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Immune cell function in the Clec16a Knock-down Mouse

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Die Promovendin ist Ärztin.

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1 Introduction

1.1 Type 1 Diabetes

The incidence of Type 1 diabetes has been rising since the 1950s: figures for Europe (1989-2008) suggest an annual median rate of increase of 3-4% among children aged 0-14[1]. Worldwide data show a similar -albeit slightly weaker- trend[2]. At the same time disease onset is shifting to an earlier age[3-5]. Since Type 1 diabetes remains an incurable disease that requires life-long treatment and -even if treated- entails considerable acute and chronic complications[6], such developments have to be taken seriously. The substantial implications for quality of life, as well as the financial burden resulting from Type 1 diabetes highlight the importance of better understanding the aetiology of this disease.

Type 1 Diabetes as defined by the American Diabetes Association is the absolute insulin deficiency and consequent hyperglycaemia resulting from pancreatic Langerhans islet β-cell destruction[7]. In the vast majority of patients this destruction is attributable to a T cell mediated autoimmune aggression, in this case it is further specified as type 1A diabetes. In contrast to this type 1B diabetes refers to those cases where insulin deficiency is idiopathic, i.e. there is no sign of immune-related destruction (ibid.). The latter form will however not be considered further in this text and for the sake of simplicity the term Type 1 Diabetes (T1D) will be used as synonymous for type 1A diabetes.

1.1.1 The role of genes and environment in T1D

Manifestation of T1D is considered to be result of the interaction of two elements: an individual's genetic susceptibility and environmental factors[8].

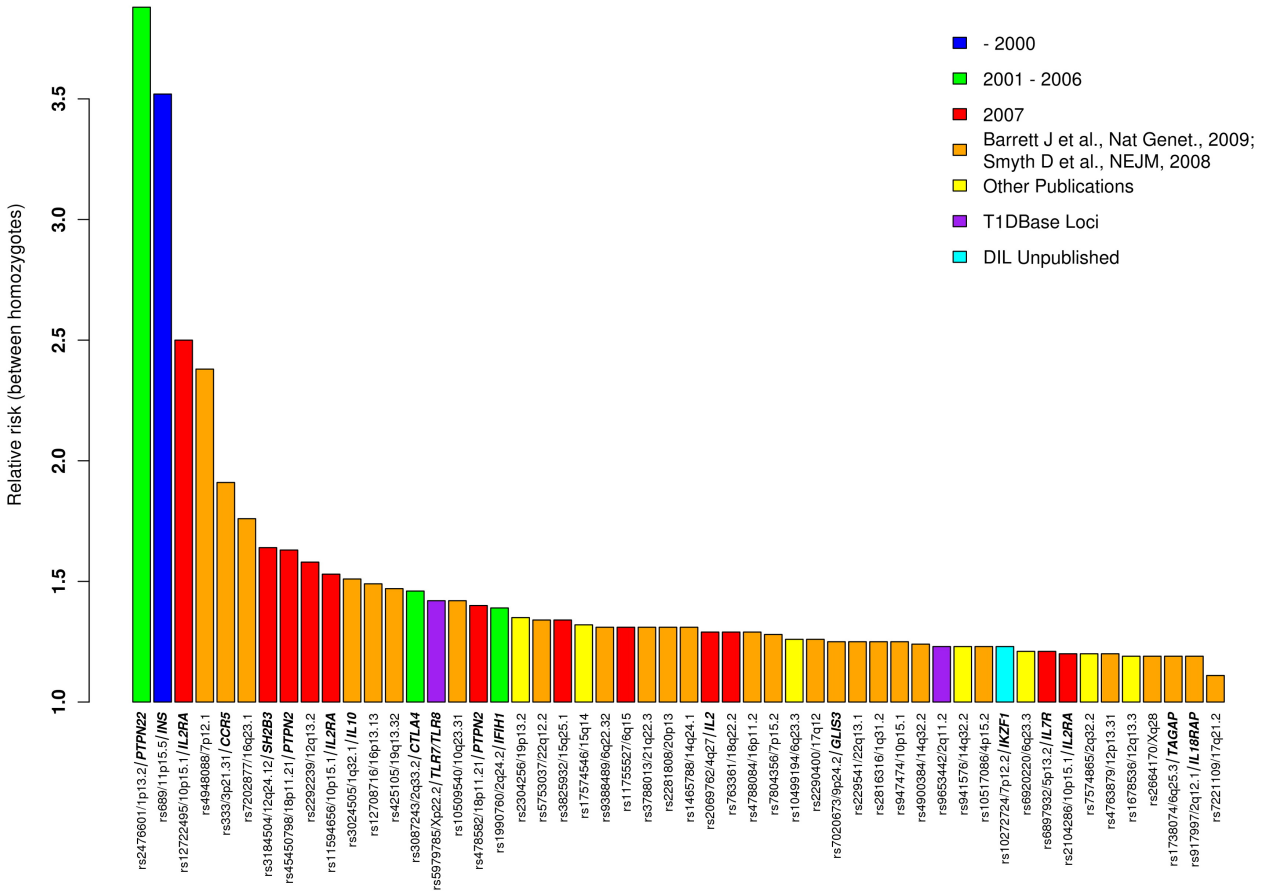

Figure 1 *Type 1 diabetes loci (SNP strongest associated with disease), their chromosome location, and, in 15 cases, the most likely causal gene (as per evidence from functional studies); (from Todd et. al. 2010[9])*

A number of genes affecting the predisposition to develop T1D have been identified (Figure 1). They are referred to as insulin-dependent diabetes mellitus (IDDM) susceptibility genes. Of far greatest importance (50% of susceptibility) is the major histocompatibility complex (MHC) HLA class II locus: depending on the haplotype it can have risk-increasing (DR3, DQ2 and DR4, DQ8 carrying the highest risk)[i] or protective effects[10, 11]. Further genetic determinants, are the gene for insulin (INS)[12], the protein tyrosine phosphatase (PTPN22)[13, 14] and the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)[15-17]. More recently genome-wide-association (GWA) studies were able to identify numerous other relevant loci (cf. Figure 1). Among these genes is the C-type lectin domain family 16, member A (Clec16a, previously known as KIAA0350) gene on chromosome 16p13.13[18, 19], the gene this thesis focuses on. Since an extensive de-

[i] DR3 = DQB1*0201, DR4 = DQB1*0302, DQ2 = DQA1*0501,DQB1*0201, DQ8= DQA1*0301,DQB1*0302

scription of all these loci and their presumed function would be beyond the scope of this introduction, only Clec16a will be considered with more detail in section 3.2.1.

Genetic alterations explain a large part of diabetes susceptibility. Still, studies of monozygotic twins only show concordance rates of about 50% with up to 30 years divergence in onset of clinical disease[20, 21]. Even though the first generation of migrants seems to exhibit the incidence rate of their country of origin[22, 23], figures for the second generation seem to converge to the incidence rate of the country of migration[24-26]. It further seems as if the recent rise in diabetes incidence is mainly attributable to haplotypes with a lower risk[27, 28]. All these findings point towards an involvement of environmental components in the pathogenesis of T1D. Nonetheless, the exact significance of environmental agents, and which exogenous trigger precisely matters, remains subject of controversy[29]. Numerous evidence highlights the role of viral infection[30], above all viruses from the Enterovirus family such as Coxsackie B Virus[31]. Intestinal flora[32] and nutritional aspects have also received extensive attention, yet their influence remains unclear[33]. Finally the hygiene hypothesis advances that the rise in allergic and auto-immune disorders observed in the last decades is at least partly attributable to better hygienic conditions and lower rates of intestinal and other infections during childhood. Early immune challenges are postulated to be immune-modulating (e.g. by inducing T regulatory cells[34]) and thus able to suppress both Th2 (e.g. asthma, atopy) and Th1 (e.g. T1D, psoriasis, rheumatoid arthritis) driven disorders[35, 36]. However, further research is warranted to identify possible underlying mechanisms[37].

1.1.2 Pathogenesis of T1D

Pathogenesis of T1D is still far from being fully understood, but there is certain consensus about key elements of this process[29, 38-40] (cf. also figure 2). Most findings have been derived from animal models, such as the Non-obese-diabetic (NOD) mouse or the Bio Breeding rat, which only partly reflect the complexity of human T1D (cf. also chapter 3.2.2). Nonetheless, given the ethical constraints of studies in human individuals, particularly in children, and the relative portability of results, they provide valuable insights[41].

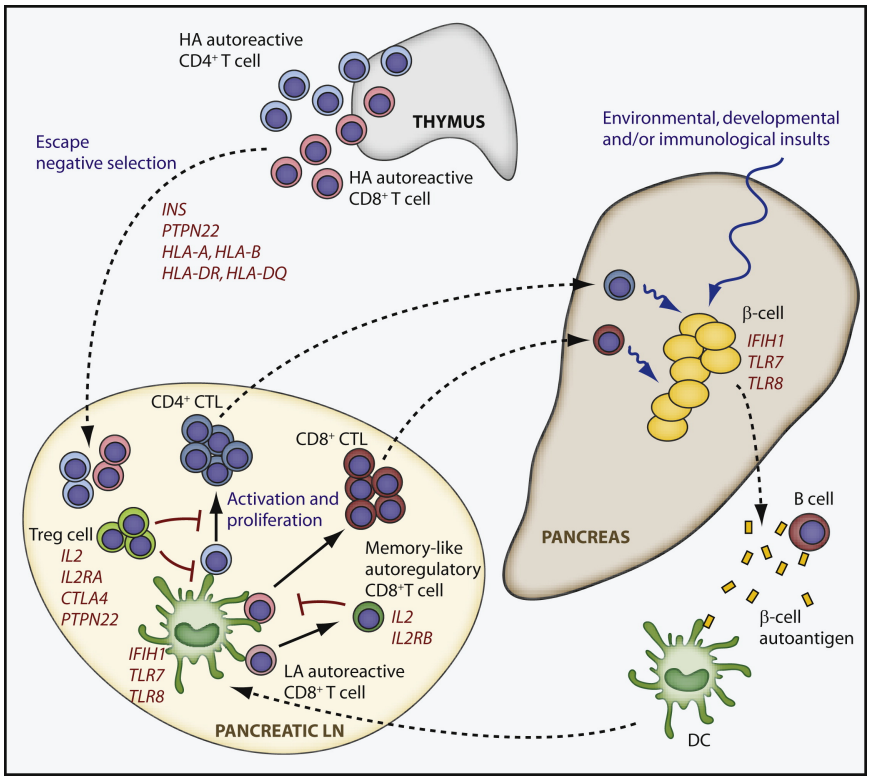

Figure 2 *Immune processes involved in the pathogenesis of T1D and role of genes associated with T1D (marked in red) (from Santamaria 2010[38])*

A leak in thymic negative selection permits high-avidity auto-reactive $CD4^+$ and $CD8^+$ T cells to survive and settle in peripheral lymphoid organs, among them pancreatic lymph nodes[25]. Different theories have been proposed to explain how auto-reactive cells escape negative selection: faulty or weak binding of certain epitopes to MHC[42-45], poor thymic expression of auto-antigen[46, 47] as well as tissue specific cleavage and post translational modifications[48].

A still unidentified β-cell insult (e.g. apoptosis[49], necrosis, autophagy or endoplasmatic reticulum stress) then leads to shedding of β-cell auto-antigen[38, 39], which is taken up by APCs such as dendritic cells (DCs)[50] or B cells[51, 52]. These migrate into pancreatic lymph nodes, where they present the antigen and aided by, $CD4^+$ T cells, IL-2 and other pro-inflammatory cytokines, activate the naive auto-reactive $CD8^+$ T cells[53-55]. Upon activation, auto-reactive T cells proliferate and develop into cytotoxic T lymphocytes (CTL) that return to pancreatic islets where they attack β-cells[56, 57]. T cell mediated β-cell destruction produces new antigen, which in turn fosters the generation of new specificities of $CD4^+$ and $CD8^+$ T cells in PLNs through epitope spreading and further fuels

aggression on islets[55, 58]. Leukocyte infiltrates of islets, also termed insulitis, are asynchronous, apparently intact islets existing alongside infiltrated and destroyed ones[57, 59].

Antigen presentation and pro-inflammatory mediators in pancreatic lymph nodes act on two further groups of immune cells. First they foster differentiation of B cells into plasma cells, which then release islet cell autoantibodies targeting insulin, insulinoma-associated antigen-2, glutamic acid decarboxylase 65 (GAD65) or Zinc Transporter 8[29]. This sero-conversion has long been described and presence and titre level of autoantibodies remain the best marker to predict future T1D manifestation (together with HLA susceptibility genes)[60, 61]. At the same time it continues to be unresolved whether autoantibodies are just a correlate of disease or actually contribute to pathogenesis[62]. In any case, the far more important role of B cells appears to be their highly efficient antigen presentation at disease onset[63, 64].

Second inflammation attracts FoxP3$^+$CD4$^+$CD25$^+$ T regulatory cells, which try to contain the autoimmune process by first expanding and being activated in PLN and then infiltrating the islets[60, 61]. Within islets, they interact with dendritic cells, the ultimate result being inhibition of CD4$^+$CD25$^-$ T effector cells[65-70]. However, in both NOD mice, as well as in humans, T effector cells exhibit increased resistance against T regulatory function[71, 72 73, 74]. Even though most studies find a normal total quantity and function of T regulatory cells in NOD mice[73, 75, 76] and human subjects[ii] with T1D[78, 79], the number of T regulatory cells within PLN and islets of NOD mice has been shown to decrease with age[80] or disease progression[65]. Some studies describe a functional impairment of T regulatory cells in PLN and islets (also in NOD mice)[81], particularly with increasing age[76]. These numerical or functional deficits in PLN and islets have repeatedly been attributed to reduced local levels of IL-2[65, 81-83]. Overall, even though the exact mechanisms remain to be determined, there is no doubt on the key role of T regulatory cells in disease progression in T1D[84, 85].

