosciences), and visualized as described above.

**Labelling with activity based probes conjugated to streptavidin**

Streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was preincubated with DCG-0N or PS457 in PBS in a molar ratio of 1:2 for 1 h. at room temperature and then added directly to the cells, which were pulsed, washed, and lysed as mentioned above. SDS reducing sample buffer (6x) supplemented with 18 mM biotin (Serva, Germany) was added to 50 µg total protein of each time-point, followed by immediate boiling. Resolution by SDS-PAGE and detection were performed as described above.

**Antisera, metabolic labelling and immunoprecipitation**

Rabbit antisera were generated against recombinant CatS and affinity-purified. Metabolic labelling and immunoprecipitation were performed exactly as described in refs[9,21,33], using the Tu36 mAb, which recognizes human MHC II complexes in a conformational-specific manner. Equal numbers of human MO-DC were incubated in 500 µl methionine/cysteine-free medium supplemented with 10% FCS (Sigma Chemical Co.). Cells then were labelled by incubation with 500 µCi/ml [$^{35}$S]methionine-cysteine (Amersham Biosciences) at 37°C for 15 min. Medium was added in a 10-20x excess, and cells were chased at 37°C for different times. Cells were washed in PBS, frozen in liquid nitrogen, and stored at -80°C. After resolution by SDS-PAGE, the labelled polypeptides retrieved were visualized using a phosphorimager.

**Synthesis of PS457**

Where appropriate removal of the 9-fluorenylmethyloxycarbonyl (Fmoc)-protecting group was accomplished by treatment of the resin-bound peptide with piperidine in N-methyl pyrrolidone (NMP; 1/4 v/v) for 20 min. Peptide coupling steps were performed by treatment of the resin with a premixed (5 min.) solution of the appropriate acid (5 eq.), 2-(-6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophos-phate(5 eq.) and *N,N*-diisopropylethylamine (DiPEA; 6 eq.) in NMP for 1 h. Coupling efficiencies were monitored with the Kaiser test and couplings were repeated if necessary. After coupling and deprotecting steps the resin was washed with NMP (5´). Fmoc Rink amide resin (78 mg, 50 µmol) was elongated using automated, standard, Fmoc-based solid-phase peptide synthesis (SPPS) to give resin-bound $(Arg(Pbf))_9$. The synthesis was continued by manual Fmoc-based SPPS to give protected and resin-bound PS457. The resin was washed extensively (alternating $CH_2Cl_2$-MeOH 3x, alternating $CH_2Cl_2$-$Et_2O$ 3x. An aliquot of resin (100 mg, 16 µmol) was transferred into a clean vial, washed with $CH_2Cl_2$ and treated with trifluoracetic acid (TFA)/ $H_2O$/trisopropylsilane (0.7 ml, 95/2.5/2.5 v/v/v) for 2h. The mixture was filtered into cold $Et_2O$ and the white precipitate was collected by centrifugation and decantation. The precipitate was washed ($Et_2O$), followed by HPLC purification of the crude product (linear gradient in Buffer B: 20-30% B in three-column volumes) to yield 4.3 mg (1.8 µmol, 11%) of product. $^1$H nuclear magnetic resonance (NMR; $D_2O$, 295 K, DMX 600): δ, 7.08 (d, $^2$H, J=7.9 Hz), 6.80 (d, $^2$H, J=8.2 Hz), 4.58-4.55 (m, $^1$H), 4.45-4.41 (m, $^1$H), 4.38-4.35 (m, $^1$H), 4.34-4.18 (m, $^{12}$H),

4.13-4.09 (m, $^{1}$H), 3.68 (br s, $^{1}$H), 3.48-3.45 (m, $^{1}$H), 3.30-3.25 (m, $^{1}$H), 3.24-3.09 (m, $^{24}$H), 3.03-2.86 (m, 4H), 2.79 (s, $^{1}$H), 2.69 (s, $^{1}$H), 2.28-2.18 (m, $^{6}$H), 1.80-1.20 (m, $^{61}$H), 0.89-0.80 (m, $^{6}$H). Electrospray interface-mass spectrometry (ESI-MS): $C_{105}H_{189}N_{45}O_{24}S + {}^{4}H^{+}$ requires 625.5, found 625.4; $C_{105}H_{189}N_{45}O_{24}S + {}^{3}H^{+}$ requires 833.7, found 833.6.

Solvents used in the SPSS, DiPEA and TFA were all of peptide-synthesis grade (Biosolve) and used as received. The protected amino acids, Rink amide p-methylbenzhydrylamine resin (0.78 mmol g–1) was obtained from NovaBiochem (Switzerland). Ethyl (2S,3S)oxirane-2,3-dicarboxylate was prepared as described. Fmoc-Lys(Mtt)-OH was from Senn Chemicals (Switzerland), SPPS was carried out using a 180° variable rate flask shaker (St. John Associates, Inc., Beltsville, MD, USA) or on a 443A peptide synthesiser (Applied Biosystems, Foster City, CA, USA). Liquid chromatography/MS analysis was performed on a Jasco HPLC system (detection simultaneously at 214 and 254 nm) coupled to a Perkin Elmer Sciex API 165 mass spectrometer equipped with a custom-made Electrospray Interface (ESI). An analytical Alltima C18 column (Alltech, 4.6 mmD , 250 mmL, 5 µm particle size) was used; buffers: A=$H_2O$; B=$CH_3CN$; C=0.5% aq TFA. For reversed-phase HPLC-purification of two and four a Biocad "Vision" automated HPLC system (PerSeptive Biosystems, inc., Framingham, MA, USA) was used. The applied buffers were A, B and C. $^{1}$H-NMR spectra were recorded with a Bruker DMX 600 instrument at 600 MHz with chemical shifts (δ) relative to tetramethylsilane.

**Quantification by densitometry**

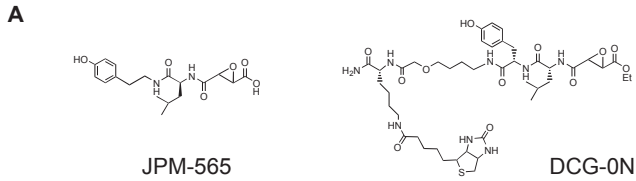
NIH ImageJ was used for quantification of the intensity of labelling for the active polypeptides visualized by affinity labelling (http://rsb.info.nih.gov/ij/).

## RESULTS

Here we applied a strategy to human DC, which allows visualization of the rendezvous between individual, endocytic, cysteine proteases and internalized, exogenous material taken up by live APC via different modes of transport. An ABP for cysteine proteases is internalized either via endocytosis or a CPP as a shuttle, reaches endocytic compartments, and interacts with the local proteolytic contents by covalent binding to the catalytic center of active cysteine proteases. As an ABP, we used DCG-0N, a derivative of the peptide epoxides JPM-565 and E-64, which targets papain-like cysteine proteases specifically. The labelling characteristics of DCG-0N are identical to its mother compounds DCG-04 and to JPM-565 (Figure 1A).[24] Proteases reached by the probe after internalization are irreversibly bound and can be visualized after cell lysis and SDS electrophoresis by streptavidin blotting against the biotin moiety of the probe so that different signal intensities of a particular protease polypeptide correspond directly to different amounts of the active protease species reached by the tracer in the living cell.

**Different patterns of active, papain-like cysteine-proteases are visualized by the active, site-directed probe DCG-0N in primary human monocytes and monocyte-derived DC**

The affinities of DCG-0N for the different papain-like cysteine proteases are in a similar order of magnitude, but not identical, and also, the total amounts of a given active protease


A
JPM-565
DCG-0N
B
Monocytes
DCG-0N
2 8 32 128 0.5 2 8 32 128
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nM
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DCG-0N-coated beads
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0.1 1 10 100 0.1 1 10 100 µM
kDa
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CatX
CatB
CatH
CatS

**Figure 1. Papain-like cysteine-proteases recognized by the active site-directed probe DCG-0N in endocytic fractions of primary monocytes and monocyte-derived DC.**
(A) Structure of the active site-directed probe JPM-565 and its biotinylated analogue DCG-0N. (B) Titration of DCG-0N for the labelling of endocytic fractions of human monocytes (upper panel) and human monocyte-derived DC (lower panel) at pH 5. Lysates were incubated with increasing concentrations of DCG-0N. Proteins were separated by SDS-Page on a 12.5% gel and reactive polypeptides were visualized by streptavidin blotting ($n$=3). (C) Effect of the immobilization of DCG-0N by conjugation to streptavidin-coated latex beads on the selectivity of labelling. Endocytic extracts of monocytes were incubated for 30 min. at RT with latex beads preincubated with different concentrations of DCG-0N, or with the soluble tracer alone at the same concentrations, prior to visualization of the labelled species ($n$=5).

vary between different types of APC. To account for differences in protease labelling, which might result solely from different affinities of the probe for various cathepsins, differences in the total amounts of an individual protease or subsaturating amounts of the probe, rather than from differential delivery of the probe to active cathepsins in intact APC, we performed titration experiments with crude endocytic fractions incubated with increasing concentrations of DCG-0N *in vitro*, followed by a streptavidin blot. The detected polypeptides in the 20- to 40-kD range were identified via immunoprecipitation with antibodies directed against individual cathepsins as well as by MS-based sequencing after pull-down with streptavidin beads[21], allowing the identification of CatB, CatH, CatL, CatS and CatX (Figure 1B). In endocytic fractions derived from primary monocytes, CatS was detected at the lowest concentration of label, followed by CatB, CatH, and CatX, with increasing amounts of DCG-0N. In endocytic fractions of human MO-DC, CatB was the protease that could be visualized at the lowest concentration of DCG-0N, and CatS was only detected with higher amounts of DCG-0N. CatX and CatL were only visualized at increasing concentrations of DCG-0N. Thus, different relative amounts of individual cathepsins in a given cell type (DC vs. monocytes) also account for differential labelling patterns of proteases, especially at low concentrations of label. Of note, in human DC, active CatS appeared to be less prominent, compared with the remaining cysteine cathepsins, than in human monocytes, especially under the limiting condition of subsaturating amounts of label. These individual labelling patterns in crude endocytic fractions served as the basis to account for the specific delivery of label to a given protease after internalization by intact cells, as analyzed in the following experiments.

DCG-04 coupled to streptavidin-coated latex beads has served as a tool to assess the delivery of exogenous material taken up by phagocytosis to endocytic proteases in murine BM-derived APC.[24] To control for the possibility that binding of DCG-0N to latex beads could affect the reactivity or selectivity of the probe, endocytic extracts from primary human monocytes were incubated either with the soluble probe or with DCG-0N coupled to streptavidin-latex beads. Labelled protease species were then visualized by SDS-PAGE and antibiotin blot (Figure 1C). The immobilization of DCG-0N on streptavidin beads favored labelling of CatS over the other cathepsins so that CatB was labelled poorly, and CatX and CatH could not be visualized at all. Thus, the immobilization of DCG-0N on latex beads affects the selectivity of labelling, which might induce artefacts when a proteolytic environment is sampled using DCG-0N immobilized on beads.

**Human MO-DC poorly internalize latex beads via phagocytosis**

When murine BM-derived DC were incubated with fluorochrome-labelled latex beads, they internalized these beads efficiently by phagocytosis at 37°C, but not at 4°C, as assessed by flowcytometry, consistent with the published data (Figure 2A).[24] By contrast, human MO-DC only very poorly ingested the same type of beads *in vitro* under otherwise identical conditions, highlighting the biological differences between both types of DC preparations. However, human MO-DC very efficiently internalized soluble fluorochrome-labelled OVA by endocytosis (1.4%-positive cells at 4°C compared to 83.12% at 37°C). As soluble antigen internalized by endocytosis represents one of the most common modes of antigen delivery to MO-DC *in vitro* in immunotherapy settings, immobilization of the ABP influences its labelling characteristics, and human MO-DC only poorly internalize exogenous material by phagocytosis *in vitro*, we analyzed the encounter of exogenous material internalized by MO-DC via endocytosis, not phagocytosis, in the following experiments.

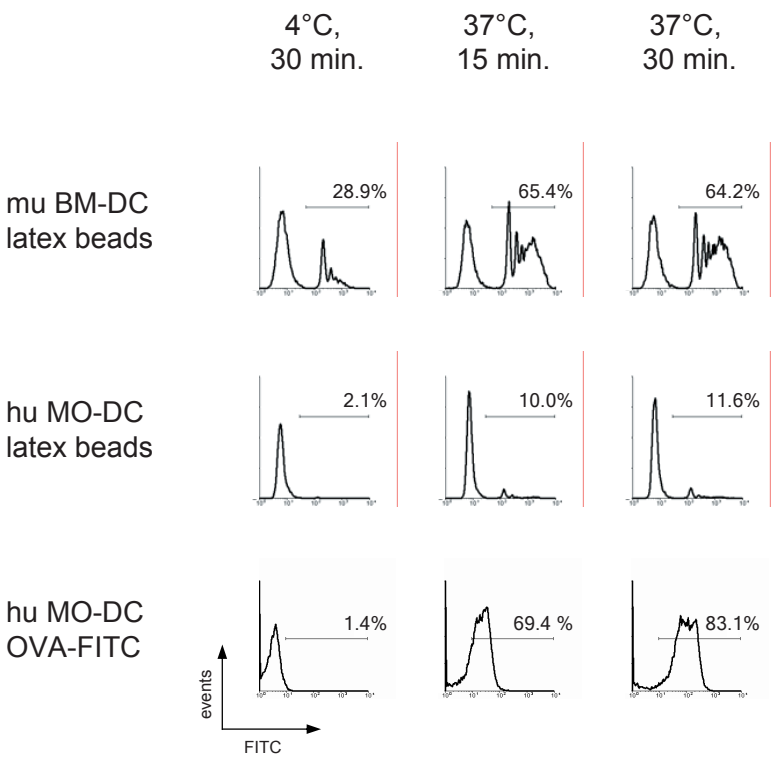



**Figure 2. Different efficiency of phagocytosis-mediated internalization between human MO-DC and murine bone-marrow derived DC.**

Murine bone-marrow derived DC (mu BM-DC) or human MO-DC (hu MO-DC) were incubated with FITC-labelled latex beads for up to 30 min. at 4°C and at 37°C, respectively. Similarly, hu MO-DC were incubated in the presence of OVA-FITC. After washing of the cells at the appropriate time points, cellular fluorescence was analysed by flowcytometry ($n$=3).

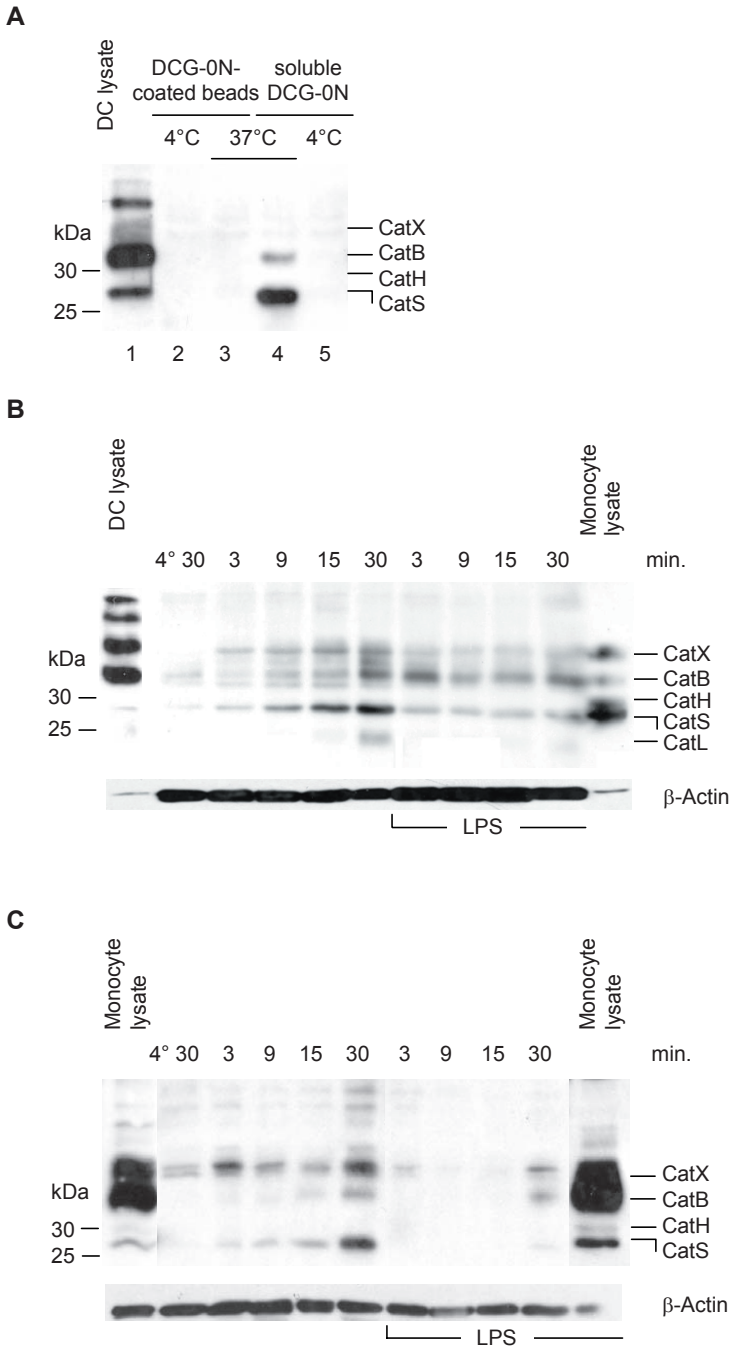
**Delivery of exogenous DCG-0N to active papain-like cathepsins by endocytosis in human DC**

To monitor the delivery of internalized material to the protease pool of MO-DC, intact DC were incubated for 1 h. at 37°C with 5 μM-soluble DCG-0N. As controls, we included DC incubated under the same conditions with streptavidin-coated latex beads previously coupled with DCG-0N, as well as the same amount of cells pulsed with soluble DCG-0N at 4°C. Excess beads were removed by extensive washing and cells were lysed at pH 7 in 2x lysis buffer containing excess, nonbiotinylated JPM-565 to largely exclude that proteases liberated upon cell lysis could bind to the probe post-lysis. For direct comparison of the fraction of active proteases labelled after internalization of the probe by MO-DC *in vivo* with the total protease pool present in these cells, a total cell lysate was prepared from a portion of the same DC sample, lysed at pH 5, and labelled with DCG-0N after cell lysis. All samples were boiled at 95°C in 6x reducing SDS sample buffer, resolved by electrophoresis, and analyzed by streptavidin blotting to visualize the DCG-0N-modified polypeptides (Figure 3A).