Eventually autoimmune processes surmount regulatory processes and once 70-90% of β-cells are destroyed, T1D manifests itself clinically with the typical signs of disturbed glucose homoeostasis: polyuria, polydipsia, weight loss, fatigue, abdominal pain[6, 86, 87]

[ii] As an exception, one study finds defects in the T regulatory function of T1D patients (relative to healthy controls)[77].

and especially in younger children, diabetic ketoacidosis[86, 88-90]. T1D treatment requires life-long insulin substitution: initiation of insulin therapy in 60% of patients results in a recovery of β-cell function, the so called "honeymoon phase"[91]. Two mechanisms are held accountable for this effect: on the one hand treatment diminishes insulin resistance caused by constant hyperglycaemia, on the other hand it relieves the stress chronic hyperglycaemia exerts on the few remaining vital β-cells[92, 93]. However this is only a transient phenomenon, and eventually residual insulin secretion will fall to very low levels or will entirely cease[93].

1.1.3 Management of T1D

Intensive treatment strategies and long term control of glycaemia (as reflected by HbA_{1c}) have proven superior in preventing long-term microvascular (neuro-, nephro- and retinopathy) and macrovascular (cardio-, cerebral- and peripheral vascular) complications[94-97]. These positive effects persist beyond the period of intensive treatment[98-100], which has promoted the concept of a so called "metabolic memory"[101]. Intensive treatment from the very beginning has proven so beneficial[94], that it is deemed to outweigh the associated increased risk of severe hypoglycaemia[94, 102, 103] and weight gain[104]. Still, therapeutic and behavioural strategies to lower the risk of the latter two are warranted[6, 99, 100].

With regard to preventive or curative therapies, no ground-braking success has been made so far. Prevention trials in high-risk patient groups have mainly relied on a desensitization approach administering oral, intranasal or sub cutaneous insulin[99, 105-110]. Desensitization aims to induce passive tolerance through anergy or removal of pathogenic T cells or to foster active tolerance via T regulatory cells. Efforts have concentrated on insulin since it is considered not only initiating antigen in NOD but also major antigen in human disease[39]. A second preventive strategy focuses on oral administration of nicotinamide[111, 112], which has previously been successful in animal models. Unfortunately neither of the two preventive approaches has proved effective. In intervention trials, desensitization strategies based on (pro-)insulin[113] and GAD[122, 123] have also been investigated. Even though initial results appear to be promising it is still too early to evaluate efficacy[29, 39]. Further intervention strategies have centred on immunosuppressive agents

such as cyclosporine A. Cyclosporine A impairs T effector and T regulator cell function by reducing the transcription of IL-2 via calcineurin inhibition. Despite success in delaying diabetes onset and stabilising β-cell function, no long-term effect of Cyclosporine A beyond treatment cessation could be confirmed. Furthermore rising concerns regarding nephrotoxicity forced discontinuation of trials[114-117]. The success of biological immune-modulators in other autoimmune diseases has prompted T1D intervention trials based on members of this substance group, particularly (FcR-non-binding) anti-CD3 monoclonal antibodies. In activated T effector cells these antibodies shift TCR mediated signal transduction towards anergy and apoptosis, preferably at sites with elevated T cell density such as in inflamed tissues, an effect that might persist beyond the direct application of the substance[118]. A single course of the anti-CD3 monoclonal Antibody (mAb) Otelixizumab succeeded in preventing loss of residual β-cell function up to two years, and reduced insulin requirements for up to 48 months[119, 120]. Teplizumab, a further anti-CD3 mAb, showed similar efficacy[121-123]. Despite this, patients remained insulin dependent throughout both studies. Furthermore safety concerns, which resulted in cessation of the Teplizumab trials, remain an important limitation[120, 123]. Numerous studies examining other immune-modulators are underway, they are reviewed in more detail elsewhere (e.g. Van Belle et al. 2011[29]).

1.2 Clec16a

Clec16a (previously termed KIAA0350) is a 233 kilo base (kb) linkage disequilibrium block that comprises 24 exons. It is located on Chromosome 16p13, in near proximity to three further genes involved in immune-regulation: the dexamethasone induced (DEXI) gene, the suppressor of cytokine signalling (SOCS1) gene and the major histocompatibility complex transactivator (CIITA) gene[18];

Genome wide association studies have established a link between Clec16a and susceptibility to numerous auto-immune disorders: not only T1D (described for the first time in 2007[18, 19, 124], later confirmed by numerous studies[125-132]), but also multiple sclerosis (MS)[128, 133-140], systemic lupus erythematosus[141], juvenile idiopathic arthritis[142], rheumatoid arthritis[138, 142], celiac disease[143], Crohn's disease[144], primary biliary cirrhosis[145], primal adrenal insufficiency (Addison's disease)[146] and alopecia areata[147]. Clec16a

SNPs associated with autoimmune disorders are largely non-coding, i.e. located in intronic regions (intron 19 and 22) of the gene, yet to date it has not been possible to detect any effect on regulatory elements. For the only coding variant in exon 23 (a non-synonymous change of one amino acid) no effect on T1D susceptibility could be proven[18].

Findings from microarray expression analysis show that mainly immune cells, in particular B cells, DCs and NKTs, express Clec16a[148]. Three splice variants have been proposed (two long and a short isoform, comprising 24, 21 and 4 exons respectively)[149]. Thus far, for whole blood samples it has not been possible to detect any significant SNP linked expression difference. This has been considered to suggest a cell type specific expression of Clec16a isoforms[150]. A trend towards a higher expression of Clec16a in a NK cell line homozygous for the T1D associated SNP rs2903692 has been described[18]. Yet hitherto only one significant correlation between a SNP and the level of expression of Clec16a has been established: expression levels of the two longer isoforms in thymic tissue samples (but not whole blood samples) correlate with a non-coding MS associated variant (rs12708716). This is also the SNP most strongly associated with T1D[150], and could hence indicate a link between the variant and thymic function. Yet a second analysis of the same samples did not confirm this result, but only found Clec16a SNPs to be associated to significantly lower SOCS and DEXI levels, which were in turn correlated to Clec16a expression[151, 152]. Similarly, Davison et al. (2012) found evidence supporting that T1D and MS associated Clec16a SNPs in intron 19 had a regulatory function on the DEXI gene by interacting with its promoter region[153]. It is hence not entirely clear whether the SNPs within Clec16a actually affect its own expression or rather that of neighbouring genes. However, the fact Clec16a lies within its own linkage disequilibrium block and that the linkage disequilibrium to the neighbouring candidate genes is low, renders it more likely that Clec16a itself is emitting the disease association signal[136]. In addition, DEXI is not significantly expressed in immune cells but mainly in liver, heart, lung and brain, which casts doubt on a possible immunoregulatory function of this gene[154]. Still, further research in this complex genetic region is needed to resolve these uncertainties.

Bioinformatical analysis predicts Clec16a to encode for a sugar-binding C-type lectin domain (CTLD)[18, 19]. Through their carbohydrate domain C-type lectins contribute to the internalization of antigen in DCs[154]. Furthermore self-recognition of NKT cells involves the expression of certain surface C-type lectins in order to interact with MHC I molecules[155]. It has been questioned whether the shortage of the CTL domain would actually allow for a carbohydrate recognition element, even though this would leave the possibility of recognition of other types of ligands such as lipids or proteins[156]. It is also important to note that none of the Clec16a orthologues share this domain[157]. The second structural domain discernible is an immuno-receptor tyrosine-based activation motif (ITAM)[154]. Since signal transduction downstream of C-type lectins is assumed to involve such ITAMs, it has been suggested that Clec16a could be important for signalling of immune-cells[156]. Two further structural domains are a potential trans-membrane region and an uncharacterised motif, termed FPL, which is highly conserved across species[157, 158].

Evidence on the function of Clec16a in humans is scarce. In contrast to this, the Clec16a orthologue in Drosophila melanogaster, ema (endosomal maturation defective) has been studied more extensively[159, 160]. Investigation of nephrocyte garland cells in ema deficient mutants revealed an expansion of the endosomal compartment due to an increased number and size of endosomal intermediates, as well as an absence of late endo- and lysosomes. Being localized in the endosomal membrane, a core function in maturation and trafficking of endosomes has been proposed for ema[159]. The same research group also unveiled that in fat body cells (as well as salivary gland, muscle and epithelial cells) ema localizes to autophagosomes. In these cells, while ema is not necessary for the maturation of autophagosomes (i.e. their fusion with endo- or lysosomes), by promoting the recruitment of Golgi vesicles to autophagosomes the gene is indispensable for the growth of the latter[160]. Since expression of the human orthologue Clec16a was able to rescue the otherwise lethal ema mutant and restored both endosomal maturation and trafficking in nephrocytic garland cells[159], as well as growth of autophagosomes in fat body cells[160], conservation of the function across orthologues has been advocated. However, of the structural domains previously mentioned, ema only shares the trans-membrane and the FPL domain, but neither the CTLD nor the ITAM sequence found in human Clec16a[156]. This is supported by recent studies in mouse fibroblast and islet cells

that identify Clec16a as membrane associated protein that is involved in late stages of autophagy. It appears to participate in the endo-lysosomal trafficking that results in the fusion of autophagosomes with the lysosomal compartment[161]. Such alterations in endosomal maturation and/or autophagosomal processes may both have implications for the function of the MHC II compartment, which in turn may affect the T cell repertoire, and hence provide a link to auto-immunity[162-166].

1.3 The NOD strain

One of the most important animal models for the study of T1D is the NOD mouse strain. It was established in the 1970s after inbreeding of the cataract-prone strain of the outbred Jcl:ICR line of mice[167]. 60-80% of females and 20-30% of male NOD mice spontaneously develop diabetes, even though both incidence and age of disease onset vary with local breeding conditions[168-170]. For example, a germ-reduced environment will result in a higher incidence rate[171, 172]. Clinical diabetes manifests itself at the age of 3-6 months, however mononuclear infiltrates in the surroundings of islets, termed peri-insulitis, can be already detected in nearly all mice at the age of 3-4 weeks[173, 174]. Subsequently, islets are invaded by immune cells ($CD4^+$- and $CD8^+$-T cells, B cells, DCs, NKCs and macrophages) resulting in severe insulitis at about 10 weeks of age (ibid.). Apart from T1D, NOD mice show a generally increased propensity to auto-immune disorders, such as autoimmune peripheral polyneuropathy[175], autoimmune thyroiditis[176], autoimmune sialadenitis[177], or the experimental induction of a disease resembling systemic lupus erythematosus[178].

Similarities between the NOD model and human T1D are numerous and comprise genetic and environmental factors affecting susceptibility, as well as pathomechanisms and markers of disease[179]. 6 of the 26 human susceptibility loci identified in GWAS have been confirmed to affect predisposition to diabetes in NOD mice[39]: among these shared loci are structural variants of the MHC II locus and IL-2 as well as genes affecting T cell regulation such as CTLA-4 and probably also PTPN22/PTPN8[29, 180]. Autoreactive $CD4^+$- and $CD8^+$-T cells directed against islet antigen are present at early stages of disease and play the dominant role in human as well as in murine disease[39]. A strong overlap of auto-antigens (principally insulin, glutamic acid decarboxylase and

zinc transporter 8) can be observed. In both cases pancreatic infiltrates in addition to T cells comprise B cells, macrophages and a small number of DCs[29].

At the same time several differences exists which call for some caution at the moment of transferring findings. NOD mice and humans share an important degree of genetic traits. Yet, it has been questioned whether the specific combination of a high-risk MHC and a large number of permissive background genes as found in the NOD strain can sufficiently represent the situation in humans[181]. Compared to humans disease onset in NOD mice is delayed[179], whereas disease progression and the degree of insulitis are far more pronounced and aggressive[29]. Inflammation even triggers β-cell proliferation[182] and leads to the formation of tertiary lymphoid structures in the pancreas[183]. As a further correlate of the more acute course of disease, nothing comparable to a "honeymoon phase" of β-cell function regeneration has been detected in NOD mice so far[29]. Gender-specific differences in disease onset and incidence exist in the NOD strain but not in humans. With a comparable degree of early insulitis in both male and female NOD mice at the age of 10 weeks, its has been advanced that this incidence difference might indicate a late regulatory event influencing disease progression[179]. Such a late checkpoint might be modulated by sex hormones (e.g. through an oestrogen mediated shift towards Th1)[184-189]. This would also explain the absence of gender-related incidence variations in humans, where disease onset usually occurs before puberty[179]. All these differences may to some extent account for the innumerable preventive and interventional therapeutic approaches that failed to prove effective in humans, despite having tested successful in the NOD model[29, 181, 190].

Despite these imperfections, it is generally acknowledged that the NOD strain remains a valuable model to improve our understanding of the pathophysiology of autoimmune diseases[179, 181, 190].

1.4 Lentiviral RNAi

Genome-wide association studies have proven to be a powerful tool to identify SNPs associated with an increased susceptibility for T1D. To be able to move from such a simple association to a truly causal relationship, it is necessary to validate results in functional studies and establish the underlying mechanisms[191]. This appears even more

warranted in the case of Clec16a, since the vast majority of disease associated SNPs is located in intronic regions of the gene and potential alternative candidate genes lie in close vicinity[18].

The traditional workhorses for this endeavour have been transgenic organisms generated through pronuclear injection of plasmid DNA into fertilized oocytes, so called knockout (or knock in) animals[192-194]. However transgenic knockout (KO) technology has a number of drawbacks[195-197]. First the gene variations found in GWAS are unlikely to be properly represented by a knockout. The likely severe consequences of such a complete loss-of-function combined with selective pressure, would render such a gene variant extremely rare[198, 199]. It is much more probable that instead the SNPs identified in GWAS only entail mild changes of gene expression, splicing or function. Likewise, if a gene has a core function, abrogating gene expression through the KO will not be reconcilable with life or will result in a premature death of the animal. This is particularly problematic in the context of autoimmune disorders since these require some time to develop. A further concern, of more technical nature, is that for the NOD strain a protocol to robustly obtain germ-line competent embryonic stem (ES) cells (based on small-molecule inhibitors) was not available until 2009[200]. However, the targeted mutagenesis of ES cells is crucial for the generation of NOD KO mice, and hence it has only been recently that the first (HLA-DM) KO NOD mice have been successfully bred[201]. An alternative to using NOD ES cells is inducing the KO within a standard strain and then backcrossing the resulting KO mice into the NOD genetic background. Yet, again the specificities of the NOD strain render such an endeavour problematic: with numerous risk loci and genetic variations contributing to the emergence of auto-immunity, the NOD strain is genetically very complex[179]. At the same time backcrossing is associated with a non-negligible risk of not only inserting the target gene but also other neighbouring gene variations[202]. These additional fragments may then confound the impact of the KO, as occurred in the case of IFN-γ signalling within the NOD strain[196]. Last but not least, the relatively low efficiency of the generation of transgenic KO mice a time-consuming and expensive endeavour[195, 197].

Given the above-mentioned limitations of KO technology, using RNA interference (RNAi) to induce a Clec16a knock down in the NOD mouse strain represents an attrac-

tive alternative. Gene knock-down based on RNAi exploits the principle that sequence-specific gene silencing can be induced by small interfering RNA (siRNA), a chemically synthesized 21 nucleotide long double strand RNA (dsRNA) molecule[203]. Analogous to endogenous mature miRNA, siRNA associates with the RNA induced silencing complex (RISC)[204]. Within this enzymatic complex the siRNA is unwound and its guide strand associates stably with the target mRNA[205, 206]; if the sequences of the siRNA and the target mRNA are identical[207], this is followed by enzymatic cleavage by Argonaute (the catalytic component of RISC) and subsequent degradation of the mRNA[206, 208]. However, due to a limited half-life of siRNA molecules and dilution effects at cell division, siRNA is unsuitable to achieve sustained gene-silencing in mammalian cells[209, 210]. This limitation is overcome by short hairpin RNA (shRNA) expressed from a promoter that is able to recruit RNA polymerase III[209, 210] or II[211-213]. ShRNA generated in this manner is converted into siRNA by Dicer, an RNAse III enzyme that usually processes pre-miRNA into mature miRNA[214].

One of the commonly used systems to deliver such shRNA into cells are replication-defective recombinant lentiviral vectors[195]. Lentiviruses are able to transduce even post-mitotic and non-dividing cells and subsequently integrate into the host genome[215]. For the generation of transgenic organisms such lentiviral vectors can be injected in the perivitelline space of fertilized oocytes[216-218]. The expression of the transgene from the lentivirus, and hence gene silencing, is maintained throughout the development of the organism until adult life and is inherited to future generations[216, 218]. It is thus possible to directly induce a gene's knock-down, even in those strains where conventional knock-out technology has proven challenging[219]. Additional flexibility can be achieved by tissue specific promoters, which ensure that transgenes are only expressed in a subset of tissues or organs[216, 220]. Inducible transgenes based on transcriptional activators or repressors bound by doxy- or tetracycline further permit a tighter control of gene silencing[221, 222].

Yet, a number of caveats have to be considered when using lentiviral RNAi[196, 197, 223]. Even though the gene silencing effect induced by RNAi is quite specific, so called off-target effects have been reported, these can be microRNA-like, operate through the immune system or through saturation of the RNAi machinery[224]. MicroRNA-like off target

effects describe the (about 1.5 to 4 fold) change in expression levels that can be induced in a target gene that exhibits a complementarity (of as few as 8 base pairs) with any of the two strands of the siRNA[225]. Such effects can result in measurable phenotypes[226-228] and may concern up to hundred genes. They preferentially affect those genes whose 3' untranslated region (UTR) is complementary to the 5' end of the siRNA guide strand[228, 229]. SiRNA can further induce a second type of off-target effects by activating the innate immune system either by eliciting the antiviral interferon response[230-234] or through toll-like receptors (TLRs) on dendritic cells[235-237]. Third, particularly shRNA may constrain the endogenous activity of micro RNA (miRNA) through saturation of certain enzymes that are used both for transport and processing of shRNA and miRNA[238]. To some extent these off-target effects can be mitigated e.g. by avoiding certain pro-inflammatory sequences or by specific chemical modifications[224]. Yet none of these measures can entirely eliminate them. Moreover, insertional effects can trigger additional unspecific impacts of RNAi: endogenous genetic elements may be disrupted by the untargeted integration of the lentiviral vector[196, 239]. To more confidently rule out that the phenotype observed is not the result of unspecific (off-target or insertional) siRNA effects but instead can be ascribed to gene knock down, two transgenic lines based on independent shRNA sequences need to be generated[240-242].

A further concern results from position-effect variegation: depending the integration site, the transgene may be subject to epigenetic regulatory mechanisms[219, 243, 244]. These may result in varying levels of expression of the transgene (and hence gene silencing) both within a specific cell type and between cell lineages[244, 245]. Achieving a stable and uniform pancellular expression of the transgene can hence prove challenging. Targeted transgenesis of ES cells, instead of lentiviral vector systems, are one remedy for this problem: it allows to choose an optimal insertion locus, which simultaneously reduces concerns from insertional effects[246]. Alternatively, improved lentiviral vector backbones have shown to successfully diminish variegation effects while enhancing the efficiency of transgenesis[195, 196, 221].

Finally, while RNAi allows to reduce gene expression in a flexible, transient and gradual manner, complete gene silencing cannot be achieved[247]. As previously noted, the

former may be a more realistic reflection of physiological processes, yet in some settings a full loss-of-function may be indispensable to study the role of a gene.

Given that adequate controls can alleviate an important share of the just discussed limitations, the use of RNA interference to study the impact of Clecl6a silencing in the NOD strain overall seems a feasible approach.

1.5 Immune regulation

The mammalian immune system is a highly flexible entity that has the ability to recognize, react to and neutralize almost any chemical structure that enters the body. Most of this flexibility is owed to the B cell and T cell receptors (BCR and TCR) that two of the main actors of the adaptive immune system, B cells and T cells express on their surface. The extreme coding diversity of these receptors is achieved with two different mechanisms of somatic genome modification. First, when T and B cells differentiate in the thymus and bone marrow, respectively (central lymphoid tissues), BCR and TCR genes are generated by combinatorial joining of (up to) three gene segments: the variable (V), diversity (D) and joining (J) genes. Second, once B cells have arrived in lymph, nodes, spleen or tonsils (peripheral lymphoid tissues), e.g. as part of an immune response, single nucleotides of BCR genes can be exchanged, a process termed somatic hypermutation. However this variability comes at a certain cost: significant shares of the thus assembled receptors either are not able to interact with peptide-MHC (pMHC) complexes at all, or bind self-antigen with high affinity. Several mechanisms that ensure functionality and self-tolerance of the T and B cell repertoire are thus in place[248, 249]. These processes occur at two distinct levels. First, during B and T cell selection and development in bone marrow and thymus (central tolerance). Second, once mature B and T cells have found their way to lymphoid and non-lymphoid organs (peripheral tolerance). In the following these two stages will be described in more detail, in doing so the main focus will be on T cells.

1.5.1 Central tolerance

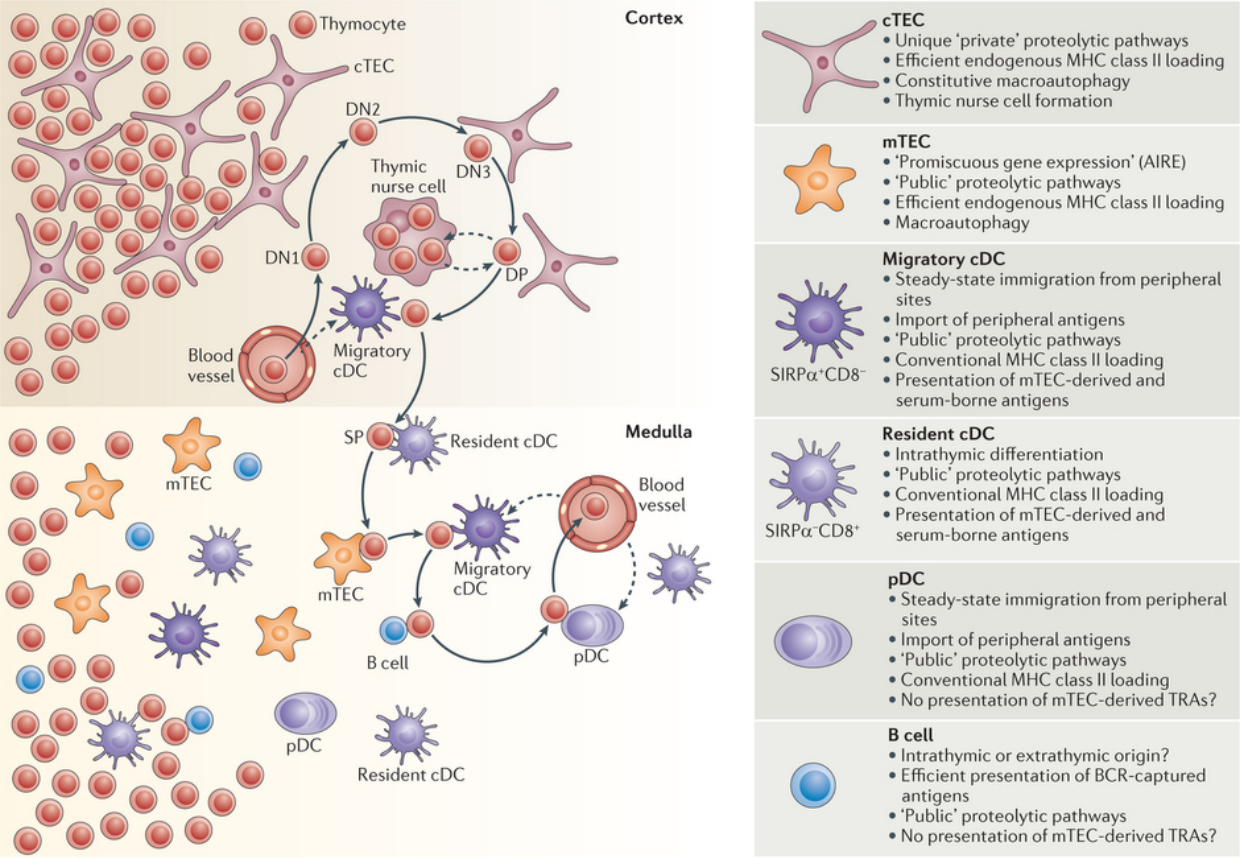

Figure 3 *Interactions between thymic stromal cells and T cells during T cell development (from Klein et al. 2014[250])*

Central tolerance of T cells is largely ensured within the thymus (cf. Figure 3): every day between 10 and 100 hematopoietic precursor cells reach the thymus through the bloodstream. Cells commit to the T cell lineage and proliferate during two weeks (mainly as $CD4^-CD8^-$ double negative T cells)[251]. During this process they move from the centre of the thymus towards the sub-capsular zone[252]. The thus generated 5 x 10^7 naive thymocytes per day then rearrange their TCR α and β-locus. Upon successful rearrangement they pass β-selection and reach the $CD4^+CD8^+$ double positive (DP) stage. Only 10% of cells are then "positively selected" while they wander through the outer cortex of the thymus: i.e. they have interactions (most likely a lower intermediate affinity and/or avidity level) with self-pMHC complexes on cortical thymic epithelial cells (cTECs) that result in their differentiation to single positive (SP) $CD4^+$ or $CD8^+$ lineage committed T cells[253-255]. In absence of such interactions (i.e. the remaining 90%), DP cells are subject to programmed cell death, they "die from neglect" after 3-4 days[256].

Positive selection is generally believed to imprint self-MHC restriction[251, 257], even though this view has been challenged by some studies[258].

Thanks to unique antigen processing pathways cTECs are able to display a repertoire of peptides on their MHC receptors (pMHC-ligandome), which is systematically different from other APCs. These distinct machineries include: a specific type of proteasome, the "thymoproteasome", which may be crucial for $CD8^+$ lineage commitment[259]; two lysosomal proteases (Cathepsin L and thymus-specific serine protease), that seem to ensure polyclonality and adequate positive selection of $CD4^+$ T cells[260, 261]; further constitutive macroautophagy, where a portion of cytoplasm is sequestered and an autophagosome (a membrane-limited compartment) formed, which subsequently fuses with a lysosome to allow degradation of its content. This process enables the endogenous (and hence "unconventional") loading of MHC II molecules, appears to shape the MHC II ligandome of cTECs, and thus to influence positive selection of $CD4^+$ T cells[262, 263]. Multiple competing theories regarding the purpose of this specific machinery have been put forward, achieving a very diverse and versatile repertoire of T cells could be one of them[250], however this question remains open to future research.

Positively selected SP cells migrate from the cortex to the medulla. In the medulla again they wander around for about 4-5 days while frequently interacting with APCs such as medullary thymic epithelial cells (mTECs) or different types of dendritic cells (DCs)[264, 265]. Medullary TECs are known to engage in 'promiscuous' or 'ectopic' gene expression: under the control of the autoimmune regulator (AIRE) protein they express antigens that are otherwise restricted to specific tissues (tissue restricted antigens or TRAs)[266-269]. TRAs are then presented on the surface of mTECs themselves, similar to cTECs they appear to use macroautophagy to accomplish lysosomal degradation of cytoplasmic constituents for presentation on MHC II receptors[164, 165, 270]. TRAs can also be handed over to nearby APCs for cross-presentation[251, 252]. If these self-peptide-MHC complexes on APCs bind too strongly to a TCR, this results in subsequent apoptosis of the corresponding thymocyte (negative selection)[252, 257]. Since almost all transcripts found in peripheral tissues are expressed somewhere in the medulla, and numerous contacts between thymocytes and APCs take place, this mechanism efficiently induces tolerance of $CD4^+$ and $CD8^+$ T cells[271-275]. This pathway may explain the association between some

genetic polymorphisms and an increased susceptibility to auto-immunity[250]. Diabetes associated SNPs in regulatory regions of the Insulin leave expression within the organ itself unaffected but have small but notable effects for expression levels within the thymus[12, 276, 277]. Such lower expression levels then may result in an inefficient negative selection of the respective auto-reactive thymocytes[277].

Of note, recent evidence seems to suggest that a non-negligible share of DP cells appears to be subject to negative selection already in the cortex: thymocytes whose TCR exhibits a high affinity for self-antigen displayed on cortical dendritic cells or cTECs undergo deletion[278, 279].

1.5.2 Peripheral tolerance

Negative selection within the thymus is relatively efficient at eliminating auto-reactive thymocytes. However, those T cells for which the strength of the interaction with self-peptide MHC complexes is too low to be deleted by negative selection and auto-reactive T cells that never encounter an APC that displays their self-antigen still manage to leak into the periphery[248, 249]. A range of additional immune system mechanisms are thus in place to preserve peripheral tolerance: immunological ignorance, peripheral deletion, T cell anergy and co-signalling, tolerogenic dendritic cells and $FOXP3^+$ T regulatory T cells[248, 280]. Traditionally the first three have been considered to be T cell intrinsic processes and the latter two T cell extrinsic. However recent evidence highlighting the complexity of T cell co-signalling may imply the lines between these two categories are much more blurred[280].