In contrast to murine BM-derived DC[24], live human MO-DC did not result in labelling of active cathepsins after coincubation with DCG-0N-coated latex beads at 37°C (Figure 3A, Lane 3), consistent with the poor uptake of latex beads by MO-DC, as judged by flowcytometry. Soluble DCG-0N, however, decorated active cathepsins when intact human monocyte-derived DC were exposed at 37°C (Figure 3A, Lane 4), in contrast to the 4°C control (Figure 3A, Lane 5). Although in crude endocytic DC-fractions, active CatB was labelled much stronger by the probe than CatS (Figure 3A, Lane 1), internalization of the probe by live human DC clearly favored labelling of CatS with little targeting of CatB and without significant visualization of CatX after endocytosis-mediated delivery (Figure 3A, Lane 4). Thus, soluble DCG-0N, internalized by macropinocytosis is routed selectively to a compartment enriched with CatS activity in human MO-DC.

To resolve the rendezvous between individual cysteine proteases and exogenous matter internalized by pinocytosis as a function of time, DC were incubated with soluble DCG-0N for up to 120 min. We observed a rather selective, time-dependent increase in labelling of active CatS between 0 and 30 min., visible already after 9 min. of uptake, consistent with an early endosomal localization of active CatS (Figure 3B, left). Longer incubation up to 120 min., did not result in further changes in the labelling results (data not shown). The labelling intensities for CatB and CatX were low and almost stable over time, indicating a negligible exposure of exogenous peptide-size matter delivered by pinocytosis to these cathepsins in live human DC. Again, incubation of cells with soluble DCG-0N at 4°C for the same time did not result in significant visualization of protease polypeptides, confirming the active uptake of DCG-0N by live DC, as opposed to postlysis artefacts or non-specific, passive transition of the plasma membrane by DCG-0N. When DC were cultured for 24 h. with LPS and then pulsed with DCG-0N, the time-dependent increase in labelling of active CatS was abolished (Figure 3B, right), consistent with the low rate of endocytosis of activated DC, as confirmed by flowcytometry analysis (not shown).

In contrast to monocytes, resting DC are capable of preserving intact antigen within endocytic compartments for several hours before antigen breakdown is initiated by DC activation, a feature that has been suggested to contribute to the ability of DC to provide antigenic memory. It was tempting to speculate that the CatS-restricted delivery of antigen to


A
DC lysate
DCG-0N-coated beads
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4° 30 3 9 15 30 3 9 15 30 min.
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4° 30 3 9 15 30 3 9 15 30 min.
kDa
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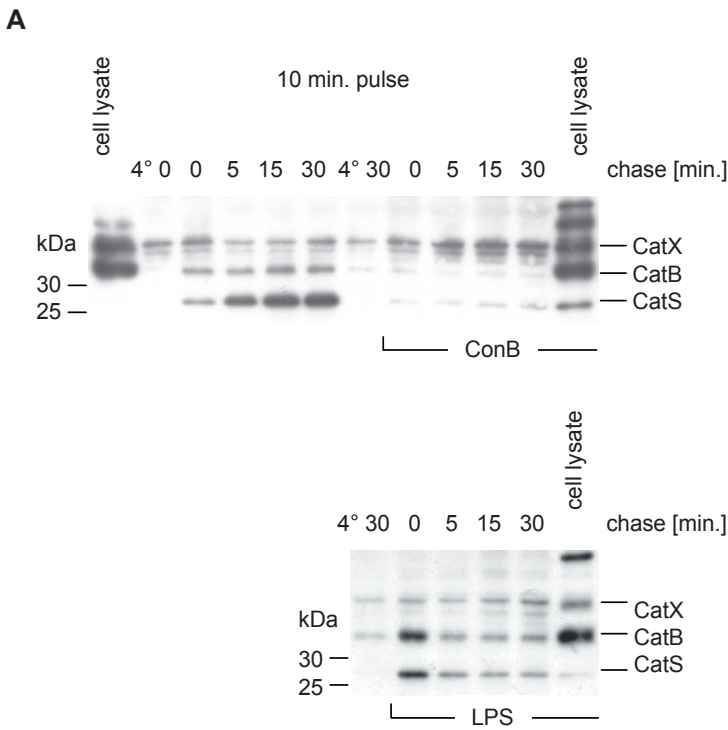
CatS
β-Actin
LPS

**Figure 3. Delivery of DCG-0N to cathepsin-containing compartments.**
(A) Live DC were incubated for 1 h. at 37°C with 25 μM soluble DCG-0N or streptavidin-coated latex beads preincubated with DCG-0N at the same concentration. After extensive washing, cells were lysed at pH7 in lysis buffer containing 100 μM JPM 565-and boiled in 6x concentrated reducing sample buffer. Incubation for 1 h. at 4°C served as control for the active internalization of the protease label. Lysates from DC labelled with DCG-0N served as standards to identify the labelled protease species and to relate their signal intensity to their relative abundance in the cell (*n*=6). (B) Intact DC were incubated with soluble DCG-0N at 37°C for different periods of time, as indicated, with or without prior exposure to LPS for 24 h. Incubation with DCG-0N for 30 min. at 4°C served as control for the active internalization of the protease label. After extensive washing, cells were lysed in 2x lysis buffer containing 100 μM JPM-565 and labelled protease species were visualized by SDS-PAGE/anti-biotin blot. DC-and monocyte-derived cell lysates served as controls, as above (*n*=5), comparable total protein load was confirmed by western blot against β-actin. (C) Intact primary monocytes enriched by percoll gradient centrifugation were incubated with soluble DCG-0N at 37°C for different periods of time, as indicated, with or without prior exposure to LPS for 24 h. as detailed above (*n*=6). Active endocytic proteases reached by the tracer were visualized as in (B). Western blot analysis for β-actin served as a loading control.

endocytic proteases observed in DC might represent the molecular basis for the low rate of antigen processing in resting DC. We therefore assessed the delivery of soluble DCG-0N to endocytic proteases in primary human monocytes enriched by Percoll gradient centrifugation in an identical manner (Figure 3C). Similar to MO-DC, CatS was reached preferentially by the internalized tracer in monocytes, and CatB and CatX were decorated relatively poorly and in variable amounts in individual experiments, most likely representing postlysis artefacts. Similar to the results observed with DC, a consistent and reproducible increase in labelling intensity was only observed for CatS, but not for CatB or CatX. Prior stimulation of monocytes by LPS markedly reduced the delivery of exogenous DCG-0N to endocytic proteases, also paralleling the results obtained with MO-DC. Thus, the preferential targeting of exogenous material to active CatS is not a unique feature of DC, but can likewise be observed in monocytes, and is therefore unlikely to represent the basis for antigen memory observed in DC.

**Activity of the vacuolar adenosine triphosphatase (ATPase) modulates the recruitment and stability of CatS targeted by internalized material**

To dissect the intracellular delivery of cathepsins to the internalized ABP, intact DC were incubated with soluble DCG-0N at 37°C for 10 min. (pulse), followed by extensive washing to remove noninternalized label and a chase at 37°C for 0-30 min., respectively, at 37°C. Control cells were incubated at 4°C for the pulse (4°, 0), or for the pulse and the chase period (4°, 30), respectively. Labelled protease species were visualized by SDS-PAGE/antibiotin blot (Figure 4A). In untreated cells, the CatS signal increased selectively during the 0- to 30-min. chase period (compare to 4°C, 30 min. control). Extending the chase to 120 min. yielded comparable results and did not result in increased labelling of additional protease polypeptides (data not shown). This indicated that active CatS, but not CatB, CatH, or CatX, was preferentially routed towards or activated within DCG-0N-containing endosomes within 30 min. after internalization by intact human MO-DC. CatS is therefore the most likely candidate for the initial proteolytic attack on newly internalized, peptide-type antigens in human MO-DC.


A
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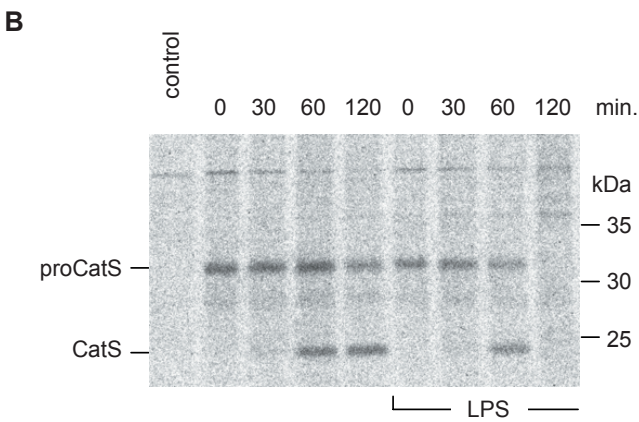
B
control
0 30 60 120 0 30 60 120 min.
kDa
35
30
25
proCatS
CatS
LPS

**Figure 4.**
(A) DC were left untreated (upper left) or either pretreated with 20 nM ConB for 60 min. (upper right) or with LPS for 24 h. (lower right). Cells were pulsed with soluble DCG-0N at 37°C for 10 min. Following extensive washing, cells were chased at 37°C for the times indicated (chase). After each time point, cells were lysed, proteins were separated by SDS-Page on a 12.5% gel, and reactive proteases were visualized by streptavidin blotting, as above (*n*=4). (B) Analysis of the biosynthesis, maturation and degradation of CatS in DC. Cells either left unstimulated or stimulated with LPS for 24 h. were pulse-labelled with $^{35}$S Met/Cys for 15 min., followed by a chase for up to 2 h. After lysis, CatS was retrieved by immunoprecipitation using a CatS antiserum. After exposure to 95°C in 6x concentrated reducing sample buffer, polypeptides retrieved were separated by SDS-PAGE, followed by autoradiography using a phosphorimager. One representative result from three independent experiments is shown (*n*=3).

The endocytic machinery of DC is regulated largely by the activity of the vacuolar $H^+$-ATPase: although low activity characterizes resting DC with high endocytic activity, DC maturation by stimuli like LPS results in ATPase activation and a drop in vacuolar pH. Addition of Concanamycin B (ConB), an inhibitor of the $H^+$-ATPase blocks orderly transport, especially at the early-to-late endosomal transition.[34] To further characterize the mechanism of the recruitment of active CatS to the internalized ABP, we added ConB to one-half of the sample prior to the pulse (Figure 4A, upper right). ConB blocked labelling of active CatS when DCG-0N was internalized by live cells but did not affect the visualization of active CatS when total cell lysates of MO-DC were treated with the same amount of the drug for 60 min. directly prior to lysis. This indicated that ConB disrupted the intracellular delivery of internalized DCG-0N to active CatS rather than interfering with cellular CatS activity. Thus, the vacuolar $H^+$-ATPase is required for the selective delivery of exogenous material to active CatS in intact human MO-DC.

LPS-induced DC maturation leads to increased activity of the endocytic ATPase. When the intracellular delivery of DCG-0N to active cathepsins was analyzed in the same type of pulse-chase experiment as above with DC stimulated with LPS, active CatS was visualized with inverse kinetics during the chase (4A, lower right): labelling intensity for CatS decreased continuously over the 30-min. chase. Because of the irreversible nature of binding of the probe to active CatS, this suggested degradation of either the bound probe, the labelled protease, or both.

To differentiate between these two possibilities, we assessed the biosynthesis, maturation, and stability of CatS in resting and LPS-stimulated DC by metabolic labelling and immunoprecipitation of CatS. DC were pulse-labelled with $^{35}$S Met/Cys for 15 min. at 37°C, followed by different chase periods in the presence of excess, nonradioactive Met/Cys. At each chasepoint, cells were washed and lysed, and CatS was retrieved by immunoprecipitation using a specific antiserum. The bound material was eluted in 95°C-reducing SDS sample buffer, followed by resolution of the retrieved polypeptides by SDS-PAGE and autoradiography (Figure 4B). In resting DC, proCatS migrating at 32 kD was visualized exclusively after the pulse. After 30 and 60 min. of chase, constant amounts of proCatS were detected. Mature CatS emerged at the 60-min. chase point, and proCatS was still present in comparable amounts, suggesting that at this time-point, proCatS was still being synthesized, and mature CatS was converted from its zymogen. After 120 min. of chase, mature CatS remained stable, and the

amounts of the labelled proform decreased. Thus, proCatS reaches the endocytic compartment roughly 60 min. after biosynthesis in resting human DC and is stable and progressively activated from its zymogen for at least another 60 min. LPS-stimulated DC were analyzed the same way: mature CatS also emerged after 60 min. of chase, suggesting that DC maturation did not grossly influence the conversion from proCatS to active CatS. However, already at the 60-min. chase-point, the amount of proCatS was reduced compared with the 30-min. chase-point or to the nonstimulated sample, and mature CatS was also present, only in lower amounts than in the nonstimulated sample. At the 120-min. chase-point, radiolabelled CatS and proCatS were consistently absent from LPS-stimulated DC, in stark contrast to nonstimulated DC, demonstrating degradation of CatS 1-2 h. after biosynthesis in LPS-stimulated DC. Complete degradation of active, newly synthesized CatS in LPS-activated human DC can therefore be observed within 60 min. after entering the endocytic compartment, in contrast to resting DC, and therefore, DC maturation controls the levels of mature CatS in an activation-dependent manner via endocytic degradation of the protease.

**Conjugation of DCG-0N to streptavidin increases CatS-targeted delivery**

Soluble peptides and whole-size proteins are being used to deliver antigen to MO-DC *in vitro* for immunotherapy. Whole-size sugars or proteins such as Dextran or HRP, at least in part, use endocytosis mediated by mannose receptor-binding, which might facilitate endocytosis and hence, delivery to endocytic proteases.[35,36] Thus, to increase the delivery of exogenous material to active cathepsins, we generated a larger protein-like tracer by conjugating DCG-0N to streptavidin prior to exposure to live DC, which were pulsed at 37°C for 10 min. using soluble 5 μM DCG-0N or the same amount of the probe prebound to 2.5 μM streptavidin prior to the pulse. After removal of noninternalized label by washing, cells were chased for up to 30 min, and labelled protease species were visualized by SDS-PAGE/antibiotin blot (Figure 5). Conjugation of DCG-0N to streptavidin significantly improved delivery of the probe to active cathepsins in intact DC, compared with soluble DCG-0N. Labelling efficiency of CatS increased nearly fivefold with streptavidin-bound DCG-0N (lower panel), compared with delivery by the nonconjugated tracer. It is important that the preferential labelling of active CatS was also preserved when the uptake of the tracer was facilitated by prior conjugation to a protein: labelling of CatS remained clearly dominant over labelling of active CatX and CatB in intact DC, in contrast to the labelling pattern observed in the total DC lysate. As expected, active cathepsins were not decorated using soluble DCG-0N or the DCG-0N-streptavidin construct for 30 min. at 4°C. Thus, protein-size cargo is transported to active cathepsins more efficiently than the peptide-size, soluble tracer in live human MO-DC. It is important that both types of delivery result in preferential targeting of exogenous material to active CatS.

**A cell-penetrating peptide increases delivery to cathepsin-containing compartments**

Cell-penetrating peptides can increase the efficiency of peptide vaccines *in vivo*, possibly by facilitating the transport of the peptide vaccine into endocytic compartments of APC. To directly assess the effect of the conjugation of a peptide to a CPP on its delivery to endocytic proteases in DC, we synthesized a DCG-0N-derivative conjugated to a nonaarginine CPP (PS457, Figure 6A). Monocyte-derived endocytic fractions were incubated consecutively with PS457 for the visualization of the labelled polypeptides. Although no protease polypeptides

were visualized by PS457 in the 95°C-preheated and the 25-μM E-64-treated samples (Figure 6B, Lanes 1 and 2), two polypeptides were weakly visualized in the sample labelled with PS457 (Figure 6B, Lane 3). The signals migrated slightly higher than the respective signals for CatS and CatB in the DCG-0N-labelled sample (Figure 6B, Lane 6), consistent with the molecular weight of the PS457 probe. Note that the intensity of labelling with DCG-0N (Figure 6B, Lane 6) is substantially higher compared with labelling with PS457 at equimolar concentrations in crude endocytic fractions (Figure 6B, Lane 3). Addition of the CatS inhibitor LHVS (25 nM) and the CatB inhibitor CA-074 (1 μM) eliminated labelling of active CatS and reduced labelling of active CatB (Figure 6B, Lane 4) and eliminated active CatB labelling (Figure 6B, Lane 5), respectively, consistent with the identity of these polypeptide species as

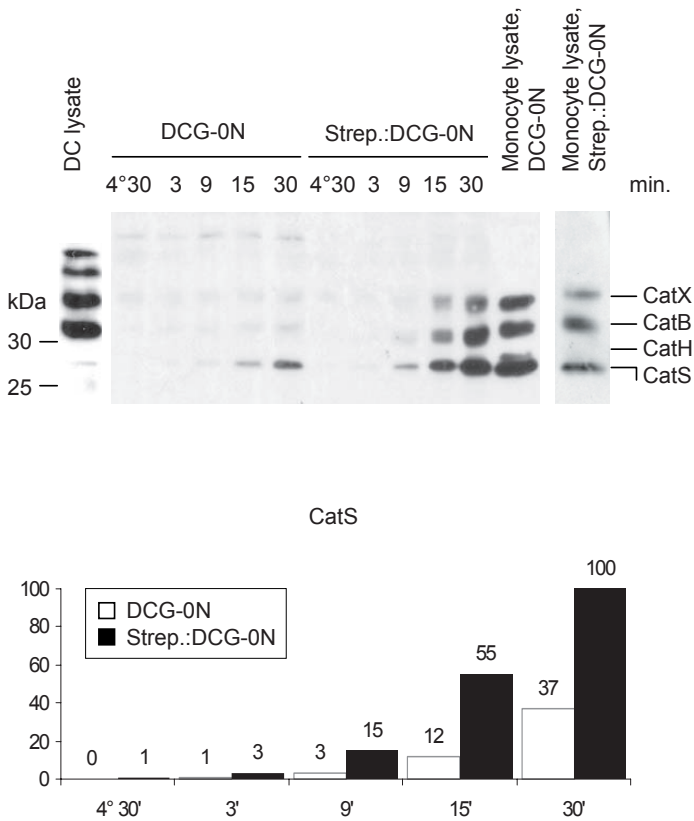


**Figure 5. Conjugation of DCG-0N to streptavidin increases the efficiency of protease delivery.**
Upper panel: DC were pulsed with soluble DCG-0N or with DCG-0N (5 μM each) preincubated with streptavidin at a 2:1 molar ratio at 37°C for different periods of time. After extensive washing, cells were lysed in 2x lysis buffer containing 100 μM JPM-565, and the targeted active polypeptides were visualized as above (*n*=2). Labelling of DC and monocyte lysates with soluble DCG-0N as well as with streptavidin-prebound DCG-0N (1 μM) served as controls.
Lower panel: Densitometric quantification of the relative amounts of active CatS targeted by either soluble or streptavidin-bound DCG-0N in live human DC, as deduced from the upper panel.