A relatively simple mechanism of peripheral tolerance is the physical separation of (potentially self-reactive) T cells and parenchymal tissues: T cells never encounter self-antigen MHC complexes, are thus immunologically "ignorant" of the existence of these[248]. Migration patterns of naive $CCR7^+$ $CD62L^+$ T cells are set up in a way that ensures that under normal circumstances T cells are not able to enter non-lymphoid peripheral tissues[281]. This changes upon first contact with self-pMHC. Cells thus differentiated into effector memory T cells are able to transit from blood into the interstitium of parenchymal organs, even more so in case of a local inflammation or infection[282-284].

Secondly, peripheral T cells constantly exposed to self-antigen MHC complexes are eliminated by apoptosis: either through a mitochondrial mechanism (pro-apoptotic BIM protein outweighs pro-survival BCL-2) or triggered by FAS/FAS-L signalling which results in activation induced cell death (AICD)[285-288]. Naive T cells mainly seem to die through the former pathway[289]. Interruption of any of the two pathways results in manifestations of auto-immunity in mice, which suggests they are relevantly engaged in maintaining peripheral tolerance[248].

Third, a pivotal role for self-tolerance accrues to the multiple co-stimulatory and co-inhibitory receptors expressed by thymocytes in addition to the TCR[290, 291]. These co-receptors control the outcome of TCR signalling, and are thus are critical for function and fate of T cells[292].

Initial activation of naive T cells ('priming') in addition to antigen recognition through the TCR requires co-stimulation of CD28 (usually through the B7 proteins CD80/CD86 on APCs)[293]. This promotes IL-2 and Bcl-x_L gene transcription and activates the AKT-mTOR pathway such that cell growth, proliferation and survival are enhanced[290]. Absent or sub-threshold stimulation of CD28 results in T cell anergy, a state of generalized inhibition of cell responsiveness, effector function and proliferation[294, 295]. In anergic T cells the IL-2 locus is actively repressed, the TCR/CD3 complex degraded, and the cell metabolism cannot be up-regulated[296]. Similar effects also seem to be induced by suboptimal TCR stimulation[295].

TCR engagements accompanied by co-inhibitory signals such as ligation of PD-1 or CTLA-4 negatively affect T cell activation, proliferation, effector function and cytokine production (IL-2) and can thus induce T cell anergy[297]. CTLA-4 and PD-1 co-stimulation also preclude ‘stop signals’ that are necessary for T cell extravasation into peripheral organs and tissue migration and thus inhibits productive TCR engagements[298, 299]. PD-1 further impairs T cell survival by inhibiting Bcl-x_L gene transcription[300]. A main ligand of PD-1, PD-L1 is constitutively expressed on non-hematopoietic cells in peripheral tissues (e.g. vascular endothelial cells, pancreatic islets)[301]. Blocking the PD-1/PD-L1 pathway results in organ-specific auto-immunity[302-304]. Thus signals transmitted via the PD-1/PD-L1 complex appear to be crucial to preserve peripheral tolerance and prevent that self-reactive T cells cause peripheral tissue

damage[280, 290]. While PD-1 is pivotal to maintain tolerance in peripheral organs, CTLA-4's foremost role seems to lie in limiting the T cell response in early stages of immunity and still within the peripheral lymphatic system[298]. As a structural homolog of CD28, CTLA-4 binds CD80/CD86 with a higher avidity[305, 306]. Yet, other than the constitutively expressed CD28, its is only up-regulated in case of T cell activation and is then able to outcompete CD28 signalling[307, 308]. Upon binding with CD80/86 it further interferes directly with intracellular signals downstream of TCR/CD28[309-311]. The finding that CTLA-4 deficient mice develop lethal post-thymic lymphproliferative disease and auto-immune disorders of the pancreas and heart highlights its importance for effective T cell regulation[312-314]. CTLA-4 has also been identified as susceptibility gene for T1D and several other auto-immune diseases[315, 316]. However, given that PD-1 and CTLA-4 receptors are not only expressed on T effector cells but also on $FOXP3^+$ T regulatory cells, the latter type of cells is likely to convey some of their tolerogenic effects[317].

Fourth, the 10% of peripheral $CD4^+$ T cells that are forkhead box P3 positive ($FOXP3^+$) hold a crucial role for peripheral immune tolerance and homeostasis. Most of these regulatory T cells (except for the so called induced $FOXP3^+$ T regulatory cells) are thymus derived, with a level of TCR self-reactivity too high for positive selection but too low for negative selection (either as self-affinity and/or per cell avidity) as a potential selecting signal[318-320]. In $CD4^+$ SP T Cells with intermediate self-reactivity of the TCR, upon interaction with thymic APC's [iii] self-peptide MHC class II complexes and co-stimulatory molecules (CD80/ CD86), the $FOXP3^+$ gene is induced (ibid.). Even though $FOXP3^+$ genes repress IL-2 and IFN-γ expression[321] for differentiation (and survival) of the $CD4^+CD25^+FOXP3^+$ T regulatory cells, the cytokine IL-2[iv] is indispensable (IL-7 and IL-15, may be relevant as well)[83, 322-326]. In the periphery, the maintenance of T regulatory cells further depends on reencounter of their cognate self-antigen[327-329]. The lymph nodes that drain tissues expressing their self-selecting self-antigen are thus their preferential location[330]. This mechanism may ensure the dynamic response of T regulatory cells in case of release of self-antigen, e.g. due to an injury, and so prevent auto-immunity[318].

[iii] mTECs and/ or bone marrow-derived APCs

[iv] the CD25 molecule is also the IL-2 receptor (IL-2R)

T regulatory cells extrinsically modulate immune responses both through direct interactions with T effector cells or APCs and by producing soluble immunosuppressing cytokines such as IL-10, IL-35 and transforming growth factor-b (TGF-b)[320, 321, 331-333]. Under exposition to antigen, they gather around APCs to prevent physical access and priming of T effector cells[334]. T regulatory cells are also the only cell type to constitutively express CTLA-4 (TCR stimuli result in further upregulation)[335, 336]. CTLA-4 mediated suppression has thus been proposed as a core mechanism of T regulatory cell function[320, 337]. CTLA-4 reduces the expression of CD80 and CD86 on immature and mature DCs[334, 338-340]: either through induction of genetic repression (via Foxo3)[341], trans-endocytosis followed by degradation within regulatory T cells[342] or potentially also trogocytosis [v] (a process of cell-contact dependent transfer of membrane fragments)[337, 344]. Interaction between CTLA-4 on regulatory cells and CD80/86 on dendritic cells further enhances the indoleamine-2,3-dioxygenase (IDO) pathway in DCs, Kynurenin production and hence promotes an immunosuppressive milieu[345]. The importance of T regulatory cells for immune homeostasis, is also reflected in the observation that humans[346] (and "scurfy" mice[347]) with defects in the FOXP3 gene develop severe multi-organ autoimmune disease. In humans this defect has been subsumed under the term IPEX syndrome (immuno-dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome)[346]. Likewise genetic polymorphisms of IL-2RA, the gene coding for the high affinity α-chain of the three chain IL-2 receptor seem to be associated with T1D susceptibility, potentially because of a resulting lower IL-2 secretion and reduced numbers and/or impaired function of T regulatory cells[348].

Tolerogenic dendritic cells are a second group of cells involved in T cell extrinsic mechanisms of immune regulation[349]. Immature dendritic cells constantly engage in uptake and processing of antigen[350]. If confronted with necrosis, mechanical trauma, pro-inflammatory cytokines or micro-biotical or viral stimuli to their Toll-like-receptors (TLR) these cells mature to immunogenic dendritic cells by up-regulating their MHC II receptors and CD80/ CD86 co-stimulatory molecules (ibid.). They then migrate to lymph nodes where they very efficiently activate T cells (ibid.). Instead when confront-

[v] However, a recent study[343] casts some doubts on whether CTLA-4 is actually involved in trogocytosis of CD80/CD86 between APCs and induced T regulatory cells.

ed with apoptotic cell material, dead or dying cells and in absence of inflammation dendritic cells mature in an incomplete manner (e.g. downregulate their TLRs) and evolve into tolerogenic APCs[351, 352]. Such tolerogenic APCs are then able to functionally inactivate (through anergy induction) or physically eliminate auto-reactive T cells that would otherwise have been activated by self-antigen[353].

In summary, preservation of self-tolerance through central and peripheral mechanism is a complex and intertwined process that is usually successful. Yet, there are cases where failure of one or several of these pathways occurs and auto-immune disorders emerges.

2 Aims

Numerous studies have shown an association between certain Clec16a SNPs and auto-immune diseases. Expression of the gene seems to be elevated in immune cells such as B cells, dendritic cells and natural killer cells and bioinformatical analysis suggests a role in ligand recognition and immune cell signalling. The drosophila orthologue of Clec16a *ema* has been shown to be involved in endosomal or autophagosomal processes, but there is not much known about the function of the gene in mammals. To explore the role of Clec16a in auto-immunity we down-regulated the gene in NOD mice by means of a lentiviral RNA interference. We therefore aim to better understand how the immune system in general and T1D development in particular will be affected by lower levels of Clec16a expression.

3 Material

3.1 Antibodies

3.1.1 Cell culture

Antigen	Clone	Concentration	Supplier
CD28	37.51	1 mg/ml	eBioscience
CD3ε	145-2C11	0,5 mg/ml	eBioscience
CD40	HM40-3	0,2 mg/ml	eBioscience
rmGM-CSF		200U/ ml	a gift by AG Prof. Lutz

3.1.2 Flow cytometry

Antigen	Fluorochrome	Clone	Dilution	Supplier
Annexin V	APC		1:100	BD
Annexin V	PE		1:100	BD
B220 (CD45R)	APC	RA3-6B2	1:300	BD
B220 (CD45R)	PE	RA3-6B2	1:300	BD
B220 (CD45R)	APC eFluor 780	RA3-6B2	1:400	eBioscience
CD11b	PE	MI/70		BD
CD11b	PE-Cy7	MI/70	1:800	BD
CD11b	eFluor 450	MI/70	1:1600	eBioscience
CD11c	APC	HL3	1:300	BD
CD11c	PE-Cy7	HL3	1:1600	BD
CD11c	eFluor 450	N418	1:200	eBioscience
CD16/32 (FcgR3/2)	APC	93		eBioscience
CD19	eFluor 450	1D3	1:1600	eBioscience
CD25	PE-Cy7	PC61		BD
CD25	PerCP-Cy5.5	PC61		BioLegend
CD25	APC	PC61.5		eBioscience
CD25	PerCP-Cy5.5	PC61.5	1:300	eBioscience
CD3ε	V500	500A2	1:800	BD
CD4	Alexa 647	RM4-5		BD
CD4	APC-Cy7	GK1.5	1:800	BD
CD4	PerCP-Cy5.5	RM4-5	1:800	BD
CD4	V500	RM4-5	1:1600	BD
CD4	APC	RM4-5	1:800	eBioscience
CD40L (CD154)	PE	MR1		BD
CD44	PE-Cy5	IM7	1:3000	BD

CD45	eFluor 450			
CD45RB	PE	C36.16A	1:1600	eBioscience
CD5	PE	53-7.3	1:300	BD
CD62L	PE	MEL-14	1:200	BD
CD62L	APC-Cy7	MEL-14		BioLegend
CD69	PE-Cy7	H1.2F3	1:300	eBioscience
CD80	APC	16-10A1	1:1600	eBioscience
CD86	APC	GL1	1:1600	eBioscience
CD86	PE	GL1	1:1600	eBioscience
CD8α	APC	53-6.7	1:800	BD
CD8α	PE	53-6.7	1:800	BD
CD8α	PE-Cy7	53-6.7	1:800	BD
CD8α	eFluor 450	53-6.7	1:1600	eBioscience
CTLA-4 (CD152)	PE	UC10-4F10-11	Intracellular: 1 µg	BD
CTLA-4 (CD152)	PE	UC10-4B9	Intracellular: 1 µg	BD
EpCAM	PE-Cy7	G8.8	1:3200	eBioscience
F4/80	PE-Cy5	BM8	1:400	eBioscience
F4/80	APC	BM8		eBioscience
GITR	APC	DTA-1	1:300	eBioscience
GITR-L	Biotin	eBio YGL386		eBioscience
Gr-1 (Ly 6G/C)	PE	RB6-8C5		BD
IgM	PE-Cy7	R6-60.2	1:200	BD
Ly51 (BP-1)	PE	6C3	1:800	eBioscience
PI	Purified/FG		1 µl/ $1x10^6$ cells	BD
RTIB (I-Ag7)	PerCP	OX-6	1:800	BD
TCR Vβ 8.3	PE	8C1	1:50	BioLegend
TCR Vβ 11	PE	RR3-15	1:100	BioLegend
TCR Vβ 12	PE	MR11-1	1:400	BioLegend
TCR Vβ 13	PE	MR12-4	1:50	BioLegend
TCR Vβ 2	PE	B20.6	1:50	BioLegend
TCR Vβ 5.1, 5.2	PE	MR9-4	1:400	BioLegend
TCR Vβ 6	PE	RR4-7	1:100	BioLegend
TCR Vβ 7	PE	TR310	1:100	BioLegend
TCR Vβ 8.1, 8.2	PE	KJ16-133.18	1:100	BioLegend
TCR Vβ 9	PE	MR10-2	1:400	BioLegend
TCR β	APC	H57-597	1:300	BD
TCR β	APC eFluor 780	H57-597		eBioscience
TCR β	PerCP-Cy5.5	H57-597	1:300	eBioscience

3.2 Biological Material

3.2.1 Mouse lines

Line	Genotype	Supplier
C57BL/6	WT	a gift from AG Zernecke
C57BL/6	Clec16 KD #3	were bred in house
NOD	WT	Taconic
NOD	Clec16 KD #3	were bred in house
NOD.SCID	WT	Taconic

3.3 Buffers and Media

3.3.1 Cell Culture

3.3.1.1 Lymphocyte Medium RPMI10:

- 10% (v/v) Fetal calf serum (FCS, Invitrogen)
- 2 mM L-Glutamin (Invitrogen)
- 10 mM HEPES (Invitrogen) 1M
- 50 U/µg /ml Penicillin / Streptomycin (Invitrogen)
- 55 µM ß-Mercaptoethanol (Invitrogen)
- 1 mM Sodium Pyrovate (Invitrogen)

in RPMI 1640 (Roswell Park Memorial Institute medium, Invitrogen)

3.3.2 Cell separation and flow cytometry

3.3.2.1 ACK lysis buffer:

- 1g KHCO3
- 8,29 g NH_4Cl
- 37,3 mg Na_2EDTA

ad 1l dH_2O

pH 7,2 – 7,4

3.3.2.2 Annexin binding buffer:

- 10 mM HEPES
- 1,8 mM $CaCl_2$
- 5 mM KCl
- 1mM $MgCl_2$
- 50 mM NaCl

ad dH_2O

pH 7,4 (NaOH)

3.3.2.3 Dynabead separation buffer:

- 0,1% BSA
- 2mM EDTA

ad PBS pH 7,2

filter sterile

3.3.2.4 MACS buffer:

- 0,5% FCS
- 2mM EDTA

ad PBS pH 7,2

filter sterile

3.4 Chemicals

Chemical	Supplier
Acetic acid glacial	Roth
ß-Mercaptoethanol	Roth
BSA (Albumin Fraction V)	Sigma
$CaCl_2$	Roth
Cyclophosphamide monohydrate	Sigma
DMSO	AppliChem
Ethanol >99,5% and denatured	Roth
EDTA	Roth
Glycerol	Roth
Glycine	Roth
HEPES	Applichem

Hydrochloric acid 37%	Roth
^{3}H-Thymidine	Hartmann analytic
Isofluran	cp-pharma
Isopropanol	Roth
Ionomycin	Sigma
KCl	Roth
$KHCO_3$	Roth
Methanol	Roth
$MgCl_2$	Roth
NaCl	Roth
Na_2EDTA	Roth
$NaHCO_3$	Sigma
$Na_2HPO_4x2H_2O$	Roth
NaOH	Roth
NH_4Cl	Roth
PFA reagent grade	Sigma
PMA	Sigma
Sodium azide	Roth

3.5 Consumable supplies

Consumable	Supplier
Cell culture plates 96 well (Flat, U, V bottom)	BD
Cell culture plates 24 well	BD
Cell culture plates 6 well	BD
Cell culture plates 10 cm	BD
Cell culture plates 10 cm	BD
Cell culture plates 15 cm	BD
Cover slips	Hartenstein
Cryotubes	Hartenstein
Diastix	Roche
Filter mats	PerkinElmer
Filter sterile 0,45µm	VWR
Glass ware	VWR
Glass slides	Hartenstein
Gloves	VWR
Injection Needles	Hartenstein
Magnets	VWR
Microseals for reaction plates	Applied Biosystem
Nitrocellulose	BioRad
Parafilm	Pechiney
Pipettes (5ml, 10ml, 25 ml)	VWR

Reaction plates 96 well white	VWR
Reaction plates 384 well clear optical	Applied Biosystem
Reaction tube 1,5 ml	Starlab
Reaction tube 15ml / 50 ml	BD
Reaction tube round bottom	BD
Syringes	VWR
Tips	TipOne, Starlab
Ultracentrifuge tubes	Beckman
Whatman paper	VWR

3.6 Enzymes

3.6.1 Cell culture Reagents and Collagenases

Reagent	Properties	Supplier
Collagenase D	Collagenase	Roche
Dispase I	Collagenase	Roche
eFluor 670	Proliferation dye	eBiosience
FCS	Fetal calf serum	Invitrogen
HEPES	Cell medium supplement	Invitrogen
L-Glutamine	Cell medium supplement	Invitrogen
Liberase Blendzyme II	Collagenase	Roche
LPS	Lipopolysaccharide	Sigma
Penicillin /Streptomycin	Antibiotics	Invitrogen
Snarf	Proliferation dye	Invitrogen
Sodium Pyrovate	Cell medium supplement	Invitrogen
TGFß	Transforming growth factor	R&D
Trypan Blue Stain	Cell dye	Invitrogen
Trypsin	Serine protease	Invitrogen

3.7 Equipment

Device	Supplier
Agarose Gel chamber	BioRad
Beta counter MicroBeta$_2$	PerkinElmer
Centrifuge MiniStar	VWR
Centrifuge 5810R	Eppendorf
Centrifuge 5424	Eppendorf

FACS Aria II	BD
Flow Cytometer FACS Canto	BD
Freezer / Fridge	Liebherr
Freezer -80°C Hera Freeze	Thermo
Gel Imager	Herolab
Harvester	PerkinElmer
Heating plate	Heidolph
Hemacytometer	Hartenstein
Homogenizer Polytron	Kinematica
Incubator Bacteria	Thermo Electron Corporation
Incubator Cell culture	Thermo Electron Corporation
Laminar Flow	Luft & Reinraumtechnik GmbH
Luminometer	bMG Labtech
Microscope Axiovert 40CFC	Zeiss
Microwave	Bomann
pH Meter	Mettler Toledo
Photometer	Implen
Pipettes (P2, 10, 200, 1000)	Gilson
Pipetus	Hirschmann Laborgeräte
Power Supply Unit	BioRad
Rocker Switch	Heidolph
Scale	Kern & Sohn GmbH
Scale (special accuracy)	Mettler Toledo
Thermoblock	Eppendorf
Thermocycler PXE 0.2	Thermo Electron Corporation
Ultracentrifuge	Beckman Coulter
Vaccuum pump	VWR
Vortex Genie 2	Scientific Industries
Waterbath	Julabo

3.8 Kits

Kit	Supplier
Cytometric Bead Array Flex Sets	BD
MACS Separation Kit CD4	Miltenyi
MACS Separation Kit CD4+ CD25+	Miltenyi
MACS Separation Kit CD43	Miltenyi
MACS Separation Kit Pan T cells	Miltenyi

3.9 Software

Software/Website	Supplier/ Web Address
ApE – A plasmid editor	M.Wayne Davis
Bio Gene Portal System	Biogps.org
Ensembl Genome Browser	www.ensembl.org
Expasy Prosite	prosite.expasy.org
FACS Diva	BD
FlowJo 9.3.2	Treestar Inc.
Mac OS X 10.6.8	Apple Inc.
Microsoft Office for Mac 2011	Microsoft Corporation
National Center for Biotechnology Information	www.ncbi.nlm.nih.gov
Prism	Graph Pad

4 Methods

4.1 Cell biological methods

4.1.1 Evaluation of cell density

A Neubauer haemocytometer was used to evaluate cell density. After re-suspending cells, trypan blue was added (usually in a 1:10 ratio). Only dead cells being permeable for this dye it hence permits to discriminate between cells that are dead and those who are alive. Per definition a volume of 0,1 µl fits into one quadrant of the haemocytometer. Having counted the number of cells in one quadrant (usually the average of four quadrants was calculated to reduce any statistical error) it is possible to deduct the cell concentration of the suspension using the following formula:

$$D = \frac{NV10^4}{Q}$$

with: D= concentration of cells per ml; N=number of cells counted; V=dilution factor of cells (usually 10), Q=number of quadrants counted;

4.1.2 Flow cytometry

Flow cytometry is a technique that allows the analysis of multiple parameters of individual cells within heterogeneous populations. These parameters include cell number, size, granularity, expression of fluorescent proteins like GFP, and presence of specific antigens (after staining with fluorescently labelled antibodies). Different scattering and refraction of light by cells is the underlying principle of this method. This is only possible if cells are in suspension, which requires homogenizing solid organs. A hydrodynamically focused stream allows cells to pass one by one through the interrogation point. Here a laser beam is directed on to the stream and several detectors at different angles collect the light reflected by cells. Information obtained is of two different types. On the one hand scattering gives information on the general characteristics of a cell: the forward scattered light (detector placed parallel to the laser beam) is relative to the size of the cell; the sideward scattered light (detector placed in 90° of the laser beam) re-

flects granularity and complexity of structures inside the cell. On the other hand fluorescence is emitted when a fluorophore (a molecule with a structure that allows excitement by a certain wavelength like GFP or fluorescently labelled antibodies) returns to its ground state after having been propelled on to a higher level.

We used BD FACS Canto II™ or BD FACS Aria III™ for all measurements and FlowJo 9.3.2 (Tree Star) software for analysis.

4.1.2.1 Extracellular staining for flow cytometry

Cells were always stained in suspension, typically in a volume of 100 µl PBS supplemented with 1% FCS (PBS + 1% FCS) at a concentration of 3-5 x 10^6/ml. If required, cells were first blocked applying Fc-receptor block for 10 minutes at 4°C. Biotinylated or directly labelled antibodies were added and incubated for 20 minutes at 4°C in the dark. Dilution for every antibody had previously been determined by titration. Once incubation had been completed, cells were washed with PBS + 1% FCS. Next, if needed, fluorescently labelled streptavidin or secondary antibodies were added to the suspension. This was then followed by another 30 minutes of incubation at 4°C in the dark. Cells having been washed for a last time, they were re-suspended in 300-400 µl PBS + 1 % FCS, we then proceeded to FACS analysis.

4.1.2.2 Intracellular staining of MHC II for flow cytometry

Cells in suspension were treated with Fc-Block and surface staining as previously explained. Then up to 10^6 cells per well were fixed with 2% paraformaldehyde (PFA) for 10 minutes at room temperature. The cell membrane was permeabilised by washing the cells in 0,03% Saponin. The staining solution, containing 0,3% Saponin, 2% FCS and the conjugated antibody (1 µg per 10^6 cells) was added to cells, well vortexed and left to incubate for 20 minutes at 4°C. After incubation cells where washed twice with 0,03% Saponin and once with PBS+1%FCS before being re-suspended in PBS+1%FCS to be finally analysed by flow cytometry.

4.1.2.3 Fluorescence-activated cell sorting

BD FACS Aria III™ and BD FACS Diva™ Software were used to sort cells. Nozzle size was adapted to the size of cells sorted. For Macrophages the 85 µm nozzle was employed, while for Lymphocytes the 70 µm nozzle was chosen. Previous to sorting the stream was fine-tuned for spill-over and drop delay. Acquisition was always performed without threshold value and/but under compensation. To minimize contamination of the subsets sorted, gates were set stringently so as to seek for maximum "purity" in sorting.

4.2 Mice

4.2.1 Diabetes monitoring (Diabetes frequency studies)

Age-matched, contemporary cohorts of mice were used for all disease studies. Diabetes onset was monitored measuring urine glucose levels with Diastix (Bayer) in a weekly (for spontaneous disease) or thrice weekly (for cyclophosphamide induction and adoptive transfer experiments) manner. A mouse was defined to be diabetic once it had tested positive for glycosuria (>250 mg/dL) at two consecutive readings (the initial of the two readings marking diabetes onset).

4.2.2 Adoptive Transfer Experiments

Homozygosity for an autosomal recessive mutation of the Prkdc gene causes severe combined immunodeficiency (SCID) in NOD.SCID mice. This immunodeficiency is characterized by an absence of intact T- and B-lymphocytes (adaptive immune system) as well as an impairment of the complement system and natural killer cells (innate immune system). For adoptive transfer experiments, 4-10x10^6 splenocytes or magnetically-sorted T or B lymphocytes once washed and resuspended in 200µl sterile PBS were injected in the tail vein of NOD.SCID or *Clec16a* KD NOD.SCID mice, as indicated.

4.2.3 Cyclophosphamide mediated diabetes induction

For simple disease acceleration 200 mg/ kg cyclophosphamide (Sigma-Aldrich) were injected intra-peritoneally into NOD WT and Clec16a KD on day 0 and 14. For adoptive cell transfer experiments, NOD.SCID received the cyclophosphamide injection 21 days after the cell transfer.

4.3 Immunological Assays

4.3.1 Primary cell suspension from lymphoid organs

Most experiments required primary cells in suspension, these had to be previously gained from lymphoid organs. Mice were sacrificed by inhalation of Isoflurane. After disinfection, the fur was opened on the front side and cervical, axillary and inguinal lymph nodes were dissected. After accessing the peritoneum, peritoneal or pancreatic lymph nodes and the spleen were removed. In order to get dendritic cells, spleens had to be perfused with Collagenase (Liberase Blendzyme, Roche) and left for incubation at 37°C for 20 minutes. For all other cell types analysed, organs were directly ground between two frosted microscope slides in 5 ml PBS + 1% FCS. The cell suspension was centrifuged for 7 min at 1500 rpm, then treated with ACK (3ml 3 min room temperature) for lysis of erythrocytes and after an additional washing step, filtered through a 40 µm nylon mesh, resulting in a single-cell-suspension, that was quantified with a haemocytometer.

4.3.2 Magnetic Beads based cell-sorting

After obtaining a single cell suspension from lymphoid organs as described above, cells were isolated with magnetic cell sorting kits, either MACS® (Milteny) or Dynabeads (Invitrogen) following the manufacturer's protocol.

4.3.3 In vitro generation of bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BMDC) were generated following a protocol kindly provided by Professor Manfred Lutz[354]. In brief femur and tibia of both hind

limbs were dissected and the surrounding muscle tissue removed. After disinfection of intact bones in 70% ethanol for about 4 minutes, the bone ends were cut. A 21 G syringe needle and PBS + 1% FCS were then used to flush out the bone marrow onto a nylon mesh filter. Clusters in the bone marrow suspension could thus be disaggregated. Cells were then washed once. One femur or tibia yielded 8-10 x 10^6 bone marrow cells.

For culture, bone marrow cells were suspended in RPMI-10 cell culture medium (=RPMI 1640, supplemented with 10% FCS, L-Glutamine, Penicillin/ Streptomycin, ß-Mercaptoethanol and Sodium Pyruvate). 2 x 10^6 cells were plated on a 100 mm dish in 10 ml RPMI-10 medium supplemented with 200 U/ml rmGM-CSF. On day 3 another 10 ml of RPMI-10 with 200 U/ml rmGM-CSF were added. On day 6 half of the supernatant was collected, centrifuged and the pellet re-suspended in 10 ml fresh RPMI-10 containing 200 U/ml rmGM-CSF. On day 8 the loosely adhering cells were collected, washed and then ready for use in further experiments like Activation or Endocytosis Assays. 2 x 10^6 bone marrow cells on day 0 resulted in 1.5-1.7 x 10^6 differentiated cells on day 8. Of these about 70% proved to be $CD11c^+$/$Cd11b^+$ in flow cytometry analysis.