CatS and CatB in PS457-labelled cell lysates.

To test protease delivery of PS457 in intact DC, we pulsed DC with 10 μM soluble DCG-0N or PS457 for 1 h. at 37°C with 4°C controls and labelling of cell lysates, respectively, as before (Figure 6C). As expected, soluble DCG-0N preferentially decorated active CatS at 37°C but did not label cathepsins at 4°C in live DC (Figure 6C, Lanes 2 and 3). By contrast, PS457 visualized the entire set of active cathepsins already at 4°C (Figure 6C, Lane 5), demonstrating that it reaches cathepsin-containing compartments efficiently, independent from endocytosis activity. In fact, PS457 labelled active cathepsins in intact DC at 4°C with efficiency comparable with DCG-0N at 37°C. Given the poor labelling characteristics of PS457 compared with DCG-0N in cell lysates, passive transition of the plasma membrane facilitated by the nonaarginine CPP is at least in the same order of magnitude as the rate of fluid-phase endocytosis of MO-DC, which is known to be particularly high. Thus, CPP represent a powerful tool to deliver peptide-sized material to endocytic compartments, independently from active endocytic transport. Uptake of PS457 at 37°C significantly increased the amounts of proteases targeted by the probe, and LPS-induced down-modulation of endocytic internalization down-regulated this delivery. PS457 is thus internalized by a combination of endocytosis and passive, transport-independent transition of the cell membranes. In stark contrast to endocytosis-mediated uptake of DCG-0N, however, passive internalization of PS457 did not target CatS-containing compartments selectively but resulted in equally efficient delivery of the probe to active CatB and CatX.

When intact DC were exposed to both compounds (DCG-0N vs. PS457) in a pulse-chase format (Figure 6D), these differences became even more obvious: although at low concentrations of label (10 μM), the signal derived from exposure of the cells to DCG-0N remained at or below the threshold of detection, robust labelling of active cathepsins could be observed after incubation of DC with 10 μM PS457, where maximum signal intensity was reached already after 15 min. of chase. Note that CatB was visualized much stronger than CatS, similar to the pattern in the DC lysate and in contrast to the pattern observed after internalization of DCG-0N (compare also Figure 3B). We conclude that the delivery of exogenous, peptide-like material to active endocytic proteases is increased significantly when this material is conjugated to the polycationic shuttle device. Under these conditions, exogenous material can enter endocytic compartments of DC independently from active transport and is routed to endocytic proteases in a nonselective manner in contrast to endocytosis-mediated delivery.

## DISCUSSION

MO-DC loaded with antigenic peptide or complex protein represent the basis for several protocols, which aim at therapeutic vaccination in clinical trials. Different strategies are being applied to tailor antigenic material in a way that best exploits the nature of the internalization and processing machinery of MO-DC so that maximum amounts of the antigenic epitope are loaded ultimately on MHC II. Growing evidence indicates that the nature and specificity of the dominant protease(s) that mediate(s) the initial proteolytic attack on antigen after internalization by APC are of critical importance for the generation of a given antigenic epitope.[19,20] Murine BM-derived macrophages selectively recruit active CatS to

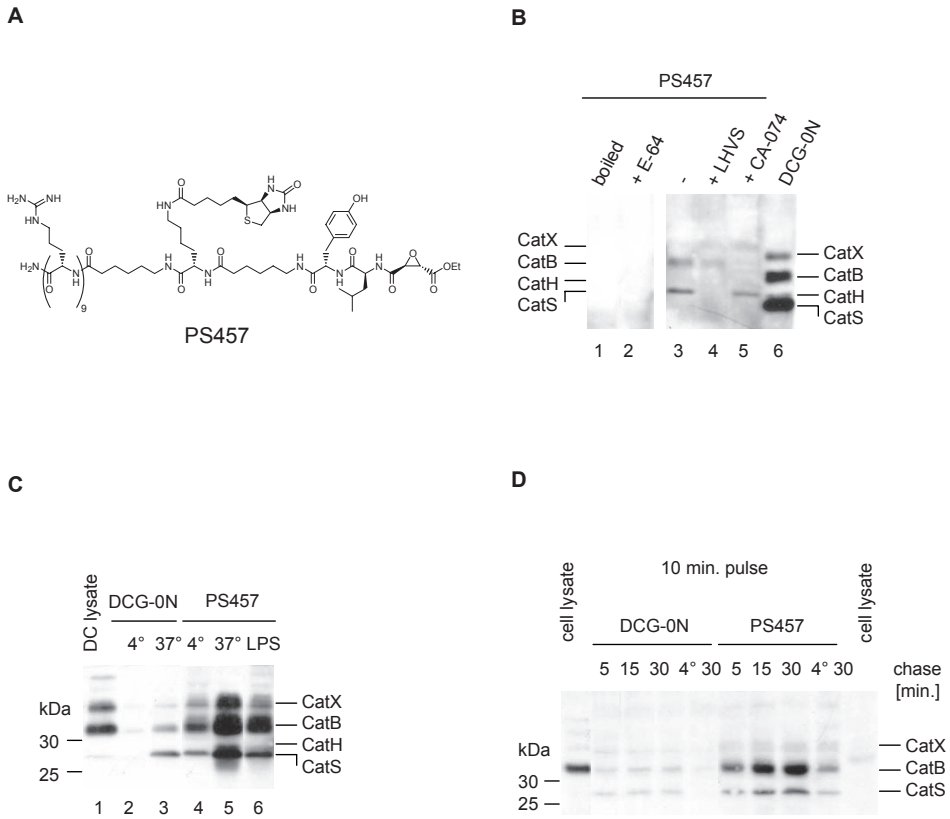


**Figure 6. Cell-penetrating peptides increase delivery to protease-containing compartments in DC.**
(A) Structure of the cell-penetrating peptide analogue PS457, consisting of a DCG-0N-core attached to a nonaarginine cell-penetrating transport device. (B) Labelling of active cysteine proteases by PS457. Cytosolic extracts of human monocytes were incubated with 10 μM DCG-0N or PS457, respectively, for 30 min. at RT. Where indicated, cells were pretreated with different inhibitors for 60 min. (25 nM LHVS, 1 μM CA-074 or 25 μM E-64). Proteins were separated by SDS-PAGE on a reducing 12.5% SDS gel and reactive proteins were visualized by streptavidin blotting ($n$=3). (C) Live human DC were incubated with either DCG-0N or PS457 at 25 μM, either at 4°C or 37°C. Where indicated, DC were matured by addition of LPS 24 h. before labelling. Labelling of DC- and monocyte-derived cell lysates at 37°C served as controls. The labelled polypeptides were visualized by anti-biotin blot after cell lysis and SDS-PAGE ($n$=4). (D) Intracellular delivery of PS457 in live DC. DC were incubated with either DCG-0N or PS457 (10 μM each) at 37°C for 10 min. (pulse), followed by extensive washing, and a 30 min. chase at 37°C. At each time point, cells were lysed and the targeted protease polypeptides visualized by streptavidin blot ($n$=3).

internalized material during phagosome maturation, in contrast to murine BM-derived DC.[24] Protease contents and the architecture of endocytic compartments likely differ among various types of APC, DC preparations, and species, so that these results cannot *a priori* be transferred to human MO-DC. We therefore here aimed at resolving the interaction between endocytic proteases and internalized material in viable human MO-DC. In addition, we evaluated

strategies that increase or modulate such delivery of exogenous material to protease-containing compartment(s).

Our results show that human MO-DC efficiently internalize the peptide-like ABP when exposed to the soluble tracer in culture medium, in contrast to internalization via phagocytosis. Exogenous material, as such incorporated, is routed selectively to active CatS, and CatB, CatL, and CatX are only poorly targeted, if at all, by a mechanism that requires activity of the vacuolar $H^+$-ATPase. This CatS-targeted delivery of exogenous, soluble matter in human DC could be observed as early as 3 min. after exposure of DC to the tracer and increased over 20-30 min. after internalization. In addition, endosomes of human MO-DC containing the internalized tracer selectively acquired active CatS during maturation. This is not a specific trait for MO-DC but was similarly observed in primary human monocytes. Our data therefore suggest that CatS is a functionally dominant active protease in early endocytic compartments of both human monocytes and MO-DC. Fractionation experiments with resolution of endocytic subcompartments revealed a differential distribution of active proteases in human DC, where CatS and CatB polypeptides were present in early endosomes in roughly equal amounts, in contrast to CatX and CatL[22], consistent with our results. The attribute the poor interaction between internalized DCG-0N and active CatB observed here to the only mildly acidic to near- neutral pH present in early endosomes, where CatB is expected to be inactive, in contrast to CatS, which shows a unique stability and activity from acidic up to neutral pH.[37] Thus, the preferential interaction of internalized material with CatS likely represents the combined result of differential distribution of cathepsins in conjunction with the pH gradient along the endocytic route. These results strongly suggest CatS as a functionally dominant endocytic protease likely to initiate antigen breakdown in human MO-DC. However, it can not be excluded formally that nonpapain proteases, such as active AEP, CatD, or CatE, which cannot be monitored with the probe used here, are reached by an exogenous tracer within a similar time window. However, unlike CatS, none of these proteases have been shown to be active or present in early endosomal compartments, consistent with their low pH optima.

The direct comparison between human MO-DC and murine BM-derived DC resulted in a 1-log lower efficiency of internalization (number of positive cells x mean fluorescence intensity) of identical fluorochrome-coupled latex beads by human MO-DC, and a similar difference was not observed for fluid phase-mediated endocytosis of a soluble protein. Thus, fluid phase-endocytosis appears to be a preferred mode of uptake of exogenous material by MO-DC *in vitro*. Although it is not entirely clear which route of antigen uptake (endocytosis vs. phagocytosis) is the preferred one for human DC *in vivo*[1], fluid-phase, mediated endocytosis has been shown to deliver antigen efficiently for T cell activation after vaccination *in vivo*.[38,39]

Proteolysis in the endocytic compartment of DC is regulated largely via the maturation state and the activity of the vacuolar ATPase. Although low ATPase activity is found in resting/ immature DC with a generally, slightly higher endosomal pH, LPS-mediated stimulation of DC leads to a drop in endocytic pH and more efficient proteolysis.[40] We here demonstrate that changes in the ATPase activity induced by the inhibitor Concanamycin B or by LPS-mediated activation indeed modulate the interaction of internalized tracer with active cathepsins. Inhibition of the ATPase abolished the delivery of the probe to active CatS. This was not a result of inactivation of the protease per se, as demonstrated by labelling of active CatS in cell

lysates treated with ConB. Treatment with LPS resulted in an increased labelling of active CatS by the internalized probe at early chase-times, in comparison to non stimulated controls. This is consistent with the model of a LPS-induced decrease in endocytic pH, which is likely to induce (auto)-catalytic activation of CatS, already in early endocytic compartments, as well as with the redistribution of active CatS to earlier endocytic compartments, as demonstrated for human DC upon LPS stimulation.[22] The intracellular maturation, activation, and degradation of newly synthesized CatS have not yet been addressed. As we here demonstrate by pulse-chase analysis and immunoprecipitation of radiolabelled, newly synthesized protein, CatS is degraded in the endocytic compartment of LPS-stimulated DC within approximately 60 min. of entry into the endocytic tract, in contrast to nonstimulated DC, and CatS biosynthesis and the rate of conversion of proCatS into its active, mature form are not influenced by DC maturation. Thus LPS-stimulated MO-DC limit the amounts of active CatS by degradation of the newly synthesized enzyme, in contrast to resting DC. This may control the (self-) destructive potential of CatS. However, the overall proteolytic environment of endocytosed antigens does not differ substantially between unstimulated and activated MO-DC.

As active CatS controls Ii maturation and thereby MHC II peptide-loading, a facilitated delivery of exogenous material to CatS-containing compartments might allow improvement of vaccination protocols. Our results demonstrate that targeting of peptide-like, synthetic compounds to active cathepsins in live human DC can be increased substantially by attaching peptide to a protein-size carrier or to CPP. As internalization of typical, fluid-phase markers such as Dextran or HRP in DC is also mediated, at least in part, by receptor-mediated endocytosis via mannose receptor-binding[35,36], we suggest that the improved internalization of the DCG-0N-streptavidin complex may also rely on this additional mode of uptake.

CPP-enhanced endocytic uptake was clearly the most efficient way of internalization into MO-DC. Despite their broad acceptance as molecular carriers, the mechanism of internalization of polycationic peptides is not well understood, and active, endocytosis-mediated as well as passive transition of the membrane are still under debate.[28] As PS457 labelled active cathepsins in intact cells also at 4°C, we strongly argue that CPP can enter the DC efficiently, independently from energy-dependent mechanisms such as endocytosis. However, endocytosis clearly improves the uptake of PS457 into cathepsin-containing compartments, as shown by appropriate changes at 37°C and after LPS-induced down-regulation of macropinocytosis, respectively. Although the selective transport to CatS is preserved when macropinocytosis is facilitated by conjugation of the probe to a protein carrier, this type of selectivity is lost when internalization is achieved by CPP. We suggest that soluble peptide or peptide attached to a protein carrier only reaches a sub-population of endocytic compartments enriched in CatS activity and separated from conventional late endosomes/lysosomes by membrane barriers. We envision that CPP may transit these separating membranes after internalization by MO-DC, and hence gain access to the entire spectrum of endocytic protease activity.

Conjugation of the immunogenic peptide to a CPP carrier prior to vaccination resulted in a vaccination success and a CD4-T cell-dependent tumor regression as a result of sustained antigen presentation in a murine model for peptide-based cancer immunotherapy, and the same peptide without a CPP carrier proved ineffective.[29] As demonstrated here, such conjugation of peptide to CPP greatly increases transport to CatS-containing endocytic compartments as well as to endocytic proteases in general. Both aspects might lead to improved antigen processing

and MHC II-peptide loading and hence, explain the sustained antigen presentation observed when antigen was delivered via CPP *in vivo*. In this sense, conjugation of antigen to CPP might serve as a universal strategy to shuttle antigenic peptide to the MHC II processing machinery. It should be noted, however, that facilitated delivery of antigenic protein to endocytic proteases (or to a broader selection of proteases) might also bear the potential to impair antigen presentation due to destructive processing. This likely depends of the nature and the processing pathway of the antigen used.[18-20]

Because we here identify CatS as a major protease reached by such constructs, conjugation of antigen to CPP via a CatS-sensitive peptide bond might be a worthwhile strategy to selectively release antigenic material in the appropriate compartment. In addition, conjugation of antigenic peptide to CPP may allow achievement of sufficient internalization of antigen and its delivery to proteases, even in LPS-activated DC, which are highly immunogenic but poorly endocytotic. Clearly, these possible applications of CPP warrant further systematic investigation with respect to the T cell response elicited *in vitro* and *in vivo*.

## ACKNOWLEDGEMENTS

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## REFERENCES

1. Trombetta ES, Mellman I. Cell biology of antigen processing *in vitro* and *in vivo*. *Annu. Rev. Immunol.* 2005;23:975-1028.
2. Mellman I, Steinman RM. Dendritic cells: specialized and regulated antigen processing machines. *Cell* 2001;106:255-258.
3. Steinman RM, Pack M, Inaba K. Dendritic cell development and maturation. *Adv. Exp. Med. Biol.* 1997;417: 1-6.
4. Rossi M, Young JW. Human dendritic cells: potent antigen-presenting cells at the crossroads of innate and adaptive immunity. *J. Immunol.* 2005;175:1373-1381.
5. Fiebiger E, Meraner P, Weber E, Fang IF, Stingl G, Ploegh H, Maurer D. Cytokines regulate proteolysis in major histocompatibility complex class II-dependent antigen presentation by dendritic cells. *J. Exp. Med.* 2001;193:881-892.
6. Burster T, Beck A, Tolosa E, Schnorrer P, Weissert R, Reich M, Kraus M, Kalbacher H, Haring HU, Weber E, Overkleeft H, Driessen C. Differential processing of autoantigens in lysosomes from human monocyte-derived and peripheral blood dendritic cells. *J. Immunol.* 2005;175:5940-5949.
7. Chain BM, Free P, Medd P, Swetman C, Tabor AB, Terrazzini N. The expression and function of cathepsin E in dendritic cells. *J. Immunol.* 2005;174:1791-1800.
8. Shi,GP, Bryant RA, Riese R, Verhelst S, Driessen C, Li Z, Bromme D, Ploegh HL, Chapman HA. Role for cathepsin F in invariant chain processing and major histocompatibility complex class II peptide loading by macrophages. *J. Exp. Med.* 2000;191:1177-1186.
9. Driessen C, Bryant RA, Lennon-Dumenil AM, Villadangos JA, Bryant PW, Shi GP, Chapman HA, Ploegh HL. Cathepsin S controls the trafficking and maturation of MHC class II molecules in dendritic cells. *J. Cell. Biol.* 1999;147:775-790.