4.3.4 Activation assays

For all activation assays cells were seeded in a 96-well (U-bottom) plate in 200 µl RPMI-10 medium.

4.3.4.1 Lymph node and T cell activation

Lymphocytes or magnetically sorted $CD4^+$ or $CD4^+CD25^-$ T cells (2.5x10^4/well) were stimulated using varying concentrations of either anti-CD3 alone or anti-CD3 plus anti-CD28. In both cases cells were co-stimulated by 3x10^5 irradiated (20 Gy) antigen presenting cells (APCs). Alternatively cells were activated with anti-CD3/anti-CD28 coupled beads or PMA/Ionomycin.

After 72 h incubation at 37°C and 5% CO_2, to allow measurement of proliferation 0.5 µCi ^{3}H-thymidine (suspended in RPMI-10) was given to each well. Once cells had been incubated for another 16 h plates could be harvested. A beta-counter (Perkin-Elmer) then quantified the radioactivity that had been up-taken by cells.

As an alternative, previous to activation, Cell Proliferation Dye eFluor 670 (eBioscience) could be applied to stain cells. In this case FACS analysis of the dye's dilution permitted to determine the degree of cell proliferation.

4.3.4.2 B cell activation

Similarly, B cells obtained from splenocytes by magnetic sorting ($2x10^5$/well) were stimulated with lipopolysaccharide (LPS) or anti-CD40 and incubated at 37°C and 5% CO_2. On Day 0 (basal activity) and after incubation for one, two or three days all cells were stained intracellularly (MHC II) or extracellularly (MHC II, CD86, B220, CD19) and analysed by flow cytometry.

4.3.5 Suppression assays

Cells were isolated from young (6-8 weeks) male NOD mice as previously described. $2.5x10^4$ MACS® sorted $CD4^+CD25^-$ T effector cells and varying numbers of $CD4^+CD25^+$ T regulatory cells (ratios of T effector to T regulatory cells ranging from 1:10 to 1:1) were seeded in a 96-well (U-bottom) plate in 200 µl RPMI-10 medium. Cells were stimulated with 1µg/ml anti-CD3 and $3x10^5$ irradiated (20 Gy) antigen presenting cells (APCs) and incubated for 72h at 37°C and 5% CO_2. Cultures were then pulsed with 0.5 µCi ^{3}H-thymidine (suspended in RPMI-10) for another 16 h, before plates were harvested and incorporated radioactivity was quantified with a beta-counter (Perkin-Elmer).

4.3.6 Mixed lymphocyte reaction

Despite T cell's MHC restriction, a remainder of between 5 and 10% of these cells are sensible to allogeneic MHC T cells and are consequently activated upon contact with this stimulus. Hence NOD WT or NOD Clec16a KD splenocytes ($3x10^5$/well) were stimulated with equal numbers of NOD WT, NOD Clec16a KD or C57BL/6 splenocytes that had been previously irradiated (20 Gy). To allow measurement of proliferation, after 72h of co-cultivation at 37° C and 5% CO_2 cells, 0.5 µCi ^{3}H-thymidine (sus-

pended in RPMI-10) was added and left for another 16h. Radioactivity up-take was quantified with a beta counter.

4.3.7 Phagocytosis assays

4.3.7.1 Fluorescently labelled E.coli particles (phRodo™)

Phagocytosis assays were performed with either peritoneal macrophages or bone marrow-derived dendritic cells. Peritoneal macrophages were obtained by rinsing the peritoneum of NOD and NOD Clec16 KD mice twice with 7 ml ice cold PBS+1%FCS. Cells were seeded in 200 µl in RPMI-10 in a 96 well-plate and non adherent cells were removed after one hour at 37° to accomplish further purification. Bone marrow-derived dendritic cells had been differentiated with rmGM-CSF for 8 days as described above.

Macrophages or bone marrow-derived dendritic cells (in both cases $2x10^5$/well) were pulsed with different concentrations (0.1 or 0.4 mg/ml) of fluorescently labelled E.coli particles (pHrodo™, Invitrogen) suspended in 200 µl RPMI-10 at exact pH 7.4 and left to incubate at 37°C. Two different variations of the experiment were carried out. Either, the phRodo™ particles were removed after a one hour pulse and this was followed by one to four hours further incubation before the reaction was stopped by putting cells on ice. Alternatively pHrodo™ particles were incubated with cells during time periods ranging from 10 minutes to 6 hours. In the latter case, putting the cells on ice to stop the reaction and removing the particles was done simultaneously. Once stained for viability (macrophages: CD11c, dendritic cells: CD11b, both: Annexin V) uptake and processing of the particles could be quantified with flow cytometry.

4.3.8 Uptake and processing of Ovalbumin Peptide and Protein by APCs

3 x 10^5 irradiated splenocytes from OT-II transgenic or Clec16a KD B6 mice were co-cultured in 200 µl RPMI-10 with OVA peptide or protein in different concentrations and 2.5 x 10^4 OT-II B6 T cells. After 48h of incubation at 37°C and 5% CO_2, 0.5 µCi ^{3}H-thymidine (in RPMI-10) was added and cells were harvested 16 h later. Radioactivity incorporation was measured with a beta-counter.

5 Results

As already mentioned, previous studies have shown an association between the Clec16a locus and an increased risk to develop T1D. In order to better understand the mechanism behind this link we decided to examine the function of Clec16a within the NOD model, its role for disease development and the immune system. These experiments were carried out by myself or together with Kay Gerold.

5.1 Lower incidence of diabetes in Clec16a KD mice

5.1.1 Spontaneous diabetes

Weekly monitoring for diabetes incidence of WT and Clec16a KD NOD mice (by measuring glycosuria for a period of 200 days) revealed that Clec16a NOD KD mice were almost entirely protected from becoming diabetic. During this time period only 2 out of 42 transgenic mice developed diabetes (Figure 4).

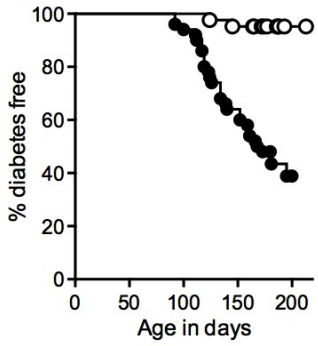

Figure 4 *Spontaneous diabetes incidence among female cohorts of WT (filled symbols, n=50) and Clec16a KD (KD, open symbols, n=42) NOD mice; Log rank test: P<0.0001.*

5.1.2 Cyclophosphamide induced diabetes

Disease onset in NOD mice can be accelerated by administration of cyclophosphamide[355, 356]. This alkylating cytotoxic agent induces a rapid, but temporary, depletion of peripheral lymphocytes which is particularly pronounced among

$CD19^+$ B cells and $CD4^+CD25^+$ T regulatory cells[356, 357]. $CD4^+CD25^+$ T regulatory cells become more sensible to apoptosis and exhibit lower homeostatic proliferation. They are also inhibited in their suppressive capacity vis-à-vis the emerging auto-reactive T cell response in NOD[356, 357]. At a later stage CY triggers a cytokine storm that shifts T helper cells towards a Th_1 dominated response[358]. All this contributes to an enhancement and synchronization of diabetes onset[355, 356].

Despite the administration of cyclophosphamide and the resulting impairment of T regulatory function Clec16a KD NOD mice did not develop diabetes in a significant way. In contrast to this in the NOD WT population cyclophosphamide induced diabetes as expected (Figure 5).

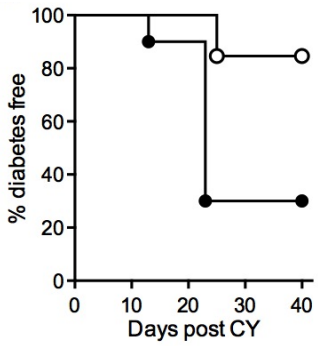

Figure 5 *Cyclophosphamide (CY) induced diabetes in female WT (filled symbols, n=10) and Clec16a KD (open symbols, n=13) NOD mice; Log rank test: P=0.0035; representative of two independent experiments.*

This may indicate that the protection of Clec16a KD NOD mice from diabetes is independent of the T regulatory function.

5.1.3 Cyclophosphamide induced diabetes in splenocyte transferred NOD.SCID mice

Results so far had left open whether the lower incidence of spontaneous and CY induced diabetes among Clec16a KD NOD mice had its origin in the immune system or rather in pancreatic islet cells. We addressed this question with adoptive transfer experiments whose aim was to determine whether the transfer of Clec16a KD splenocytes was sufficient to confer the protection observed in Clec16a KD NOD mice. NOD.SCID

mice received either splenocytes from WT or from Clec16a KD NOD mice and once these splenocytes had been allowed to populate peripheral lymphoid organs, mice were challenged with cyclophosphamide (Figure 6).

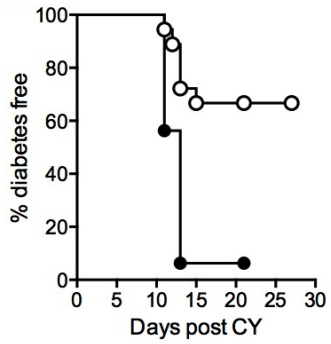

Figure 6 *Cyclophosphamide (CY) induced diabetes in female WT NOD.SCID mice reconstituted with WT (filled symbols, n=16) or Clec16a KD (open symbols, n=18) NOD splenocytes; Log rank test: P=0.0002.*

Results showed that CY induces diabetes in the majority of the NOD.SCID mice that had received WT NOD donor splenocytes, but in very few of the NOD.SCID mice that had received Clec16a KD NOD donor splenocytes. Thus the transfer of Clec16a KD NOD donor splenocytes appears to be able to impede islet destruction and diabetes development in NOD.SCID mice. This points to an immune related origin of the diabetes protection of Clec16a KD mice.

However this last experiment did not rule out that Clec16a KD NOD mice exhibit alterations in pancreatic islet cells that equally contribute to the protection from diabetes. Hence a reverse experiment with transfer of WT and Clec16a KD NOD donor splenocytes into NOD.SCID Clec16a KD mice was carried out. NOD.SCID Clec16a KD mice that had received NOD WT splenocytes developed diabetes in a similar manner as NOD WT mice (data not shown). This reinforces the notion that lower diabetes incidence in Clec16a KD mice is caused by changes in the immune system.

5.1.4 Cyclophosphamide induced diabetes in B and T cell transferred NOD.SCID mice

The previous experiments having shown that Clec16a KD NOD donor splenocytes convey protection against development of CY induced diabetes, we were interested in identifying the exact population of immune cells responsible for this effect. We thus transferred different combinations of WT and Clec16a KD NOD donor B and T cells into NOD.SCID mice as shown in table 1. Both cell subsets were obtained by negative selection with MACS, and then $2x10^6$ of each cell type were transferred into NOD.SCID mice. After 3 weeks mice received cyclophosphamide to induce diabetes.

		T cells	
		WT	Clec16a KD
B cells	WT	1	2
	Clec16a KD	3	4

Table 1 *Combinations of T and B cells from WT or Clec16a KD NOD donors for reconstitution of female NOD.SCID*

Figure 7 illustrates that diabetes incidence in mice that had been transferred pure WT or Clec16a KD subset combinations proved to be comparable to the incidence observed in the previous splenocyte transfer experiments. WT NOD.SCID mice that had received both NOD T and B cells (i.e. subgroup 1) quickly developed diabetes. WT NOD.SCID with both Clec16a KD T and B cells (i.e. subgroup 4) failed to develop disease. In the mixed subgroups, only those mice that had obtained Clec16a KD T cells (and WT B cells, i.e. subgroup 2) remained protected from diabetes development whereas diabetes incidence among those who had obtained Clec16a KD B cells (but WT T cells, i.e. subgroup 3) was comparable to the pure WT T and B cell case (subgroup 1). Hence the origin of transferred T cells appears to be the decisive factor for the development of diabetes in this experiment, Clec16a KD T cells conferring the protective effect, regardless of the co-transferred B cells.

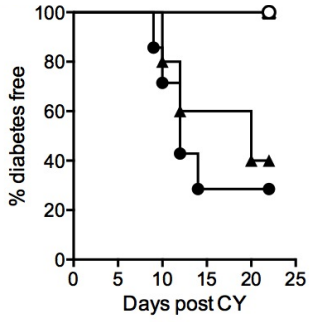

Figure 7 *Cyclophosphamide induced diabetes in female NOD.SCID mice reconstituted with the following combinations of T and B cells from either WT or Clec16a KD NOD: WT T cells/WT B cells (subgroup 1, closed circles, n=7), WT T cells/KD B cells (subgroup 3, closed triangles, n=5), KD T cells/WT B cells (subgroup 2, open circles, n=5), KD T cells /KD B cells (subgroup 4, open triangles, n=4); Fisher's exact test: P=0.0046 for WT T cell groups vs. KD T cell groups.*

5.2 T cell characterisation

As the preceding *in vivo* data shows, a T cell associated mechanism could be an important contributor to the lower incidence of diabetes observed in Clec16a KD NOD mice. Seeking to better understand the underlying mechanisms, we decided to further assess T cell function *in vitro*.

5.2.1 T cell activation status

For an initial idea of the repertoire and activation status of T cells in Clec16a KD NOD mice we used FACS to analyse surface receptor on T cells from freshly gained PLN and spleens. This revealed that compared to WT NOD, Clec16a KD NOD mice have similar shares of CD4+/CD8- and CD8+/CD4- T cells. Equally CD25, CD44, CD62L and CD69 surface receptor expression on these two T cell subsets did not differ between WT and Clec16a KD NOD.

5.2.2 Ln proliferation

We then examined the proliferation of peripheral lymph node T cells. In a first step we stimulated peripheral lymph node cells of WT and Clec16a KD NOD mice with differing concentrations of anti-CD3.