10. Driessen C, Lennon-Dumenil AM, Ploegh HL. Individual cathepsins degrade immune complexes internalized by antigen-presenting cells via Fcgamma receptors. *Eur. J. Immunol.* 2001;31:1592-1601.
11. Riese RJ, Wolf PR, Bromme D, Natkin LR, Villadangos JA, Ploegh HL, Chapman HA. Essential role for cathepsin S in MHC class II-associated invariant chain processing and peptide loading. *Immunity* 1996;4: 357-366.
12. Nakagawa T, Roth W, Wong P, Nelson A, Farr A, Deussing J, Villadangos JA, Ploegh H, Peters C, Rudensky AY. Cathepsin L: critical role in li degradation and CD4 T cell selection in the thymus. *Science* 1998;280:450-453.
13. Riese RJ, Mitchell RN, Villadangos JA, Shi GP, Palmer JT, Karp ER, De Sanctis GT, Ploegh HL, Chapman HA. Cathepsin S activity regulates antigen presentation and immunity. *J. Clin. Invest.* 1998;101:2351-2363.
14. Shi GP, Villadangos JA, Dranoff G, Small C, Gu L, Haley KJ, Riese R, Ploegh HL, Chapman HA. Cathepsin S required for normal MHC class II peptide loading and germinal center development. *Immunity* 1999;10: 197-206.
15. Nakagawa TY, Brissette WH, Lira PD, Griffiths RJ, Petrushova N, Stock J, McNeish JD, Eastman SE, Howard ED, Clarke SR, Rosloniec EF, Elliott EA, Rudensky AY. Impaired invariant chain degradation and antigen presentation and diminished collagen-induced arthritis in cathepsin S null mice. *Immunity* 1999;10: 207-217.
16. Honey K, Nakagawa T, Peters C, Rudensky A. Cathepsin L regulates CD4+ T cell selection independently of its effect on invariant chain: a role in the generation of positively selecting peptide ligands. *J. Exp. Med.* 2002;195:1349 -1358.
17. Pierre P, Mellman I. Developmental regulation of invariant chain proteolysis controls MHC class II trafficking in mouse dendritic cells. *Cell* 1998;93:1135-1145.
18. Manoury B, Hewitt EW, Morrice N, Dando PM, Barrett AJ, Watts C. An asparaginyl endopeptidase processes a microbial antigen for class II MHC presentation. *Nature* 1998;396:695-699.
19. Manoury B, Mazzeo D, Fugger L, Viner N, Ponsford M, Streeter H, Mazza G, Wraith DC, Watts C. Destructive processing by asparagine endopeptidase limits presentation of a dominant T cell epitope in MBP. *Nat. Immunol.* 2002;3:169-174.
20. Watts C, Moss CX, Mazzeo D, West MA, Matthews SP, Li DN, Manoury B. Creation versus destruction of T cell epitopes in the class II MHC pathway. *Ann. N. Y. Acad. Sci.* 2003;987:9-14.
21. Lautwein A, Kraus M, Reich M, Burster T, Brandenburg J, Overkleeft HS, Schwarz G, Kammer W, Weber E, Kalbacher H, Nordheim A, Driessen C. Human B lymphoblastoid cells contain distinct patterns of cathepsin activity in endocytic compartments and regulate MHC class II transport in a cathepsin S-independent manner. *J. Leukoc. Biol.* 2004;75:844-855.
22. Lautwein A, Burster T, Lennon-Dumenil AM, Overkleeft HS, Weber E, Kalbacher H, Driessen C. Inflammatory stimuli recruit cathepsin activity to late endosomal compartments in human dendritic cells. *Eur. J. Immunol.* 2002;32:3348-3357.
23. Greiner A, Lautwein A, Overkleeft HS, Weber E, Driessen C. Activity and subcellular distribution of cathepsins in primary human monocytes. *J. Leukoc. Biol.* 2003;73:235-242.
24. Lennon-Dumenil AM, Bakker AH, Maehr R, Fiebiger E, Overkleeft HS, Rosemblatt M, Ploegh HL, Lagaudriere-Gesbert C. Analysis of protease activity in live antigen-presenting cells shows regulation of the phagosomal proteolytic contents during dendritic cell activation. *J. Exp. Med.* 2002;196:529-540.
25. Greenbaum D, Medzihradszky KF, Burlingame A, Bogyo M. Epoxide electrophiles as activity-dependent cysteine protease profiling and discovery tools. *Chem. Biol.* 2000;7:569-581.
26. Tolosa E, Li W, Yasuda Y, Wienhold W, Denzin LK, Lautwein A, Driessen C, Schnorrer P, Weber E, Stevanovic S, Kurek R, Melms A, Bromme D. Cathepsin V is involved in the degradation of invariant chain in human thymus and is overexpressed in myasthenia gravis. *J. Clin. Invest.* 2003;112:517-526.
27. Burster T, Beck A, Tolosa E, Marin-Esteban V, Rotzschke O, Falk K, Lautwein A, Reich M, Brandenburg J, Schwarz G, Wiendl H, Melms A, Lehmann R, Stevanovic S, Kalbacher H, Driessen C. Cathepsin G, and not the asparagine-specific endoprotease, controls the processing of myelin basic protein in lysosomes from human B lymphocytes. *J. Immunol.* 2004;172:5495-5503.
28. Fischer R, Fotin-Mleczek M, Hufnagel H, Brock R. Break on through to the other side-biophysics and cell biology shed light on cell-penetrating peptides. *Chembiochem.* 2005;6:2126-2142.
29. Wang RF, Wang HY. Enhancement of antitumor immunity by prolonging antigen presentation on dendritic cells. *Nat. Biotechnol.* 2002;20:149-154.

30. Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, Schuler G. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* 1999;223:77-92.
31. Van Swieten PF, Maehr R, Van Den Nieuwendijk AM, Kessler BM, Reich M, Wong CS, Kalbacher H, Leeuwenburgh MA, Driessen C, Van Der Marel GA, Ploegh HL, Overkleeft HS. Development of an isotope-coded activity-based probe for the quantitative profiling of cysteine proteases. *Bioorg. Med. Chem. Lett.* 2004;14:3131-3134.
32. Palmer JT, Rasnick D, Klaus JL, Bromme D. Vinyl sulfones as mechanism-based cysteine protease inhibitors. *J. Med. Chem.* 1995;38:3193-3196.
33. Wiendl H, Lautwein A, Mitsdorffer M, Krause S, Erfurth S, Wienhold W, Morgalla M, Weber E, Overkleeft HS, Lochmuller H, Melms A, Tolosa E, Driessen C. Antigen processing and presentation in human muscle: cathepsin S is critical for MHC class II expression and upregulated in inflammatory myopathies. *J. Neuroimmunol.* 2003;138:132-143.
34. Benaroch P, Yilla M, Raposo G, Ito K, Miwa K, Geuze HJ, Ploegh HL. How MHC class II molecules reach the endocytic pathway. *EMBO J.* 1995;14:37-49.
35. Sallusto F, Cella M, Danieli C, Lanzavecchia A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J. Exp. Med.* 1995;182:389-400.
36. Garrett WS, Chen LM, Kroschewski R, Ebersold M, Turley S, Trombetta S, Galan JE, Mellman I. Developmental control of endocytosis in dendritic cells by Cdc42. *Cell* 2000;102:325-334.
37. Chapman HA, Riese RJ, Shi GP. Emerging roles for cysteine proteases in human biology. *Annu. Rev. Physiol.* 1997;59:63-88.
38. Delamarre L, Pack M, Chang H, Mellman I, Trombetta ES. Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science* 2005;307:1630-1634.
39. Zhong G, Reis e Sousa C, Germain RN. Antigen-unspecific B cells and lymphoid dendritic cells both show extensive surface expression of processed antigen-major histocompatibility complex class II complexes after soluble protein exposure *in vivo* or *in vitro*. *J. Exp. Med.* 1997;186:673-682.
40. Trombetta ES, Ebersold M, Garrett W, Pypaert M, Mellman I. Activation of lysosomal function during dendritic cell maturation. *Science* 2003;299:1400-1403.

# VI

Conclusion and Discussion

## 6.1. CONCLUSIONS

The following conclusions can be drawn from our studies:

1. DC cultured from bone-marrow precursors show several defects in the BB-DP rat model of endocrine autoimmunity:
   a. Bone-marrow precursors of DC show a higher apoptosis during culture to DC, leading to a lower yield of DC after 7 days of culture in GM-CSF/IL-4. This defect is present in BB-DP rats, but absent in BB-DR rats.
   b. Immature DC showed a reduced MHC class II cell surface expression in 3 BB-DP sub-lines studied as compared to control rats. This defect was present in BB-DP rats, but absent in BB-DR rats.
   c. Immature DC of both BB-DP and BB-DR rats show a normal T cell stimulatory capacity as compared to DC of control rats.
   d. Immature DC of BB-DP rats showed a poor capability to differentiate into fully mature DC, i.e. DC with a clearly raised MHC class II and a superb T cell stimulatory capacity. This defect was also found to a certain extent in BB-DR rats, which only showed a poor T cell stimulatory capacity of mature DC.
   e. Immature DC of both BB-DP and BB-DR rats showed a reduced production of IL-10 as compared to control rats.

2. To study the gene linkage of the DC defects of the BB rat we used in addition F344 rats congenic for the region of the BB-DP rat chromosome 4, on which the *lyp* gene is located, designated F344.*lyp* rats.
   Here it must be noted that BB-DP/Seattle (S) and BB-DR/S rats differ in chromosome 4 with a relatively small region (1.4 Mb), which included the *lyp* gene (*iddm2*, the mutated *Ian5* gene), while F344.*lyp* rats differ from F344.+/+ rats in chromosome 4 with a much larger region (51.1 Mb), not only including the *lyp* gene (*iddm2*, the mutated *Ian5* gene), but also other potential diabetogenic genes, in particular *iddm4*.
   We found that
   a. DC precursors of F344.*lyp* animals show a higher apoptosis during culture to DC as compared to precursors of control F344.+/+, leading to a lower yield of DC after 7 days of culture in GM-CSF/IL-4.
   b. Immature DC of F344.*lyp* animals have a low MHC class II expression as compared to DC of control F344.+/+.
   c. Immature DC of F344.*lyp* animals have a reduced capability to stimulate T cells in allogeneic MLR as compared to DC of control F344.+/+.
   d. Immature DC of F344.*lyp* animals have a normal capability to differentiate into fully mature DC, a low MHC class II expression as compared to DC of control F344.+/+, i.e. DC with a clearly raised MHC class II and a superb T cell stimulatory capacity.
   e. Immature DC of F344.*lyp* animals have a reduced production of IL-10 (but only when *lyp*/*lyp*) as compared to control rats.

3. In a comparison of the abnormalities in the BB-DP/S versus BB-DR/S and those in F344.*lyp*/*lyp* rats versus the F344.+/+ rat we were able to construct a relatively detailed linkage of *lyp* and other BB genes to the aberrancies typical of the BB rat model of diabetes (see Table below).

**Linkage of DC phenotype with the genetic background of the various sub-lines of the F344 and BB rats.**

| Genes involved | | F344.+/+ | F344.*lyp/lyp* | BB.+/+ | BB.*lyp*/*lyp* |
|---|---|---|---|---|---|
| | | normal genes | normal genes plus BB genes on chromosome 4 (42.5-93.6 Mb) | BB genes minus *iddm2* | BB genes plus *iddm2* |
| *Iddm2 (lyp)* | Apoptosis precursors | normal | **increased** | normal | increased |
| | MHC class II expression | normal | **decreased** | normal | decreased |
| BB genes not on the 42.5 Mb-93.6 Mb region of chromosome 4 | Yield of DC | normal | normal | **increased** | **increased** |
| | *In vitro* maturation capacity after LPS | normal | normal | **decreased** | **decreased** |
| *Iddm4* (candidate) | T cell stimulation | normal | **decreased** | normal | normal |
| Complex linkage | IL-10 production | normal | **decreased** | **decreased** | **decreased (but equal to BB.+/+)** |

4. Bone-marrow precursor derived DC of BB rats and control rats did not express mRNA for *Ian5* or other *Ian* genes.

5. In an attempt to study thymus DC in the BB rat we used a protocol to isolate non-adherent low-density cells (LDC), a protocol that has been successfully used to isolate spleen DC and a population of APC from the thyroid. However after careful phenotyping of the cells these thymus LDC (unlike spleen DC) turned out to be $ED2^+$ branched cortical macrophages, and not $OX62^+$ thymus cortico-medullary and medullary dendritic cells. These $ED2^+$ branched cortical macrophages were excellent T cell stimulators, but more importantly rescued double-positive thymocytes from apoptosis. The $ED2^+$ branched cortical macrophages of lymphopenic F344.*lyp*/*lyp* and BB-DP rats showed a reduced T cell stimulatory capacity as compared to non-lymphopenic F344.+/+ and BB-DR/S rats. LDC of lymphopenic rats were poor inducers of $ART2^+$ regulatory T cells as compared to LDC of non-lymphopenic rats.
   $ED2^+$ branched cortical macrophages did express mRNA for *Ian5* and showed a reduced expression of *Ian5* in *lyp*/*lyp* animals (comparable to what was found in *lyp*/*lyp* thymocytes). Our study suggests that branched cortical macrophages play a role in positive selection and particular of naturally occurring Treg cells and that these cells are defective in BB-DP rats perhaps due to the *lyp* mutation (Chapter 4).

6. We simulated the rendezvous between antigen and endocytic proteases in human MO-DC. The most important endocytic proteases are the cysteine cathepsins, which we can identify with one tool showing all the active cysteine proteases.
   After internalization of the peptide-size tracer by macropinocytosis only cathepsin S was targeted. Blocking of the vacuolar ATPase abolished this CatS-selective targeting, while LPS-induced maturation of DC resulted in degradation of active CatS, in keeping with the concept that immature DC change from antigen-uptake cells to antigen-presenting cells during maturation. Conjugation of the ABP to a protein facilitated the delivery to endocytic proteases and resulted in labelling of sizable amounts of CatB and CatX, although CatS still remained the major protease reached by this construct. Conjugation of the probe to a cell-penetrating peptide (CPP) routed the tracer to the entire panel of intracellular cathepsins, independently from endocytosis or LPS-stimulation. Thus, different means of internalization result in differential targeting of active cathepsins in live MO-DC. CPP may serve as vehicles to target antigen more efficiently to protease-containing endocytic compartments (Chapter 5).

## 6.2. DEFECTS IN APC FUNCTION IN ANOTHER MODEL OF AUTOIMMUNE THYROIDITIS/DIABETES (THE NOD MOUSE) AND IN TYPE-1 DIABETES/ AUTOIMMUNE THYROIDITIS PATIENTS

The here found defects in the differentiation and function of DC are not only found in the BB rat model of autoimmune thyroiditis/diabetes. Similar defects have been found in the other important rodent model of autoimmune diabetes, the NOD mouse, and in type-1 diabetes patients. But apart from similarities there are also many dissimilarities.

### 6.2.1. Similarities and dissimilarities of DC between the BB-DP rat model and the NOD mouse model

In the NOD mouse model many studies have focused on growth factor induced development of DC from bone-marrow precursors. In studies in our own lab we used GM-CSF without IL-4 to culture DC from NOD bone-marrow precursors. These experiments showed a poor generation of immature and mature DC from precursors and these DC had a reduced capability to stimulate T cells and showed a more macrophage-like phenotype being strongly positive for acid phosphatase.[1] In not published experiments I also found the bone-marrow derived DC of the BB-DP rat to have a higher expression of the typical macrophage marker ED3 (see Figure 1).

Thus, NOD mouse bone-marrow derived DC show very similar defects as BB-DP rat bone-marrow derived DC being poor APC with a macrophage-like character.

It must be noted however, that, when bone-marrow precursors of the NOD mouse are cultured in GM-CSF plus IL-4, the defective and macrophage-skewed differentiation is restored resulting in DC with a superb T cell stimulatory capacity. This is thus different from BB-DP rat bone-marrow derived DC which show defects in differentiation also in the presence of additional IL-4 in the culture fluid.

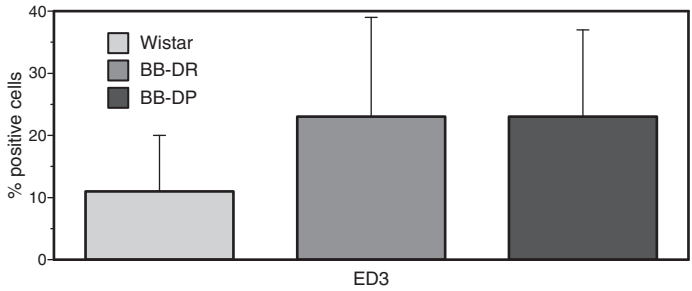


**Figure 1. Increased expression of the macrophage marker ED3 by bone-marrow derived DC of the BB-DP and BB-DR rat.**
The expression of the macrophage marker ED3 (CD169) on bone-marrow derived DC of BB-DP, BB-DR and Wistar rats was analyzed by flowcytometry. Bone-marrow derived DC of the BB rat, both BB-DP and BB-DR, showed a trend towards higher expression of the macrophage marker ED3 as compared to bone-marrow derived DC of the control Wistar rat (*n*=3-5).

A similarity between NOD and BB-DP rat DC is that both are in a pro-inflammatory state, yet a difference is that NOD mouse bone-marrow derived DC reach this pro-inflammatory state in a different way as the BB-DP rat DC. While NOD DC show an increased IL-12p70 production (due to increased levels of NF-κB)[2,3,4], bone-marrow derived DC of the BB-DP rat show a decreased IL-10 production.

Also the genes involved in the aberrancies of DC in the NOD and BB-DP rat are different. We here show in this thesis that *iddm2*, another gene on chromosome 4 (perhaps *iddm4*) and genes on other locations are involved in the various abnormalities of the BB-DP DC. These gene abnormalities are not present in the NOD mouse and studies suggest that *idd10*, *idd17* and *idd18* are involved in the DC abnormalities of the NOD mouse.[5]

### 6.2.2. Similarities and dissimilarities of DC between the BB-DP rat model and human type-1 diabetes

As elaborated before, in the rodent animal model spleen DC, thyroid DC and in particular bone-marrow derived DC populations have been studied. This has not been possible in human type-1 diabetes: in patients it is only possible to study monocyte-derived DC and circulating myeloid and plasmacytoid DC.

In studies on such DC an inconsistent picture has arisen. While in initial studies there was a poor differentiation of DC from monocytes in type-1 diabetes patients and individuals at risk to develop diabetes (i.e. those with multiple islet-reactive antibodies present)[6,7], later studies were unable to confirm these defects[8] (this is also our experience), or even found an enhanced differentiation of DC from precursors. Also with regard to numbers and function of circulating mDC and pDC there is confusion: a first report showed increased levels of mDC and pDC[9], while another report showed reduced numbers and function in mDC and pDC.[10] Explanations for these discrepant results have been suggested to reside in the fact that in the different studies different isolation and culture methods have been used to obtain monocyte-derived DC. There is, however, also the issue of patient heterogeneity. It is very well possible that

type-1 diabetes starting in childhood is different from type-1 diabetes starting in adulthood. In one of the reports it was found that the reduced numbers of mDC and pDC in the circulation were in particular present in childhood onset diabetes, while age was a confounding factor for these numbers.[7]

Interestingly, our group has detected more consistent defects regarding other myeloid circulating APC populations in type-1 diabetes and autoimmune thyroiditis patients: The generation of an APC population very similar to the monocyte-derived DC, i.e. of monocyte-derived veiled macrophage-like cells, was found hampered in type-1 diabetes and autoimmune thyroiditis patients, particularly after fibronectin adhesion of the monocytes, which normally up-regulates the generation of such veiled APC from monocytes. This hampered differentiation resulted in a low T cell stimulatory capacity of the APC.[11,12]

With regard to IL-10 and IL-12 production abnormalities of human APC, recent reports show a decreased *in vitro* IL-10 (and IL-4) production by peripheral blood cells of first-degree relatives at a high risk of type-1 diabetes.[13] Additional evidence for an IL-10 production aberrancy comes from twin studies: type-1 diabetes-discordant twins with a raised risk (i.e. islet cell antibody-positive) show a reduced IL-10 response to hsp60 as compared to twins at low risk.[14] In addition there appears to be a defect in the production of IL-10 (and IL-4) in the pancreas of recently diagnosed type-1 diabetes mellitus patients.[15]

### 6.2.3. A general blueprint of APC defects in rodent models and type-1 diabetes patients

Collectively our rat data and those of others in mice and humans thus show a general blueprint regarding the aberrant function of professional myeloid derived APC, i.e. of DC and veiled macrophages: The differentiation of such APC from precursors is defective, resulting in a pro-inflammatory state of these cells and a defective capability of the cells to induce Treg cells and/or to induce tolerance via AITCD (Figure 2). However the genes governing these defects, the pathways via which these defects are reached and the actual APC forms affected differ between the rodent models and patients. This suggests heterogeneity of the immune pathogenesis of autoimmune diabetes between the two rodent models and between the rodent models and the human disease, at least at the level of the initiation of the autoimmune response.

## 6.3. DO DEFECTIVE MYELOID APC PLAY AN ESSENTIAL ROLE IN DIABETES DEVELOPMENT?

Presently it is known that semi-mature and mature DCs are required for optimal tolerance induction. As discussed in the introduction section various mechanisms play a role in such tolerance induction; I here want to highlight the induction and maintenance of Treg cells in the thymus and periphery respectively.

### 6.3.1. The induction of ART2$^+$ regulatory T cells in the thymus

We here report that thymus branched cortical macrophages of BB-DP rats were poor rescuers of regulatory ART2$^+$ T cells as compared to those of BB-DR rats.

Previous work has shown that transfers of thymus APC of BB-DR rats to BB-DP rats are

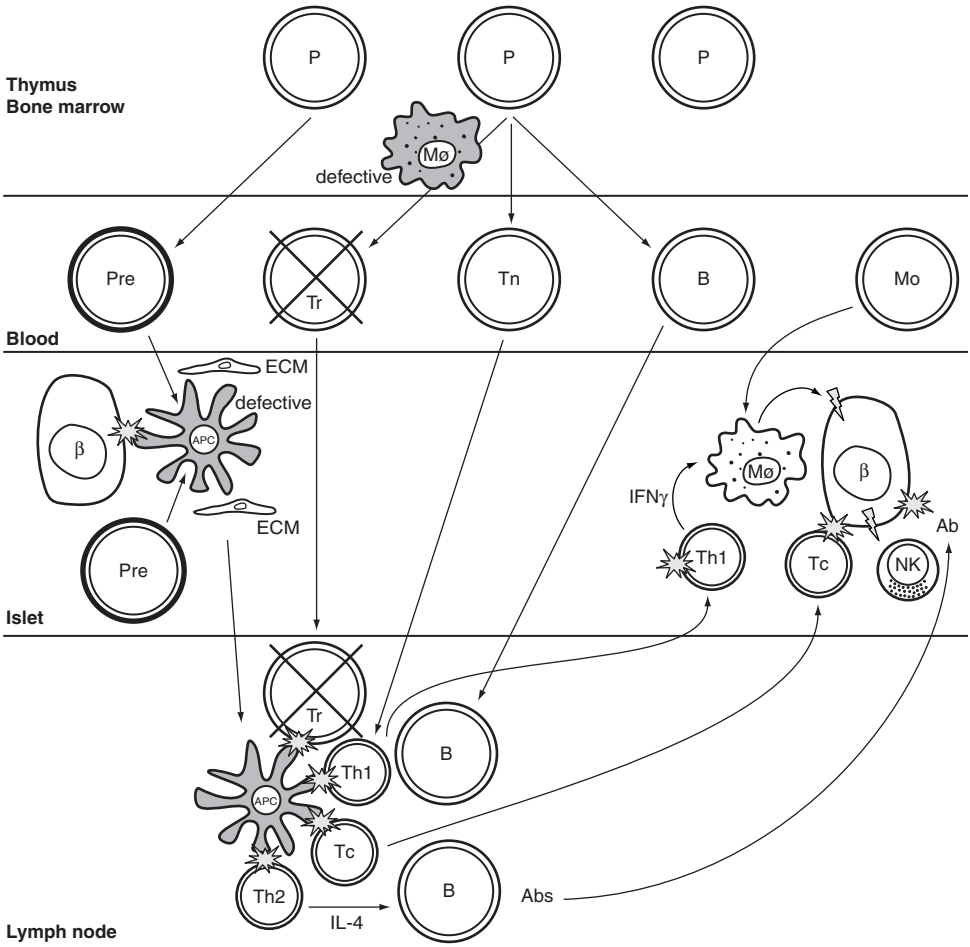


**Figure 2. General blueprint for the animal models of type-1 diabetes.**
Our rat data and those of others in mice and humans show a general blueprint regarding the aberrant function of professional myeloid derived APC, i.e. of DC, thymic branched cortical macrophages and veiled macrophages. The differentiation of such APC from precursors is defective, resulting in a pro-inflammatory state of these cells and a defective capability of the cells to induce regulatory T cells and/or to induce tolerance via AITCD. This loss of tolerance contributes to the destruction of the β cell. Ab, Antibody; B, B cell; β, beta cell; APC, antigen-presenting cell; ECM, extracellular matrix; Mo, monocyte; Mϕ, macrophage; NK, natural killer cell; P, progenitor; Pre, precursor; Th1, T helper 1 cell; Th2, T helper 2 cell; Tc, cytotoxic T cell; Tn, naïve T cell; Tr, regulatory T cell.

partially able to restore the defects of ART2$^+$ T in BB-DP rats and to prevent insulitis and diabetes.[16,17,18] Similar results were found when thymus macrophages from control CBA mice were adoptively transferred into neonatal NOD mice, preventing autoimmunity.[19,20]

Thus, it is very likely that the here described ART2$^+$ regulatory T cell induction defects of APC at the level of the thymus play a role in the faulty tolerance induction of the BB-DP rat

towards various endocrine autoantigens, though this needs now to be studied further.

Intrathymic tolerance induction is dependent on the local expression of autoantigens in the thymus. With regard to diabetes, different thymic cells express the insulin peptide family. All members of the insulin family are expressed in the thymus stroma according to a specific hierarchy and cellular topography. IGF-2 expression by TEC is the highest followed by IGF-1 by macrophages and insulin (INS) by thymic medullary epithelial cells and/or dendritic cells. This specific hierarchy in gene expression pattern is in accord with the observation that IGF-2 is better tolerated than INS. In the BB-DP rat model, IGF-2 expression was defective in 11 of 15 thymuses in close concordance with the diabetes incidence in their rat strain (while IGF-1 and insulin expression were intact).[21] Thus apart from the branched cortical macrophage defect autoantigen expression defects may contribute to the build up of the autoantigenic effector T cell repertoire of the BB-DP rat. However it needs further exploration how the defect in the branched cortical macrophages in the BB-DP rat relates to the putative defect of these cells to express members of the insulin antigen family.

A study in humans showed that IGF-2 peptide 11-25, which binds with the same affinity to DQ8 as insulin B chain peptide 9-23, induced an anti-inflammatory T cell response.[22] These studies provide an interesting view that not insulin, but IGF-2 should be the target autoantigen to establish a tolerogenic state in diabetes-prone individuals.

### 6.3.2. The maintenance and induction of regulatory T cells in the periphery

At an earlier occasion our group reported on a poor capability of the spleen DC of the BB-DP rat to support the expansion of ART2$^+$ T cells. Tolerance is partly dependent on survival and proliferation of regulatory T cells. At present a general consensus exists that IL-2 is a key stimulatory factor in this process. An additional factor, which seems to be important for the peripheral maintenance of regulatory T cells is CD28. At particularly this point peripheral DC of BB-DP rats seem to provide an insufficient stimulatory signal for proper peripheral Treg maintenance. The defect as described in this thesis of immature DC of the BB-DP rat to fully mature into MHC class II, CD80 and CD86 expressing mature DC results in a low capacity of these defectively maturated APC to stimulate T cells via the MHC class II-TCR and CD80-CD28 route.[23-29] Precisely such triggering via the latter route is essential to prevent the development of diabetes in the BB-DP rat and NOD mouse model. *In vivo* treatment of BB-DP rats with stimulatory anti-CD28 monoclonal Abs completely prevents the development of both insulitis and diabetes. Recent experiments show that this treatment expands in particular CD4$^+$CD25$^+$ Treg cells in the BB-DP rat (Visser, personal communication).

Apart from the defective APC signaling required for natural Treg cell maintenance, the pro-inflammatory state of the aberrant bone-marrow derived DC of the BB-DP rat, i.e. their reduced IL-10 production, must lead to a defective induction of at least the inducible forms of Treg cells, since IL-10 has been described as an important stimulator for Tr1 cell induction. However this needs further study.

*In sum*, the defective development of APC in BB-DP rats resulting in poor APC with a pro-inflammatory set point most likely contributes to the poor tolerogenic capability of the animals and the heightened aggressiveness to endocrine glandular cells.

## 6.4. FUTURE DIRECTIONS

### 6.4.1. *In vivo* effects of functionally restored DC and cortical branched macrophages

Future work in the BB-DP rat regarding DC defects should study their functional consequences *in vivo*. We expect that transfer of fully mature bone-marrow derived DC (of e.g. Wistar rats) to BB-DP rats would reduce/prevent diabetes. Such transfers have been shown to be effective in the NOD mouse model.[30,31]

Another approach would be to study the *in vitro* and *in vivo* suppressive function of regulatory $ART2^+$ T cells, after their *in vitro* generation in APC driven systems, e.g. via Wistar bone-marrow derived DC or thymus LDC. The generated $ART2^+$ regulatory T cells should suppress the proliferation of $ART2^-$ T cells *in vitro*. Final proof that the thymus LDC and fully mature bone-marrow derived DC are important in the prevention of diabetes could be best shown by purification of the induced $ART2^+$ T cells followed by adoptive transfer into pre-diabetic BB-DP rats to show that insulitis/diabetes can be prevented. Alternatively selective depletion of BB-DP LDC and the adoptive transfer of BB-DR LDC would give direct evidence that these cells are involved in the prevention of diabetes; a similar approach using thymus APC has been performed previously[16], although investigators were unable to phenotype the APC as branched cortical macrophages at that time.

### 6.4.2. Plasmacytoid DC

Future work should also focus on the other major DC population, the plasmacytoid DC. Plasmacytoid DC (pDC) are derived from plasmacytoid pre-DC, formerly called plasmacytoid cells or interferon-producing cells (IPC). Indeed these cells are specialized in a high production of type I IFN (IFN-α and β) upon viral/bacterial stimulation. First discovered in 1958 by Lennert en Remmele, they were mistakingly designated plasmacytoid T cells based on their plasma cell-like morphology (eccentric nucleus) and their presence in T cell areas of lymphoid organs.[32] At that time they were thought to be the T cell counterpart of plasma cells. In fact, their plasma cell-like appearance is an indication for their high production of proteins, which turned out to be the production of type I IFN. In comparison to other cells they are the cells producing the largest amount of type I IFN.[33] The human pDC lineage expresses CD45RA, BDCA-2, BDCA-4, CD123 (IL-3Rα) and survival is mainly dependent on IL-3. The mouse pDC lineage, in contrast to the human pDC lineage, does not express CD123. The phenotype of mouse pDC is $CD11b^-CD11c^{low}B220^+Gr\text{-}1^+$. The complete phenotype of rat pDC is $CD3^-CD4^+CD5^+CD90^+$ $CD45R^+CD45RC^+$ $CD11b^-CD11c^-CD161a^+CD200^+CD172^+$ $CD32^+CD86^+OX62^-$.[34] They appear homologous to human pDC as they produced type I IFN upon viral/bacterial stimulation via restricted expression of TLRs, namely TLR7 and TLR9. TLR9 confers responsiveness to bacterial DNA[35], while TLR7 responds to single stranded RNA from viruses (ssRNA). Other cytokines produced by rat pDC include IL-12 and IL-6.[34]

Interestingly Summers et al found that pDCs in human type-1 diabetes produced less IFN-α.[36] Could it be that a defective anti-viral response by pDC is the trigger for diabetes development? These studies could be performed in the BB rat model using both BB-DP and BB-DR rats, the latter with or without infection with Kilham virus or after poly I:C treatment.

### 6.4.3. Further studies on autoantigen effects and processing

A drawback of the studies described in this thesis is that none of the experiments has been done in the presence of diabetes relevant antigens. Particularly the T cell stimulation assays by faulty APC of the BB-DP rat should be carried out with relevant autoantigen(s). These include for the BB-DP rat IGF-2.

Also further work on the processing and presentation of diabetic autoantigens must consist of studies using such antigens, which are *in vitro* digested by isolated endolysosomal endocytic proteases of primary human and rat APC. This will provide information about the endocytic proteases involved and the actual peptide fragments generated from the autoantigens. Functional studies must then address the actual effect of the generated peptide fragment on the stimulation of autoantigen-specific T cells.

In our study in Chapter 5 we used ABP to address the delivery of exogenous material to active, papain-like cathepsins in human MO-DC. Cell-penetrating peptides (CPP) modulated the patterns and efficiency of the delivery of exogenous antigenic material to endocytic proteases in intact viable DC. Especially the use of such CPPs to deliver antigen efficiently inside the cells is an interesting approach, since this does not require an active uptake and antigen can reach MHC class I and MHC class II pathways in high amounts in both non-professional and professional APC. The use of cell-penetrating peptides coupled to autoantigens could be a promising approach to activate both $CD4^+$ regulatory T cells and $CD8^+$ regulatory T cells, since both MHC class II and MHC class I pathway are targeted by CPP coupled antigens. Such a strategy could be used to design a vaccine-like method to deliver autoantigens efficiently into (tolerogenic) DC to induce regulatory T cells, which are able to prevent the development of autoimmunity. The BB-DP rat could be a model to test such approaches.

## 6.5. SO IN THE END: WHAT DO WE LEARN FROM THE BB RAT MODEL FOR ENDOCRINE ORGAN-SPECIFIC AUTOIMMUNE DISEASES IN THE CLINIC?

It must be emphasized again that studying the BB rat 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, immunopathogenic pathways and genes involved seen in the BB-DP rat and the NOD mouse, that the causes of the diseases in the human and the involvement of various genes and environmental factors may differ from patient sub-set to patient sub-set. Yet in the study of particularly the pre-autoimmune phases of the diseases there is hardly an alternative than to study the animal models, the BB-DP rat and the NOD mouse. Only limited series of experiments can be carried out in individuals who are at risk to develop endocrine autoimmune diseases. Moreover the etio-pathogenesis found in the animal models may provide novel information of mechanisms that are relevant to human studies. Defects present in both the BB-DP rat, the NOD mouse and diabetes-prone individuals should in particular be considered for designing prophylactic and therapeutic therapies.