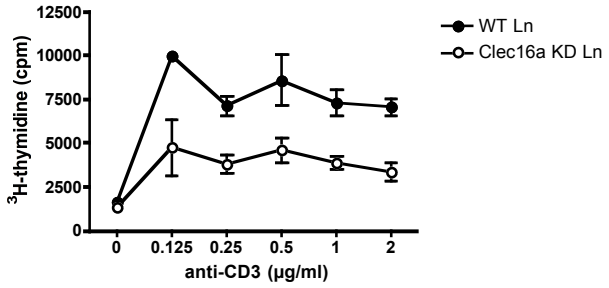

Figure 8 *^{3}H-Thymidine uptake of 3.0 x 105 lymph node cells after 3 days activation with anti-CD3 in different concentrations, both figures show representative results of three independent experiments.*

Even though both genotypes proliferated, lymph node cells from Clec16a KD mice did this in a considerably weaker manner, irrespective of the concentration of anti-CD3 employed (Figure 8).

5.2.3 Function of regulatory T cells

With an impaired T regulatory cell function as a potential cause for the reduced proliferation exhibited by Clec16a KD lymph node T cells, we decided to examine the function of T regulatory cells in a conventional suppression assay. Under stimulation with anti-CD3, $CD4^+CD25^-$ T effector cells and APCs of the same genotype were co-cultured with different ratios of either WT or Clec16a KD $CD4^+CD25^+$ T regulatory cells. Higher shares of T regulatory cells would be expected to increasingly suppress proliferation of T effector cells.

Clec16a KD T regulatory cells displayed an undistorted capacity to suppress T effector cells at all ratios (Figure 9).

An alternative explanation for the observed effect in lymph node proliferation could be that T regulatory cells generally more easily suppress the proliferation of Clec16a KD T cells. We therefore performed an additional suppression assay where we tested how easily WT T regulatory cells could suppress the proliferation of different combinations of WT and Clec16a KD T effector cells and APCs.

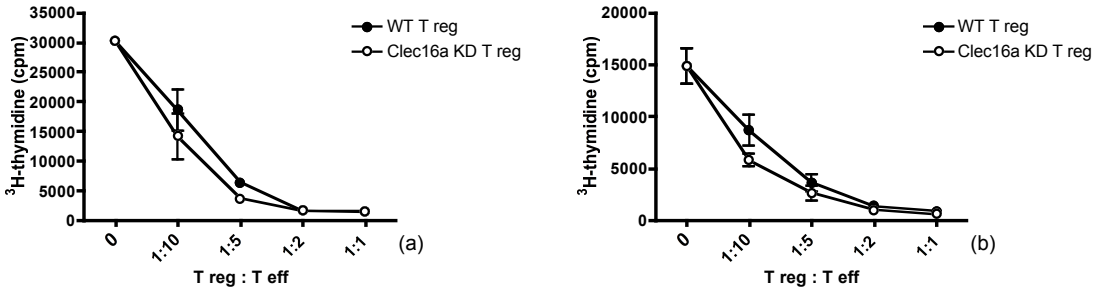

Figure 9 *Suppression of T effector cell proliferation by varying shares of T regulatory cells of both genotypes, proliferation measured as ^{3}H-Thymidine uptake of 2.5 x 104 CD4+CD25- T cells and 3.0 x 105 APCs of both either WT (a) or Clec 16a KD (b) genotype after 3 days activation with anti-CD3=1 µg/ml, both figures show results representative of three independent experiments.*

Both WT and Clec16a KD cells proved to be suppressed in a comparable manner by WT T regulatory cells (Figure 10).

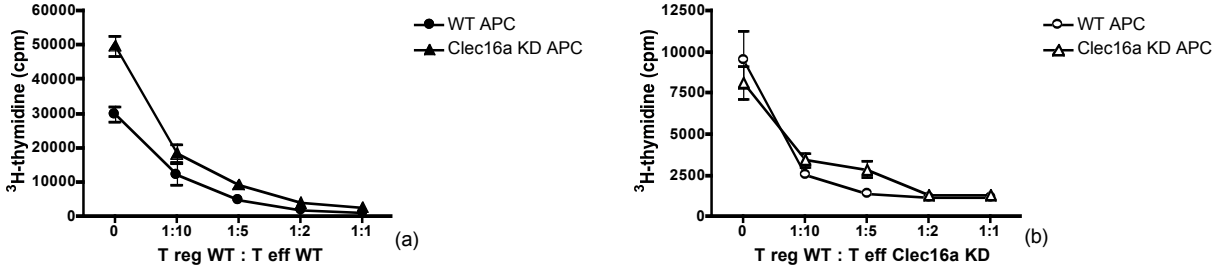

Figure 10 *Suppression of T effector cell proliferation by varying shares of CD4+CD25+ WT T regulatory cells, proliferation measured as ^{3}H-Thymidine uptake of 2.5 x 104 CD4+CD25- T effector cells of either WT (a) or Clec 16a KD (b) genotype and 3.0 x 105 APCs of both genotypes after 3 days activation with anti-CD3=1 µg/ml, both figures show results representative of three independent experiments.*

5.2.4 T cell proliferation

5.2.4.1 Activation with anti-CD3 and APCs

Having observed that the function of Clec16a KD and WT T regulatory cells was comparable, we wondered whether a lack of proliferative capacity of T effector cells itself could explain the weaker proliferation of lymph node cells. We hence stimulated

CD4+CD25- T effector cells from spleens and peripheral lymph nodes of WT and Clec16a KD NOD mice with anti-CD3 and antigen presenting cells (APC) of both genotypes.

Results confirmed that even though T effector cells from both genotypes proliferated, T effector cells from Clec16a KD demonstrated a clear disadvantage (Figure 11).

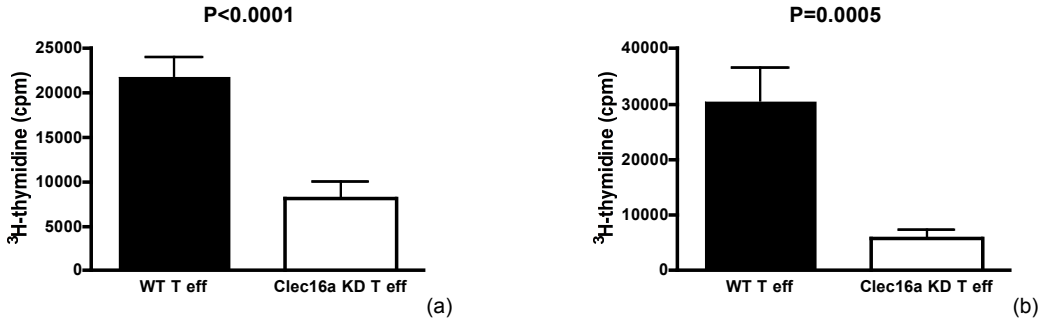

Figure 11 *^{3}H-Thymidine uptake of 2.5 x 10^4 $CD4^+CD25^-$ T cells after 3 days activation with anti-CD3=1 µg/ml and 3.0 x 10^5 WT APCs (a) or Clec16a KD APCs (b), both figures show combined results of six independent experiments.*

This T effector cell hyporeactivity proved to be independent of the genotype (and number) of irradiated APC as well as the concentration of anti-CD3 (Figure 12).

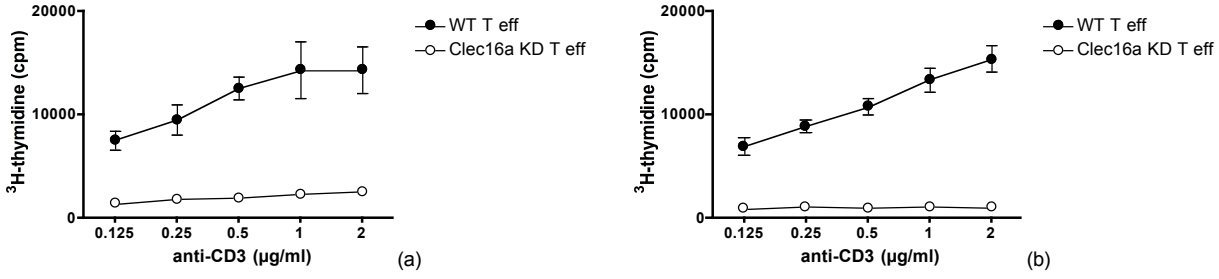

Figure 12 *^{3}H-Thymidine uptake of 2.5 x 104 CD4+CD25- T cells after 3 days activation with different concentrations of anti-CD3 and 3.0 x 105 WT APCs (a) or Clec16a KD APCs (b) both figures show combined results of three independent experiments.*

We were then interested in whether this hyporeactivity could be overcome by co-stimulation of CD28 (with soluble anti-CD28) in addition to the basic TCR stimulus (anti-CD3 and irradiated APCs). CD28 receptors engage in similar pathways as TCR receptors, and are assumed to increase TCR induced T cell proliferation through a quantitative amplification of TCR signals (among others by activating nuclear factor κB (NF- κB) and nuclear factor of activated T cells (NFAT)) and possibly also through an own qualitative effect [359, 360]. Both WT and Clec16a KD T effector cells proliferated significantly stronger in response to CD28 co-stimulation (Figure 13). However, compared to their WT counterparts, transgenic T effector cells exhibited a significantly weaker (absolute) increase in proliferation in response to aCD28. As a result a significant proliferative disadvantage of Clec16a KD T effector cells persisted under co-stimulation with aCD28.

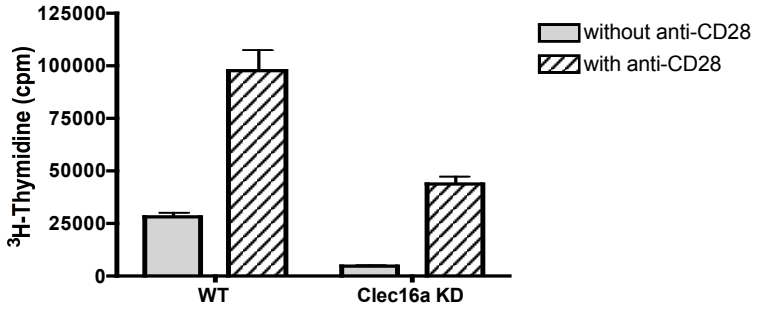

Figure 13 *^{3}H-Thymidine uptake of 2.5 x 104 CD4+CD25- T cells after 3 days activation with 3.0 x 105 WT APCs, CD3 antibody (at concentration of 1 µg/ml) and with or without additional CD28 antibody (at concentration of 1 µg/ml), results representative of three independent experiments.*

Activation of the entire $CD4^+$ subset, without previous removal of $CD4^+CD25^+$ cells yielded analogous results (data not shown).

In contrast to this, the genotype of APCs did not appear to have a significant effect on the proliferative capacity of neither WT nor Clec16a KD T effector cells (Figure 14).

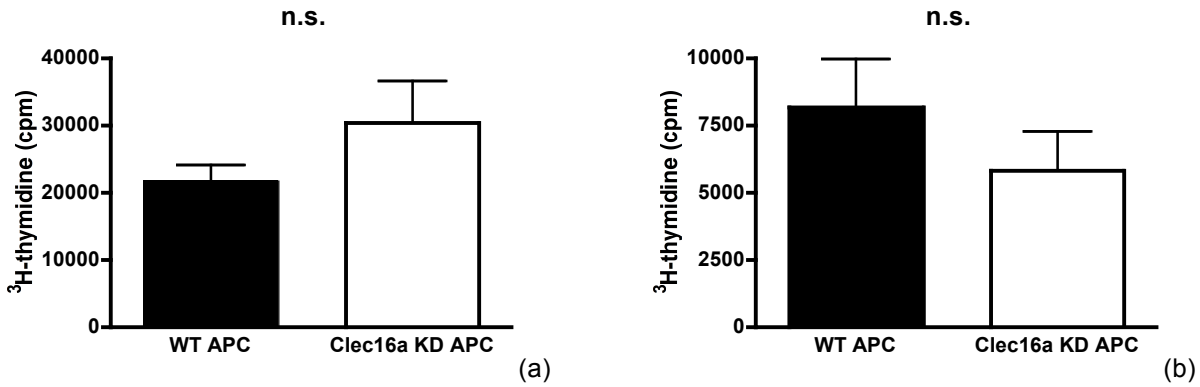

Figure 14 *^{3}H-Thymidine uptake of 2.5 x 104 CD4+CD25- T cells after 3 days activation with anti-CD3=1 µg/ml and 3.0 x 105 WT APCs (a) or Clec16a KD APCs (b) both figures show combined results of six independent experiments.*

Knowing that the lentiviral transgene (shRNA plus GFP) is expressed by only about 70% of T lymphocytes, we were interested in investigating whether proliferative capacity of Clec16a KD T effector cells varied depending on the transgene expression level. We hence separated Clec16a KD T effector cells into GFP+ and GFP- cells by flow cytometry. Surprisingly, GFP+ and GFP- cells showed similar impairment in their proliferation (Figure 15), indicating that T cell hyporeactivity was independent of the level of transgene expression within the cell and hence unrelated to T cell-intrinsic effects of gene silencing.

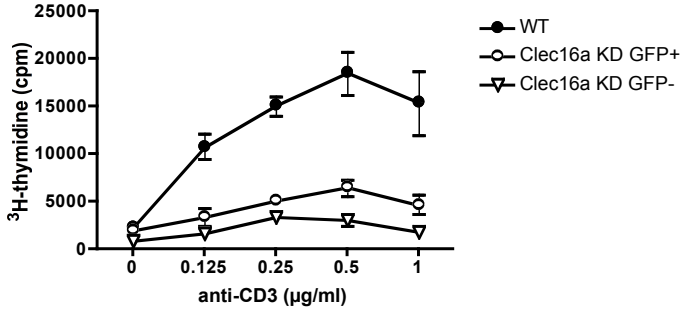

Figure 15 *^{3}H-Thymidine uptake of 2.5 x 10^{4} $CD4^{+}CD25^{-}$ T cells after 3 days activation with different concentrations of anti-CD3 and 3.0 x 10^{5} WT APCs, Clec16a KD $CD4^{+}CD25^{-}$ T cell had been previously sorted in GFP+ and GFP- cells, figure representative of two independent experiments.*

5.2.4.2 Mixed lymphocyte reaction

Mixed lymphocyte reaction (MLR) offers a further possibility to assess T cell proliferation. 5-10% of T cells are allo-reactive, i.e. they are able to recognize allogeneic MHC. This TCR-MHC contact then leads to activation and proliferation without requiring further activating signals such as anti-CD3 or anti-CD28.