From this thesis it is clear that such target defects are the shared aberrancies of APC (poor generation, poor T cell stimulatory function, pro-inflammatory state), since they appear as cornerstones in the development of endocrine autoimmunity.

In summary, we believe that the BB rat model of endocrine organ-specific autoimmune disease still holds 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.

## REFERENCES


1. Nikolic T, Bunk M, Drexhage HA, Leenen PJ. Bone marrow precursors of nonobese diabetic mice develop into defective macrophage-like dendritic cells *in vitro*. *J. Immunol.* 2004;173:4342-4351.
2. Weaver DJ Jr, Poligone B, Bui T, Abdel-Motal UM, Baldwin AS Jr, Tisch R. Dendritic cells from diabetic mice exhibit a defect in NF-kappa B regulation due to a hyperactive I kappa B kinase. *J. Immunol.* 2001;167: 1461-1468.
3. Marleau A, Singh B. Myeloid dendritic cells in non-obese diabetic mice have elevated costimulatory and T helper-1-inducing abilities. *J. Autoimmun*. 2002;19:23-35.
4. Poligone B, Weaver Jr DJ, Sen P, Baldwin Jr AS, Tisch R. Elevated NF-κB activation in nonobese diabetic mouse dendritic cells results in enhanced APC function. *J. Immunol*. 2002;168:188-196.
5. Peng R, Bathjat K, Li Y, Clare-Salzler MJ. Defective maturation of myeloid dendritic cell (DC) in NOD mice is controlled by IDD10/17/18. *Ann. N. Y. Acad. Sci.* 2003;1005:184-186.
6. Takahashi K, Honeyman MC, Harrison LC. Impaired yield, phenotype, and function of monocyte-derived dendritic cells in humans at risk for insulin-dependent diabetes. *J. Immunol.* 1998;161:2629-2635.
7. Skarsvik S, Tiittanen M, Lindström A, Casas R, Ludvigsson J, Vaarala O. Poor *in vitro* maturation and pro-inflammatory cytokine response of dendritic cells in children at genetic risk of type 1 diabetes. *Scand. J. Immunol*. 2004;60:647-652.
8. Zacher T, Knerr I, Rascher W, Kalden JR, Wassmuth R. Characterization of monocyte-derived dendritic cells in recent-onset diabetes mellitus type 1. *Clin. Immunol.* 2002;105:17-24.
9. Peng R, Li Y, Brezner K, Litherland S, Clare-Salzler MJ. Abnormal peripheral blood dendritic cell populations in type 1 diabetes. *Ann. N. Y. Acad. Sci*. 2003;1005:222-225.
10. Vuckovic S, Withers G, Harris M, Khalil D, Gardiner D, Flesch I, Tepes S, Greer R, Cowley D, Cotterill A, Hart DN. Decreased blood dendritic cell counts in type 1 diabetic children. *Clin. Immunol.* 2007;123:281-288.
11. Jansen A, van Hagen M, Drexhage HA. Defective maturation and function of antigen-presenting cells in type 1 diabetes. *Lancet* 1995;25:491-492.
12. Canning MO, Ruwhof C, Drexhage HA. Aberrancies in antigen-presenting cells and T cells in autoimmune thyroid disease. A role in faulty tolerance induction. *Autoimmunity* 2003;36:429-442.
13. Szelachowska M, Kretowski A, Kinalska I. Decreased *in vitro* IL-4 [corrected] and IL-10 production by peripheral blood in first degree relatives at high risk of diabetes type-I. *Horm. Metab. Res*. 1998;30:526-530.
14. Kallmann BA, Lampeter EF, Hanifi-Moghaddam P, Hawa M, Leslie RD, Kolb H. Cytokine secretion patterns in twins discordant for type I diabetes. *Diabetologia* 1999;42:1080-1085.
15. Farilla L, Dotta F, Di Mario U, Rapoport B, McLachlan SM. Presence of interleukin 4 or interleukin 10, but not both cytokines, in pancreatic tissue of two patients with recently diagnosed diabetes mellitus type I. *Autoimmunity* 2000;32:161-166.
16. Georgiou HM, Bellgrau D. Thymus transplantation and disease prevention in the diabetes-prone bio-breeding rat. *J. Immunol.* 1989;142:3400-3405.
17. Georgiou HM, Lagarde AC, Bellgrau D. T cell dysfunction in the diabetes-prone BB rat: a role for thymic migrants that are not T cell precursors. *J. Exp. Med.* 1988;167:132-148.
18. Georgiou HM, Bellgrau D. Modulation of insulitis following thymus transplantation. *Transplant. Proc.* 1989;21: 215-217.
19. Georgiou HM, Constantinou D, Mandel TE. Neonatal transfer of allogeneic thymic macrophages prevents autoimmunity in nonobese diabetic mice. *Transplant. Proc.* 1995;27:2168-2169.
20. Georgiou HM, Constantinou D, Mandel TE. Prevention of autoimmunity in nonobese diabetic (NOD) mice by neonatal transfer of allogeneic thymic macrophages. *Autoimmunity* 1995;21:89-97.

21. Kecha-Kamoun O, Achour I, Martens H, Collette J, Lefebvre PJ, Greiner DL, Geenen V. Thymic expression of insulin-related genes in an animal model of autoimmune type 1 diabetes. *Diabetes Metab. Res. Rev.* 2001;17: 146-152.
22. Geenen V, Louis C, Martens H, The Belgian Diabetes Registry. An insulin-like growth factor 2-derived self-antigen inducing a regulatory cytokine profile after presentation to peripheral blood mononuclear cells from DQ8+ type 1 diabetic adolescents: preliminary design of a thymus-based tolerogenic self-vaccination. *Ann. N. Y. Acad. Sci.* 2004;1037:59-64.
23. Dahlen E, Hedlund G, Dawe K. Low CD86 expression in the nonobese diabetic mouse results in the impairment of both T cell activation and CTLA-4 up-regulation. *J. Immunol.* 2000;164:2444-2456.
24. Delemarre FG, Simons PJ, de Heer HJ, Drexhage HA. Signs of immaturity of splenic dendritic cells from the autoimmune prone biobreeding rat: consequences for the *in vitro* expansion of regulator and effector T cells. *J. Immunol.* 1999;162:1795-1801.
25. Delemarre FG, Hoogeveen PG, De Haan-Meulman M, Simons PJ, Drexhage HA. Homotypic cluster formation of dendritic cells, a close correlate of their state of maturation. Defects in the biobreeding diabetes-prone rat. *J. Leukoc. Biol.* 2001;69:373-380.
26. Feili-Hariri M, Morel PA. Phenotypic and functional characteristics of BM-derived DC from NOD and non-diabetes-prone strains. *Clin. Immunol.* 2001,98:133-142.
27. Beaudette-Zlatanova BC, Whalen B, Zipris D, Yagita H, Rozing J, Groen H, Benjamin CD, Hunig T, Drexhage HA, Ansari MJ, Leif J, Mordes JP, Greiner DL, Sayegh MH, Rossini AA. Costimulation and autoimmune diabetes in BB rats. *Am. J. Transplant.* 2006;6:894-902.
28. Boudaly S, Morin J, Berthier R, Marche P, Boitard C. Altered dendritic cells (DC) might be responsible for regulatory T cell imbalance and autoimmunity in nonobese diabetic (NOD) mice. *Eur. Cytokine Netw*. 2002;13: 29-37.
29. Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, Bluestone JA. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 2000;2:431-440.
30. Feili-Hariri M, Dong X, Alber SM, Watkins SC, Salter RD, Morel PA. Immunotherapy of NOD mice with bone marrow-derived dendritic cells. *Diabetes* 1999;48:2300-2308.
31. Feili-Hariri M, Falkner DH, Morel PA. Regulatory Th2 response induced following adoptive transfer of dendritic cells in prediabetic NOD mice. *Eur. J. Immunol.* 2002;32:2021-2030.
32. Lennert K, Remmele W. Karyometric research on lymph node cells in man. I. Germinoblasts, lymphoblasts & lymphocytes. *Acta Haematol*. 1958;19:99-113.
33. Siegal FP, Kadowaki N, Shodell M, Fitzgerald-Bocarsly PA, Shah K, Ho S, Antonenko S, Liu YJ. The nature of the principal type 1 interferon-producing cells in human blood. *Science* 1999;284:1835-1837.
34. Hubert FX, Voisine C, Louvet C, Heslan M, Josien R. Rat plasmacytoid dendritic cells are an abundant subset of MHC class II+ CD4+CD11b-OX62- and type I IFN-producing cells that exhibit selective expression of Toll-like receptors 7 and 9 and strong responsiveness to CpG. *J. Immunol.* 2004;172:7485-7494.
35. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S. A Toll-like receptor recognizes bacterial DNA. *Nature* 2000;408:740-745.
36. Summers KL, Marleau AM, Mahon JL, McManus R, Hramiak I, Singh B. Reduced IFN-alpha secretion by blood dendritic cells in human diabetes. *Clin. Immunol*. 2006;121:81-89.

# ABBREVIATIONS

| | |
|---|---|
| ABP | Activity-based probe |
| Ag | Antigen |
| AITCD | Activation Induced T Cell Death |
| Aire | Autoimmune regulator |
| APC | Antigen-presenting cell |
| APS | Autoimmune polyglandular syndrome |
| ART2 | ADP-ribosyltransferase-2 |
| BB-DP | Biobreeding-Diabetes Prone |
| BB-DR | Biobreeding-Diabetes Resistant |
| BM | Bone-marrow |
| CPP | Cell-penetrating peptide |
| CTLA-4 | Cytotoxic T-lymphocyte-associated protein 4 |
| DC | Dendritic cell |
| DN | Double-negative |
| EAE | Experimental Autoimmune Encephalomyelitis |
| ECM | Extracellular matrix |
| EGFP | Enhanced green fluorescence protein |
| ER | Endoplasmatic reticulum |
| Foxp3 | Forkhead box p3 |
| GITR | Glucocorticoid-induced TNF receptor |
| HSA | Heat stable antigen |
| Ian | Immune associated nucleotide |
| *Iddm* | Insulin-dependent diabetes mellitus gene (rat) |
| IFN | Interferon |
| IGF | Insulin-like growth factor |
| IL | Interleukin |
| INS | Insulin gene |
| LDC | Low-density cell |
| LPS | Lipopolysaccharide |
| MBT | *Mycobacterium tuberculosis* |
| MHC | Major histocompatibility complex |
| MO-DC | Monocyte-derived DC |
| NKT | Natural Killer T cell |
| NO | Nitric oxide |
| NOD | Non-obese diabetic |
| nTreg | Naturally occurring regulatory T cell |
| OS | Obese strain |
| Poly I:C | Polyinosinic-polycytidylic acid |
| PTPN22 | Protein tyrosine phosphatase non-receptor type 22 |
| PVS | Peri-vascular spaces |
| RAG | Recombination activating gene |
| RIP-LCMV | Rat insulin promoter-lymphocytic choriomeningitis virus |

| | |
|---|---|
| RTE | Recent thymic emigrant |
| SAC | *Staphylococcus aureus* Cowan |
| Scid | Severe combined immunodeficiency |
| SP | Single-positive |
| T1D | Type-1 diabetes |
| TCR | T cell receptor |
| Tg | Transgenic |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| Treg | Regulatory T cells |
| TSLP | Thymic stromal lymphopoietin |

# SUMMARY

Type-1 diabetes is the result of a T cell mediated immune response against the insulin-producing β cells in the islet of Langerhans. In humans, until now, the disease is only clearly detectable at the onset of the disease. Therefore studies to identify initial factors involved in the etio-pathogenesis are impossible in humans prone to develop diabetes. In order to study the early, prodromal phases of type-1 diabetes we used a spontaneous rodent animal model of the disease, the Biobreeding-Diabetes Prone (BB-DP) rat. This rat develops diabetes, because it is in particular defective for a population of regulatory T cells, the ART2$^+$ regulatory T cells and because it possesses the disease-prone MHC haplotype RT1$^u$ *(iddm1)*.

We investigated (**Chapter 2**) the myeloid dendritic cells (DC) in this animal model, since DC, the antigen-presenting cells *par excellence*, are able to elicit immune responses from naïve T cells and are known to be involved in autoimmune responses because they are capable of modulating immunity versus tolerance. We studied bone-marrow precursor derived myeloid DC of three BB-DP rat sub-lines (Worcester, Groningen, Seattle) to identify defects in these DC, which could be responsible for the defective tolerance induction towards diabetes-associated islet autoantigens in this rat model. We found that the myeloid DC generated from bone-marrow precursors were defective in these three BB-DP rat sub-lines, showing an immature, more macrophage-like phenotype, a low MHC class II expression on their surface, a reduced T cell stimulatory capacity, a reduced capability to differentiate into fully mature DC and a reduced production of the immunosuppressive cytokine IL-10 as compared to two control rat strains (Wistar, F344). We assume that such DC defects contribute to the decreased tolerance towards islet autoantigens in the autoimmune diabetes of the BB-DP rat, since such defective DC are in particular defective to stimulate ART2$^+$ regulatory T cells sufficiently.

We went on to study the gene linkage of the DC defects in the BB rat model (**Chapter 2** and **Chapter 3**) and firstly we studied the DC development from bone marrow precursors in BB-diabetes resistant (DR) rats of the Seattle (S) sub-line. The BB-DP/S rat develops diabetes, the BB-DR/S rat not. The BB-DP/S rat is lymphopenic (particularly for the ART2$^+$ regulatory T cells), the BB-DR/S rat is not. Genetically the BB-DP/S and DR/S rat differ for the *Ian5* gene at the *lyp* gene locus *(iddm2)*. This gene regulates the apoptosis of recent thymic emigrants and particularly induces the lymphopenia of the ART2$^+$ Treg cells in the rat. In this model we found almost similar defects as in the generation of DC from bone-marrow precursors, but there was a difference with the BB-DP rat in that the DC had a normal MHC class II expression.

Secondly we used F344.*lyp* rats congenic for a large stretch of chromosome 4 of the BB-DP/S rat which harbours the *lyp* gene (*iddm2*), but also other diabetes-linked genes such as *iddm4*. This F344.*lyp* rat resembles the BB-DP rat in that it has a strong lymphopenia, but in contrast to the BB-DP rat does not develop diabetes, due to the lack of other important diabetes susceptibility genes (such as *iddm1*). The DC of these rats showed almost similar defects as found in the BB-DP rat model, but in contrast had a normal capability to differentiate into fully mature DC.

From these observations in the various BB-related models we were able to construct a relatively detailed linkage of *lyp* and other BB genes with the DC differentiation defects

present in the BB-DP rat:

1. the low MHC class II expression is linked to *lyp* (*iddm2)*,
2. the reduced T cell stimulatory capacity is linked to other genes on chromosome 4 (candidate gene: *iddm4*) and
3. the reduced IL-10 production has a complex linkage pattern, including *iddm2*.

In **Chapter 4** we set out to isolate the thymus DC of the BB-DP rats and isolated the low-density non-adherent cells from rat thymuses. For other tissues (lymph nodes, spleen, thyroid) this procedure results in a (relatively) pure population of DC. To our surprise the thymus isolated low-density cells were not DC, but appeared to be a relatively unknown population of accessory cells in the thymus, the branched cortical macrophages. These cortical macrophages are involved in positive selection in the thymus cortex. The branched cortical macrophages of the BB-DP and the F344.*lyp* rat appeared to be defective in function and less capable of rescuing double-positive thymocytes from apoptosis as compared to BB-DR and F344.+/+ rats. This defect was thus linked at least in part to the *lyp* gene. Interestingly the defective branched cortical macrophages of the BB-DP and F344.*lyp* rat were also poor rescuers of the thymus generated $ART2^+$ Treg cells. Hence defects in the branched cortical macrophages, as described here in the BB-DP rat model, most likely contribute both to the T cell lymphopenia of the rat and its deficient tolerogenic state.

In experiments described in **Chapter 5** we simulated the rendezvous between antigen and endocytic proteases in human monocyte-derived DC. The most important endocytic proteases are the cysteine cathepsins, which we can identify with a chemical tool, called activity-based probe (ABP), which identifies all the active cysteine cathepsins. By the action of these cysteine cathepsins exogenous proteins/peptides are digested into small peptides, which are eligible for loading onto MHC class II molecules followed by presentation to T cells. After internalization of ABP by macropinocytosis only cathepsin S is targeted. Blocking of the vacuolar ATPase abolishes this CatS-selective targeting, while LPS-induced maturation of DC results in degradation of active CatS, in keeping with the concept that immature DC change from antigen-uptake cells to antigen-presenting cells during maturation. Conjugation of the ABP to a protein facilitated the delivery to endocytic proteases and resulted in labelling of sizable amounts of CatB and CatX, although CatS still remained the major protease reached by this construct. Conjugation of the probe to a cell-penetrating peptide (CPP) routed the tracer to the entire panel of intracellular cathepsins, independently from endocytosis or LPS-stimulation. Thus, different means of internalization result in differential targeting of active cathepsins in live monocyte-derived DC. We also concluded that CPP may serve as vehicles to target antigen more efficiently into cells.

*Taken together*, when our DC data obtained in the BB rat model of type-1 diabetes are compared to DC data previously obtained in a mouse model of type-1 diabetes (the NOD mouse) and to those in type-1 diabetic patients, a blueprint regarding the aberrant function of professional myeloid APC (DC and accessory macrophages) becomes evident: The T cell stimulatory function of such cells is defective, while the cells are more macrophage-like and in a pro-inflammatory state. The pro-inflammatory state of the APC skews naïve T effector cells into predominantly "dangerous" Th1 cells. Due to their defective T cell stimulatory capability the APC support/induce Treg cells insufficiently and are less capable of inducing tolerance via activation induced T cell death (AITCD). We therefore consider the here described defects in

myeloid DC as cornerstones in the etio-pathogenesis of T1D contributing to the breakdown of tolerance towards islet autoantigens finally leading to the destruction of the insulin-producing β cells.

# SAMENVATTING

Type-1 diabetes is het gevolg van een T cel gemedieerde autoimmuun reactie tegen de insuline-producerende β cellen in de eilandjes van Langerhans. In de mens is, op dit moment, de ziekte alleen bij aanvang duidelijk detecteerbaar en kan de vaak lange (enkele jaren durende) prodromale fase niet worden geïdentificeerd. Daarom zijn studies die factoren willen onderzoeken, die betrokken zijn bij het vroege ontstaan van de ziekte, vrijwel onmogelijk bij patiënten of individuen die de aandoening onder de leden hebben. Om in staat te zijn deze vroege, prodromale fase van type-1 diabetes te bestuderen, hebben wij gebruik gemaakt van een spontaan knaagdier model van de ziekte, de Biobreeding-Diabetes Prone (BB-DP) rat.

Deze rat ontwikkelt een autoimmuun vorm van diabetes, omdat de rat

1. Een tekort heeft aan een populatie van regulatoire T cellen, de ART2$^+$ regulatoire (reg) T cellen, die bij normale ratten in staat is de autoimmuun reactie te onderdrukken, en
2. Positief is voor een bepaald MHC haplotype, nl. RT1$^u$, een specifiek molekuul op zogenaamde antigeen-presenterende cellen (APC) dat bepaalde stukken van eiwitten (antigeen peptiden, vermoedelijk diabetes gerelateerde lichaamseigen antigeen peptiden) beter aan het afweer systeem (de T cellen) aanbiedt.

We onderzochten in eerste instantie (**Hoofdstuk 2**) de dendritische cellen (DC) in dit diermodel, aangezien

1. De DC, antigeen-presenterende cellen *par excellence*, in staat zijn een afweer reactie op te starten vanuit naïve T cellen, en
2. De DC bekend staan om hun invloed op autoimmuun reacties: Ze zijn in staat afweer versus tolerantie (het vermijden van afweer tegen lichaamseigen antigene peptiden) te moduleren.

We bestudeerden DC ontstaan uit voorlopercellen in het beenmerg van drie BB-DP rat lijnen (Worcester, Groningen, Seattle) met het doel defecten in deze DC te identificeren, die verantwoordelijk zouden kunnen zijn voor de defecte tolerantie jegens diabetes geassocieerde eiland autoantigenen in dit rat model. We vonden dat DC gegenereerd uit beenmerg voorlopercellen defecten vertoonden in deze drie BB-DP rat stammen in vergelijking met twee gezonde controle rattenstammen (Wistar, F344): De DC vertoonden een onrijp, meer macrofaag-achtig fenotype, een lage MHC klasse II expressie op hun oppervlak, een verlaagde T cel stimulatie capaciteit, een verlaagde mogelijkheid te differentiëren naar volledig rijpe DC en een verminderde produktie van het immunosuppressieve cytokine IL-10. We hebben aanwijzingen dat zulke defecten van de DC bijdragen aan de verlaagde tolerantie ten opzichte van eiland autoantigenen in de autoimmuun diabetes van de BB-DP rat, aangezien zulke defecte DC in het bijzonder defect zijn in het voldoende stimuleren van de ART2$^+$ regulatoire T cellen.

We bestudeerden verder de genetische associatie van de bovengenoemde DC defecten in het BB rat model van type-1 diabetes (**Hoofdstuk 2** en **Hoofdstuk 3**). Ten eerste bestudeerden we de DC onwikkeling vanuit beenmerg voorlopercellen in een andere BB rat stam, de BB-diabetes resistente (DR) ratten van de Seattle (S) sub-lijn. Terwijl de BB-DP/S rat diabetes ontwikkelt, doet de BB-DR/S rat dat niet. De BB-DP/S rat heeft een sterk gebrek aan T cellen (in het bijzonder aan de ART2$^+$ regulatoire T cellen), de BB-DR/S rat heeft dat niet.

Genetisch onderscheiden de BB-DP/S en de DR/S rat zich van elkaar wat betreft een "fout" *Ian5* gen. Dit foute gen veroorzaakt een "fout" versneld afsterven (apoptose) van T cellen die uit de thymus (de ontstaansplaats van T cellen) naar het bloed migreren en in het bijzonder het versneld afsterven van de ART2$^+$ Treg cellen. In het BB-DR/S model vonden we bijna vergelijkbare defecten in het ontstaan van DC vanuit beenmerg voorlopercellen, maar er was één verschil met de BB-DP/S rat: de DC van de BB-DR/S ratten hadden een normale MHC klasse II expressie op hun DC. Dit betekent dat dit defect in de DC gerelateerd is aan het foute *Ian5* gen.

Ten tweede maakten we gebruik van F344.*lyp* ratten, dit zijn gezonde F344 ratten die een stuk gen hebben gekregen van de BB-DP/S rat. Dit stuk is een tamelijk groot stuk van chromosoom 4 van de BB-DP/S rat en dit stuk bevat zowel het foute *Ian5* gen (ook wel het *lyp* gen of *iddm2* genoemd) alsook andere diabetes vatbaarheidgenen, zoals het *iddm4*. Deze F344.*lyp* rat lijkt op de BB-DP/S rat in dat de rat een sterk gebrek aan T cellen (en vooral de ART2$^+$ Treg cellen) bezit; maar in tegenstelling tot de BB-DP/S rat ontwikkelt de F344.*lyp* rat geen diabetes door de afwezigheid van andere belangrijke diabetes vatbaarheidgenen genen die de BB-DP/S rat wel bezit, zoals het RT1$^u$ (ook wel *iddm1* genoemd). De DC van deze F344.*lyp* ratten vertoonden ook weer bijna dezelfde defecten als die in het BB-DP/S rat model, maar er was nu geen gestoorde differentiatiemogelijkheid naar volledig rijpe DC.

Uit deze observaties in de diverse BB rat-gerelateerde modellen van type-1 diabetes waren we in staat een relatief gedetailleerde associatie te construeren van de invloed van het foute *Ian5* gen en andere BB rat vatbaarheidgenen op de DC differentiatie defecten aanwezig in de BB-DP rat:

1. de lage MHC klasse II expressie op BB-DP rat DC is geassocieerd met het *lyp* (*iddm2)* gen,
2. de verlaagde T cel stimulatoire capaciteit van de BB-DP rat DC is geassocieerd met andere genen op chromosoom 4 (kandidaat gen: *iddm4*) en
3. de verminderde productie van het immuun suppressieve IL-10 door de DC heeft een complex associatie patroon, waarbij het *lyp* (*iddm2)* gen ook een rol speelt.

In **Hoofdstuk 4** beschrijven we experimenten waarin wij van plan waren om DC van de thymus van BB-DP/S ratten te isoleren. Wij isoleerden de lage-dichtheid niet-adherente cellen uit ratten thymussen. Voor andere weefsels (lymfeklier, milt, schildklier) genereert deze procedure een (relatief) zuivere populatie van DC vanuit die weefsels. Tot onze verbazing waren de uit de thymus geïsoleerde lage-dichtheidscellen geen DC, maar waren het een relatief onbekende populatie van andere antigeen-presenterende cellen uit de thymus, de zogenaamde dendritische corticale thymus macrofagen. Deze vertakte corticale macrofagen zijn betrokken bij het "redden" van geschikte (bruikbare) T cellen die ontstaan in de cortex (schors) van de thymus en die anders zouden afsterven. De vertakte corticale macrofagen van de BB-DP en de F344.*lyp* rat bleken afwijkend in functie en slechter in staat deze geschikte T cellen van de dood te redden. Ook waren de defecte vertakte corticale macrofagen van de BB-DP en F344.*lyp* rat slechte redders van de in de thymus gegenereerde ART2$^+$ Treg cellen. Defecten in de vertakte corticale macrofagen, als beschreven in het BB-DP rat model, dragen dus hoogst waarschijnlijk bij aan de deficiënte tolerogene staat van de rat.

In experimenten beschreven in **Hoofdstuk 5** simuleerden we de rendezvous tussen antigeen en endocytische proteasen in DC die we uit menselijke monocyten hadden opgekweekt.

Endocytische proteasen zijn enzymen die betrokken zijn bij het zodanig knippen van antigenen dat zij goed aangeboden kunnen worden door DC aan T cellen in het immuniseringproces. Door de werking van deze enzymen worden de antigene eiwitten verteerd tot kleine peptiden, die geschikt zijn voor belading van MHC klasse II molekulen gevolgd door presentatie aan T cellen. De meest belangrijke endocytische proteasen zijn de cysteïne cathepsinen, die we kunnen identificeren met behulp van een chemisch instrument, een soort model antigeen, het "activity-based probe" (ABP) genoemd. Dit ABP bindt zich aan alle actieve cysteïne cathepsinen na opname door een APC. Na opname van ABP door de monocyt-afkomstige DC via macropinocytose bleek alleen het cathepsine S door dit ABP bereikt te worden. Blokkade van de vacuolaire ATPase bleek deze CatS-selectieve "targeting", te voorkomen, terwijl LPS-geïnduceerde rijping van DC in degradatie van het actieve CatS resulteerde. Dit is in overeenstemming met het concept dat onrijpe DC veranderen van antigeen-opnemende cellen naar antigeen-presenterende cellen tijdens de rijping. Conjugatie van de ABP aan een eiwit vergemakkelijkte het transport naar de endocytische proteasen en resulteerde in het binden van ABP aan ook behoorlijke hoeveelheden van andere cysteïne cathepsinen, het CatB en CatX, alhoewel CatS toch de belangrijkste protease bleef die bereikt werd door ABP. Conjugatie van ABP aan "Cel-Penetrerend Peptide" (CPP) bracht de probe naar het gehele scala aan intracellulaire cathepsinen, onafhankelijk van endocytose of LPS-stimulatie. In conclusie: Verschillende manieren van antigeen opname resulteren in het verschillend "targeten" van actieve cathepsinen in monocyt-afkomstige DC. We concludeerden verder dat CPP kan dienen als een vehikel dat antigenen efficiënter in APC kan brengen.

Wanneer wij al de experimenten in het BB rat model samenvatten en vergelijken met de gegevens voorheen verkregen met DC in het muis model van type-1 diabetes (de NOD muis) en de gegevens gevonden in type-1 diabetes patiënten, wordt een blauwdruk (sjabloon) duidelijk wat betreft een gestoorde functie van APC in autoimmuun vormen van diabetes: de T cel stimulerende functie van APC is defectieus, de cellen zijn meer macrofaag-achtig en zij staan in een ontstekingsbevorderende (pro-inflammatoire) staat afgesteld. De pro-inflammatoire staat van de APC drijft naïve T cellen voornamelijk in de richting naar de-voor-de- ontwikkeling-van-diabetes "gevaarlijke" Th1 cellen. Door hun defecte T cel stimulerende capaciteit ondersteunen/induceren de defecte APC regulatoire T cellen onvoldoende en zijn zij ook minder in staat tot inductie van tolerantie door middel van andere processen, zoals de "Activation Induced T Cell Death" (AITCD). De in mijn proefschrift beschreven defecten van de DC kunnen daarom als een hoeksteen in de ontstaanswijze van type-1 diabetes worden beschouwd.

# DANKWOORD

De totstandkoming van dit proefschrift vond plaats bij de sectie autoimmuunziekten van de afdeling Immunologie van het Erasmus MC.

Beste Hemmo, mijn promotor, bedankt voor het begeleiden tijdens mijn promotieonderzoek. Je hebt me als kritische begeleider de fijne kneepjes van het vak bijgebracht.

Ik wil verder de mensen bedanken die het onderzoeksobject, de BB rat, aan mij verstrekt hebben. Zonder hun hulp was dit onderzoek absoluut niet mogelijk. Het verkrijgen van dit dier was de eerste horde die overwonnen moest worden bij het onderzoek, aangezien de bestaande kolonie naar de groep van dr. Jan Rozing in Groningen was verhuisd. Tijdens het project bleef dit een punt van aandacht, aangezien dit proefdier niet eenvoudig verkrijgbaar is. Eerst werden de dieren verkregen bij BRM Inc. te Massachussettes, U.S.A. Nadat dit niet meer mogelijk was, werden de BB ratten uit Groningen gehaald. Hiervoor wil ik dan ook speciaal dr. Jan Rozing uit Groningen bedanken voor het verstrekken van de BB ratten. Uiteindelijk werden ook BB ratten uit Seattle (University of Washington) gehaald. Prof. Åke Lernmark thanks for the very pleasant and nice cooperation, without your support I would be lost! I would also like to thank Jessica Fuller for genotyping and shipping these precious rats to us, and Brian Van Yserloo and Elizabeth Rutledge for determining the expression levels of *Ian5* mRNA.

Prof. Rob Benner, bedankt dat je het mogelijk voor mij maakte om dit onderzoek uit te voeren op jouw afdeling. Verder wil ik onze analist Pieter Sijrier en hoofdanalist Harm de Wit, tevens mijn paranimf, in het bijzonder bedanken voor hun hulp tijdens mijn onderzoek. De samenwerking heb ik altijd als prettig ervaren. Ik wil tevens de medewerkers van het proefdiercentrum van het Erasmus MC, het EDC, bedanken. In het bijzonder John Mahabier, Henk Dronk en Ed Lansbergen, die het uitvoeren van de proefdierexperimenten überhaupt mogelijk maakten. De leden van de leescommissie prof. Herbert Hooijkaas, prof. Åke Lernmark en prof. Vincent Geenen wil ik bedanken voor het corrigeren van het proefschrift. Mijn dank gaat ook uit naar alle (ex-)medewerkers van de afdeling Immunologie die me behulpzaam zijn geweest tijdens mijn periode als promovendus: dr. Pieter Leenen, dr. Marjan Versnel, Roos Padmos, Joey Riepsaame, Manon Wildenberg, Marcel Dupasquier, Hui Wan, dr. Wai-Kwan Lam-Tse, dr. Dariusz Stepniak en dr. Tanja Nikolic.

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Vinod Bhagwanbali wil ik bedanken dat hij mijn paranimf wil zijn. Mijn familie, mijn moeder, mijn broer Rajesh en mijn zussen Patricia en Sita, ben ik een enorme dank verschuldigd voor hun steun. Tevens wil ik mijn vrienden bedanken voor hun begrip en support. Tot slot wil ik Kirtie bedanken, zonder jouw steun en toeverlaat was deze uitdaging niet mogelijk geweest. Bedankt voor alles wat je voor me gedaan hebt!

# CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren te Paramaribo, Suriname. Hij volgde zijn voortgezet onderwijs (atheneum) aan het Christelijk Lyceum te Alphen aan den Rijn. Na zijn atheneum besloot hij de studie Biologie aan de Rijksuniversiteit Leiden te volgen. Als specialisatie werd de richting Medische Biologie gekozen. Het doctoraal examen in de Medische Biologie werd behaald met als hoofdvakken dierfysiologie en immunopathologie.

Na de studie Medische Biologie werd begonnen aan het promotieonderzoek op de afdeling Immunologie van het Erasmus MC onder leiding van prof. dr. H.A. Drexhage. Tijdens het promotieonderzoek werden defecten in de antigeen-presenterende cellen in het BB rat model voor type-1 diabetes onderzocht. Tijdens deze periode werden verschillende cursussen gevolgd: Medische Immunologie, stralingshygiëne 5B, proefdierkunde, cursus Moleculaire Biologie en Oxford English Exam. Na een korte periode docent Biologie te zijn geweest, werd in oktober 2005 aangevangen als Postdoc/Research Scientist aan de Eberhard Karls Universiteit te Tübingen onder leiding van dr. C. Driessen. Het onderzoeksproject werd gefinancieerd door het Marie Curie Research Training Network Project 'Drugs for therapy'. Het doel van dit onderzoeksproject is de rol van endolysosomale proteasen (cathepsinen) in antigeen-processering en antigeen-presentatie te elucideren.

The writer of this thesis was born in Paramaribo, Suriname. He followed his High School education (atheneum) at the Christian Lyceum in Alphen aan den Rijn. After his high school education, he decided to study Biology at the University of Leiden. As a specialization Medical Biology was chosen. His doctoral degree in Medical Biology was achieved with majors in animal physiology and immunopathology.

After studying Medical Biology he started his PhD research at the department of Immunology at the Erasmus MC under supervision of Prof.Dr. H.A. Drexhage. During his PhD research he investigated the defects in the antigen-presenting cells in the BB rat model of type-1 diabetes. During this period several courses were followed: Medical Immunology, radiation course, experimental animal course, Molecular Biology and Oxford English Exam. After a short period working as a Biology teacher, he started his current Postdoc/Research Scientist position in October 2005 at the Eberhard Karls University in Tübingen under supervision of Dr. C. Driessen. The research project was financed by the Marie Curie Research Training Network 'Drugs for therapy'. The aim of this research project is to elucidate the role of endolysosomal proteases (cathepsins) in antigen-processing and antigen-presentation.

# LIST OF PUBLICATIONS

1. Van Ginneken VJT, Balm P, Sommandas V, Onderwater M, Van den Thillart G. Acute stress syndrome of the yellow European eel (*Anguilla Anguilla* Linnaeus) when exposed to a graded swimming-load. *Netherlands Journal of Zoology* 2002;52:29-42.

2. Sommandas V, Rutledge EA, Van Yserloo B, Fuller J, Lernmark Å, Drexhage HA. Aberrancies in the differentiation and maturation of dendritic cells from bone-marrow precursors are linked to various genes on chromosome 4 and other chromosomes of the BB-DP rat. *Journal of Autoimmunity* 2005;25:1-12.

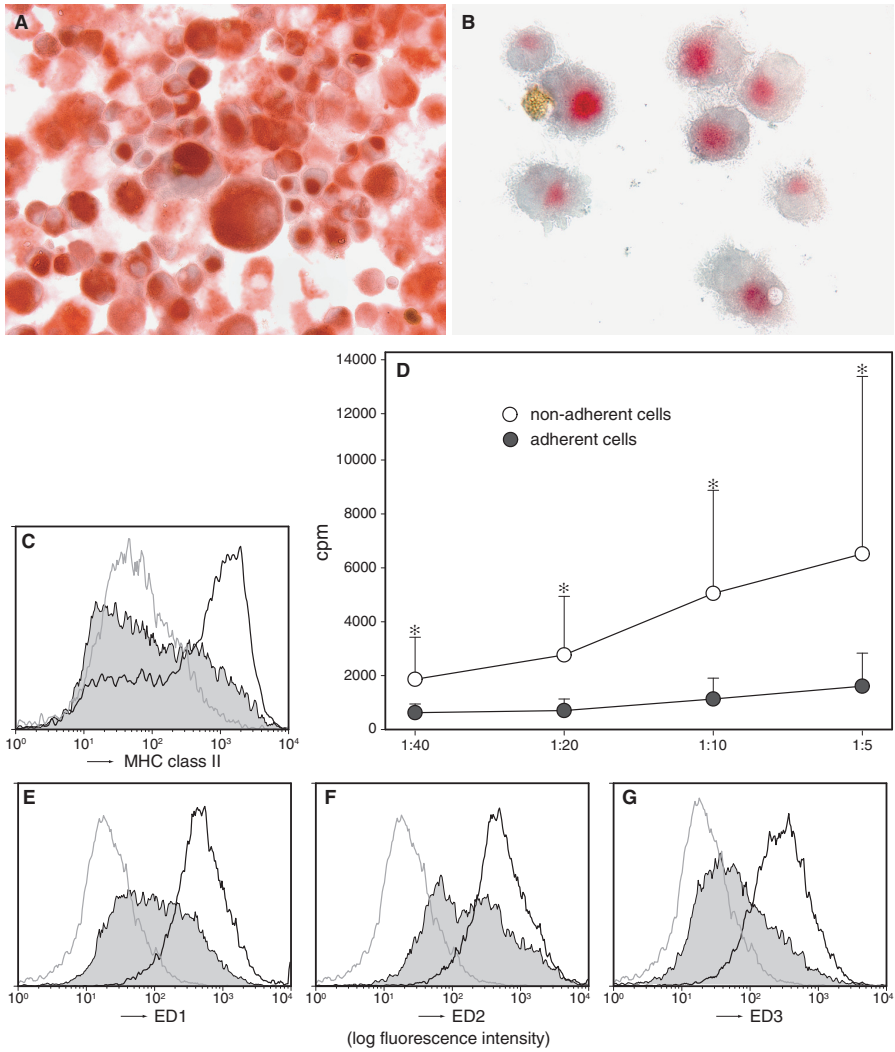
3. Sommandas V, Rutledge EA, Van Yserloo B, Fuller J, Lernmark Å, Drexhage HA. Defects in differentiation of bone-marrow derived dendritic cells of the BB rat are partly associated with *iddm2* (the *lyp* gene) and partly associated with other genes in the BB rat background. *Journal of Autoimmunity* 2005;25:46-56.

4. Sommandas V, Rutledge EA, Van Yserloo B, Fuller J, Lernmark Å, Drexhage HA. Low-density cells isolated from the BB-DP rat resembling branched cortical macrophages have a reduced capability of rescuing double-positive thymocytes from apoptosis. *Journal of Leukocyte Biology* 2007;82:869-876.

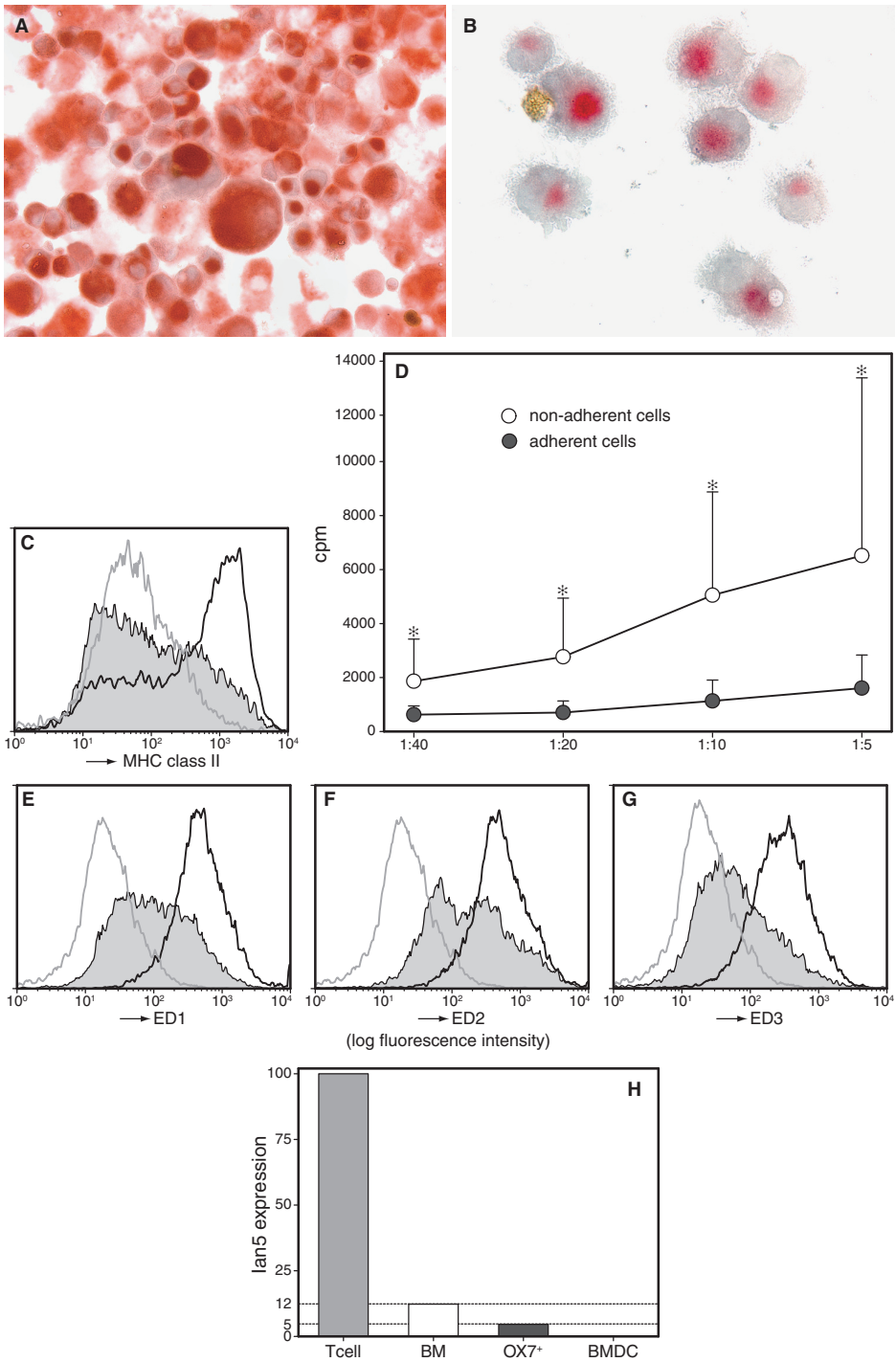
5. Reich M, Van Swieten PF, Sommandas V, Kraus M, Fischer R, Weber E, Kabacher H, Overkleeft H, Driessen C. Endocytosis targets exogenous material selectively to cathepsin S in live human dendritic cells, while cell-penetrating peptides mediate nonselective transport to cysteine cathepsins. *Journal of Leukocyte Biology* 2007;81:990-1001.

6. Zaidi N, Burster T, Sommandas V, Herrmann T, Boehm BO, Driessen C, Voelter W, Kalbacher H. A novel cell penetrating aspartic protease inhibitor block processing and presentation of tetanus toxoid more efficiently than pepstatin A. *Biochemical and Biophysical Research Communications* 2007;364:243-249.

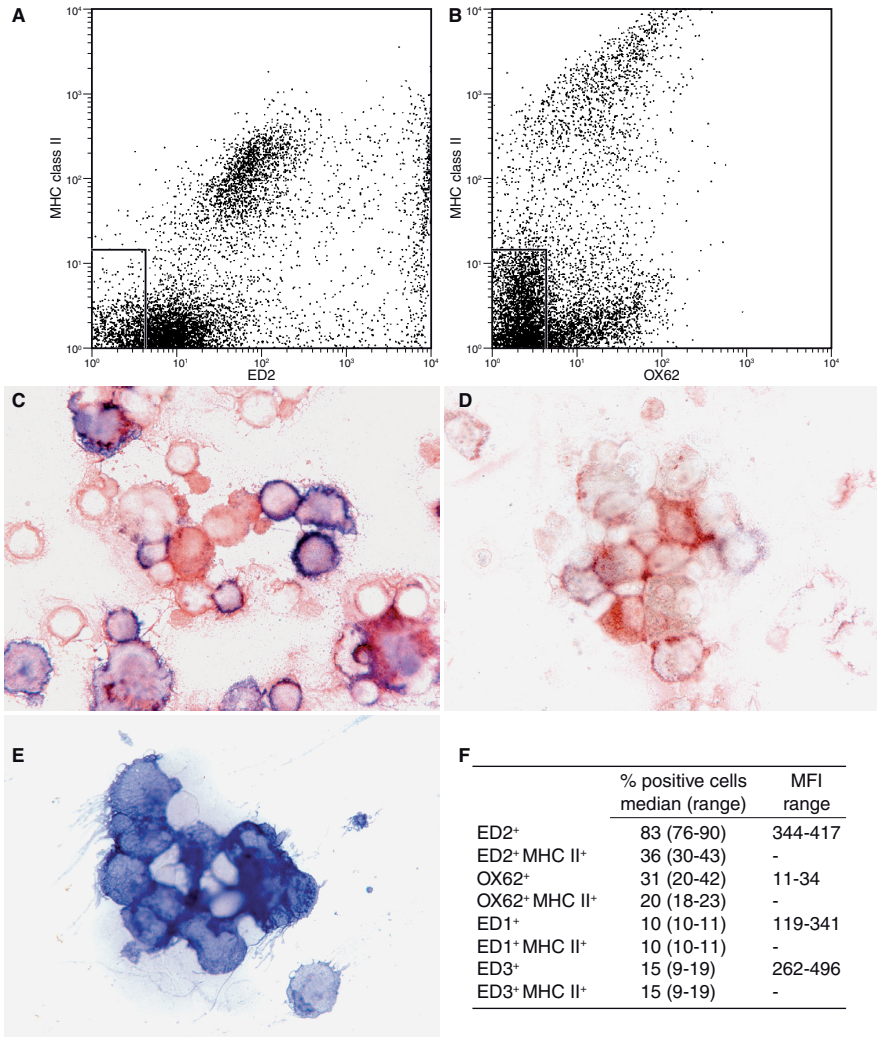
# APPENDIX




**Chapter II Figure 1. Characteristics of rat bone-marrow derived DC.**
When bone-marrow precursors are cultured in GM-CSF/IL-4 two $OX62^{+}CD11c^{+}$ populations are obtained: (A) a population of adherent (colony-forming) cells and (B) a non-adherent population of floating single cells. Although the non-adherent cells show a definite, but lower expression of MHC class II (C) as compared to adherent cells, the cells have a much higher capability to stimulate T cells to proliferate (D, mean ± standard deviations are shown; *n*=10). The T cell stimulatory capacity was measured in syngeneic MLR and expressed as the incorporation of $^{3}$H-thymidine (c.p.m.) in the stimulated T cells. The adherent cells also have various macrophage characteristics like a higher ED1 (E), ED2 (F) and ED3 (G) expression and a stronger and ''spotty'' acid phosphatase activity (A). We therefore consider the non-adherent cells as the typical DC. The grey tone in (C, E, F and G) represents the non-adherent cells. The black line in (C, E, F and G) represents the adherent cells. The isotype control is depicted as a grey line. Representative figures are shown in (C, E, F and G) (*n*=18). Wistar rat DC are shown (similar results are found for BB and Fischer rats; data not shown).


A
B
C
MHC class II
D
non-adherent cells
adherent cells
cpm
14000
12000
10000
8000
6000
4000
2000
0
1:40
1:20
1:10
1:5
E
ED1
F
ED2
G
ED3
(log fluorescence intensity)
H
Ian5 expression
100
75
50
25
12
5
0
Tcell
BM
OX7+
BMDC

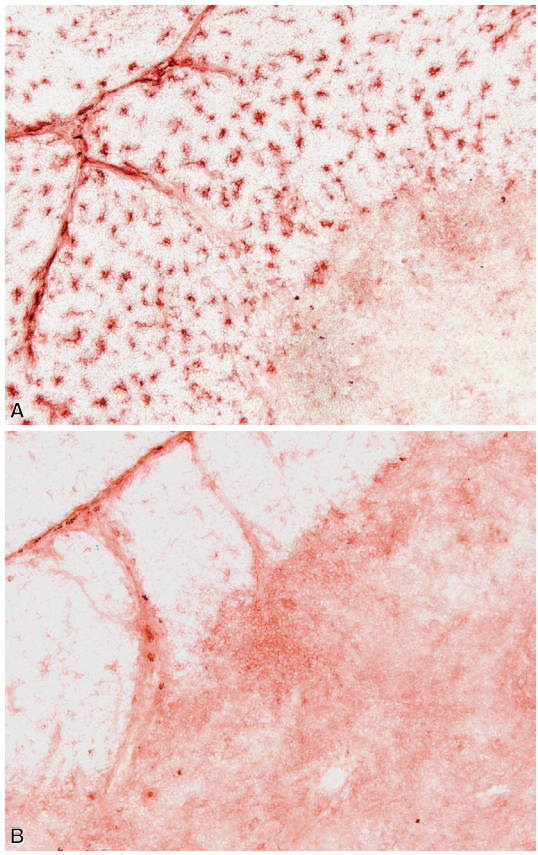
**Chapter III Figure 1. Characteristics of rat bone-marrow derived DC. When bone-marrow precursors are cultured in GM-CSF/IL-4 two $OX62^{+}CD11c^{+}$ populations are obtained: a population of adherent (colony-forming) cells and a non-adherent population of floating single cells.**
Although the non-adherent cells show a definite, but lower expression of MHC class II (C) as compared to adherent cells, the cells have a much higher capability to stimulate T cells to proliferate (D; *n*=10). The adherent cells also have more macrophage characteristics like a higher ED1 (E), ED2 (F) and ED3 (G) expression and a stronger and ''spotty'' acid phosphatase activity (A). We therefore consider the non-adherent cells as the typical DC. Interestingly, *Ian5* mRNA is not expressed in myeloid DC (H). The grey tone in (C), (E)-(G) represents the non-adherent cells. The dotted line represents isotype control. Representative figures are shown in (C), (E)-(G) (*n*=18). Experiments shown were performed in Wistar rats. The magnification for (A) and (B) is 64x and 96x, respectively.




F

| | % positive cells median (range) | MFI range |
|---|---|---|
| ED2$^+$ | 83 (76-90) | 344-417 |
| ED2$^+$ MHC II$^+$ | 36 (30-43) | - |
| OX62$^+$ | 31 (20-42) | 11-34 |
| OX62$^+$ MHC II$^+$ | 20 (18-23) | - |
| ED1$^+$ | 10 (10-11) | 119-341 |
| ED1$^+$ MHC II$^+$ | 10 (10-11) | - |
| ED3$^+$ | 15 (9-19) | 262-496 |
| ED3$^+$ MHC II$^+$ | 15 (9-19) | - |

**Chapter IV Figure 1.**
(A) Dot plot of thymus low-density cells (LDC) with ED2 expression on the *x*-axis and MHC class II on the *y*-axis. Negative cells are in the boxed area. Note that the majority of the LDC are ED2$^+$ and approximately half are MHC class II$^+$. The latter population is also the strongest positive for ED2 (for quantitative data, see Figure 1F). (B) Dot plot of thymus LDC with OX62 expression on the *x*-axis and MHC class II on the *y*-axis. Note that only a minority of LDC is intermediately positive for OX62 (for quantitative data see Figure 1F). (C) Immune cytology of thymus LDC with ED2 (red) and MHC class II (blue). Magnification X 1000; no counterstaining. It can clearly be seen that the majority of cells is ED2$^+$, and that about half of them stain double for MHC class II (particularly the large cells). (D) Immune cytology of thymus LDC with ED2 (red) and OX62 (blue). Magnification X 1000. A weaker staining was used for ED2 as compared with Figure 1C by shorter development of the staining step, since OX62 staining appeared rather faint; no counterstaining. It shows that part of the ED2$^+$ cells are also faintly OX62$^+$. (E) Immune cytology of thymus LDC with MHC class II (blue). Magnification X 1000. Overstaining was used by longer development of the staining step to clearly visualize the long, cellular protrusions (branches/dendrites). No counterstaining. (F) Quantification of marker positive LDC in flowcytometric analysis.




**Chapter IV Figure 2.**
(A) Immunohistochemistry of a F344.+/+ rat thymus stained for ED2 (magnification X 150). The $ED2^{+}$ cells in the cortex (i.e. the branched cortical macrophages) are clearly visible. (B) Immunohistochemistry of a F344.+/+ rat thymus stained for OX62 (magnification X 150). The positivity for OX62 of the cortico-medullary and medullary dendritic cells can clearly be seen.