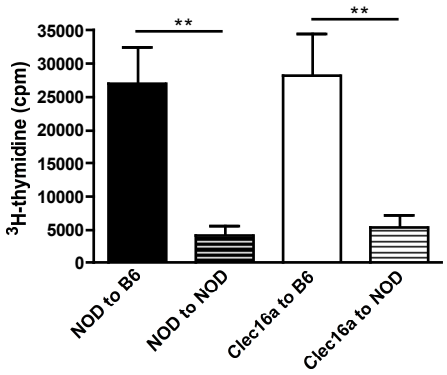

Figure 16 *^{3}H-Thymidin uptake of 2 x 105 splenocytes (APCs) after 3 days incubation with same number of respective irradiated splenocytes (APCs), graph is a summary of three independent experiments.*

In contrast to the observations made in the previously described anti-CD3/APC experiment, in MLR Clec16a KD T cells exhibited a proliferative response equivalent to that of WT NOD T cells (Figure 16). This observation also applied for other ratios of responder to irradiated APCs, 10:1, 1:2 and 1:5) (data not shown). Yet it has to be considered that, as just mentioned, only a small share of allo-reactive T cells responds in such an experiment. Yet, these results indicate that when confronted with certain activating signals Clec16 KD T cells are able to behave analogous to WT T cells. Since our experimental setting did not include any previous treatment or separation of the responder splenocytes, we are however not able to tell whether activated T cells belonged to the CD4+ or the CD8+ subset.

5.2.4.3 T cell activation without APCs

Previous experiments all having included APCs, we were interested how Clec16a KD T cells responded to APC independent stimulation. We therefore activated T cells with either anti-CD3/anti-CD28 coupled beads (Invitrogen) or PMA/Ionomycin. While the former gives a combined first and second signal, the latter directly triggers the Protein-kinase Cθ (PKCθ)/Ca^{2+} pathway and thus circumvents the T Cell Receptor (TCR).

While stimulation with anti-CD3/anti-CD28 coupled beads reproduced the previously found proliferative disadvantage of Clec16a KD T cells, direct activation of the PKCθ/Ca^{2+} pathway was followed by a very strong proliferative response of Clec16a KD T cells (Figure 17).

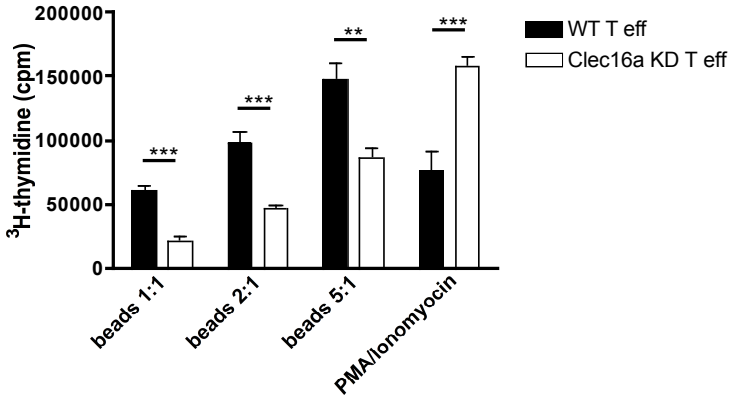

Figure 17 *^{3}H-Thymidine uptake of 2.5 x 104 CD4+CD25- T cells after 3 days activation with anti-CD3/anti-CD28 coupled beads in different ratios or with PMA/Ionomycin, figure representative of three independent experiments.*

5.3 Characterisation of Antigen Presenting Cells

Considering the just described in vivo and in vitro experiments an impaired T cell function appears to be the most likely cause for the lower diabetes incidence in Clec16a KD mice. Yet these results do not finally rule out that this effect may be attributable to some deficit in the function of antigen presenting cells. This appears even more important bearing in mind that previous evidence has both demonstrated the expression of

Clec16a particularly in antigen presenting cells[361] and the importance of the Drosophila orthologue for the process of endosome maturation[159, 160].

5.3.1 Antigen presenting cell subsets and surface receptor staining

As a first step we used FACS analysis to compare the subsets of antigen presenting cells (B cells, DCs and Macrophages) in freshly gained WT and Clec16a KD peripheral lymph nodes and spleens. Both genotypes appeared to have similar shares of B220+/CD19+ B cells, CD11b+/CD11c- Macrophages, and CD11b+/CD11c+ Dendritic cells (DCs). Clec16a KD B cells exhibited levels of IgM and FcgRII/III on the surface comparable to WT. Furthermore all three cells types did not differ in their expression of CD54, PD-L1/2 (=CD274/CD273), GITR-L and CD40. Figure 18 displays exemplary data for CD54 and CD273 surface receptors.

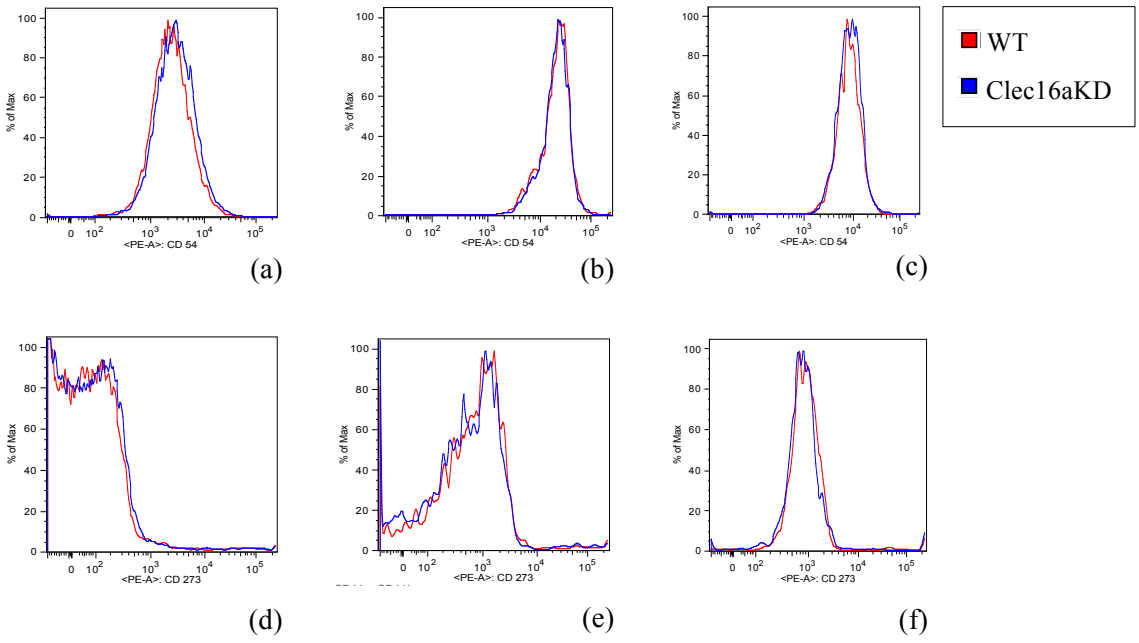

Figure 18 *CD54 (a,b,c) and CD273 (d,e,f) expression on B cells (a, d), CD11c+/Cd11b+ DCs (b, e) and CD11c-/Cd11b+ Macrophages (c, f).*

5.3.2 B cell and dendritic cell activation

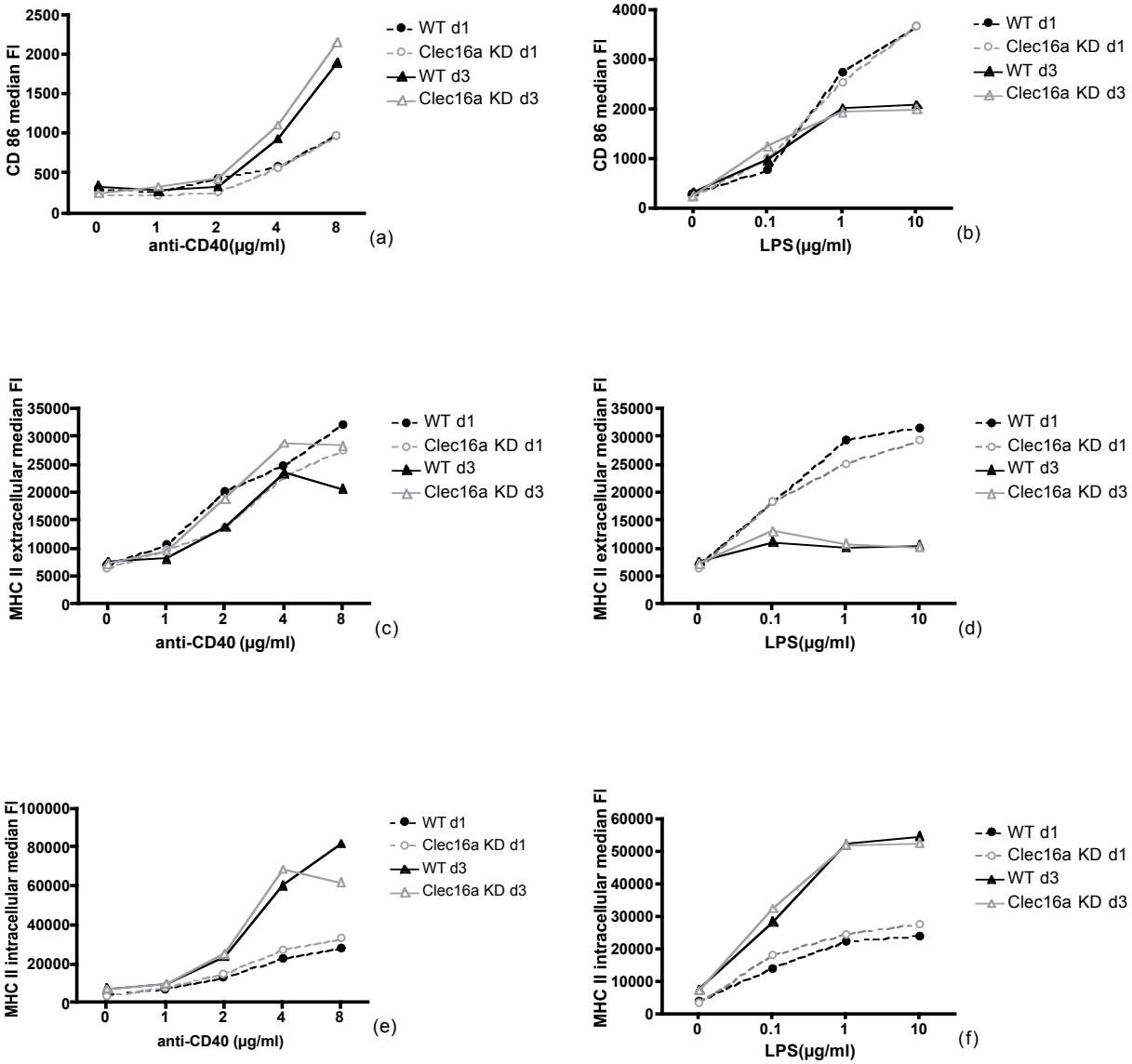

Figure 19 *Expression of CD86 (a and b), MHC II extra- (c and d) and intracellular (e and f) as measured by FACS after activation of 2x 106 MACS isolated B cells with different concentrations of anti-CD40 (a, c and e) or Lipopolysaccharide (LPS) (b, d and f) for 1 to 3 days; CD86, MHC II extra- and intracellular given as median fluorescence intensity (FI), anti-CD40 and Lipopolysaccharide (LPS) given in µg/ml.*

To investigate APC function we first evaluated in vitro activation of peripheral B cells and dendritic cells, with particular regard for receptors used in interaction with T cells. The idea was that a lower responsiveness of B cells might be responsible for the ob-

served decrease in T cell activation. We hence stimulated these cells, once they had been isolated from freshly obtained splenocytes with MACS, with varying concentrations of anti-CD40 or Lipopolysaccharide (LPS) for up to three days. At day 0, 1, 2 and 3 of activation we studied CD86 and MHC II surface receptor status as well as the intracellular levels of MHC II, both using fluorescence cytometry.

Similar levels of CD86, MHC II extra- and intracellular were found for WT and Clec16a KD B cells during the entire time course (Figure 19), thus indicating comparable capacity of B cells of both genotypes to be activated.

We conducted analogous experiments with BMDCs activated on Day 8 of the differentiation protocol. Again levels of up-regulation of CD86, CD80 and MHC II extracellular proved to be comparable in WT and Clec16a KD BMDCs (data not shown).

5.3.3 Uptake of fluorescently labelled E.coli particles by Macrophages and BMDCs

As referred to earlier, studies of the Clec16a Drosophila orthologue *ema,* have indicated that this protein plays an important role in the maturation process of endosomes[160]. It thus seemed reasonable to explore whether uptake and processing of endosomes by Clec16a KD Macrophages and BMDCs showed any particularities. We tested this with fluorescently labelled E.coli particles (pHrodo™ , Invitrogen). These were given to either macrophages that had been obtained by peritoneal lavage and activated by contact with tissue culture plates or to BMDCs on day 8 of the differentiation protocol. The fluorescent pHrodo™ SE dye has the advantage of only giving a fluorescent signal at pH lower than 7. Thanks to this non-detectability at extracellular pH, there is no further need for quenching. At low pH, as it can be observed during acidification in maturing endosomes, it is possible to detect a fluorescent signal in the PE channel of FACS, which increases with decreasing pH. This made it possible to follow the time course of antigen uptake and processing in NOD WT and CLEC16A KD Macrophages and BMDCs.

Macrophages of both genotypes showed equal capacity to take up and process phRodo™ labelled particles during the entire observed time course (Figure 20a-c). This

proved to be independent of the GFP status of Clec16a KD cells (Figure 20c), of the concentration of particles, (Figure 20b) of whether particles were left for the entire time course or removed after 60 min (Figure 20a). Likewise we could detect no difference in endosomal processing of BMDCs of both genotypes (Figure 20d). Further inspection with light microscopy revealed no remarkable difference in the number and appearance of phRodo containing phagocytosis vacuoles (data not shown). This suggests endosomal uptake and processing in NOD Clec16a KD macrophages and BMDCs is comparable to NOD WT.

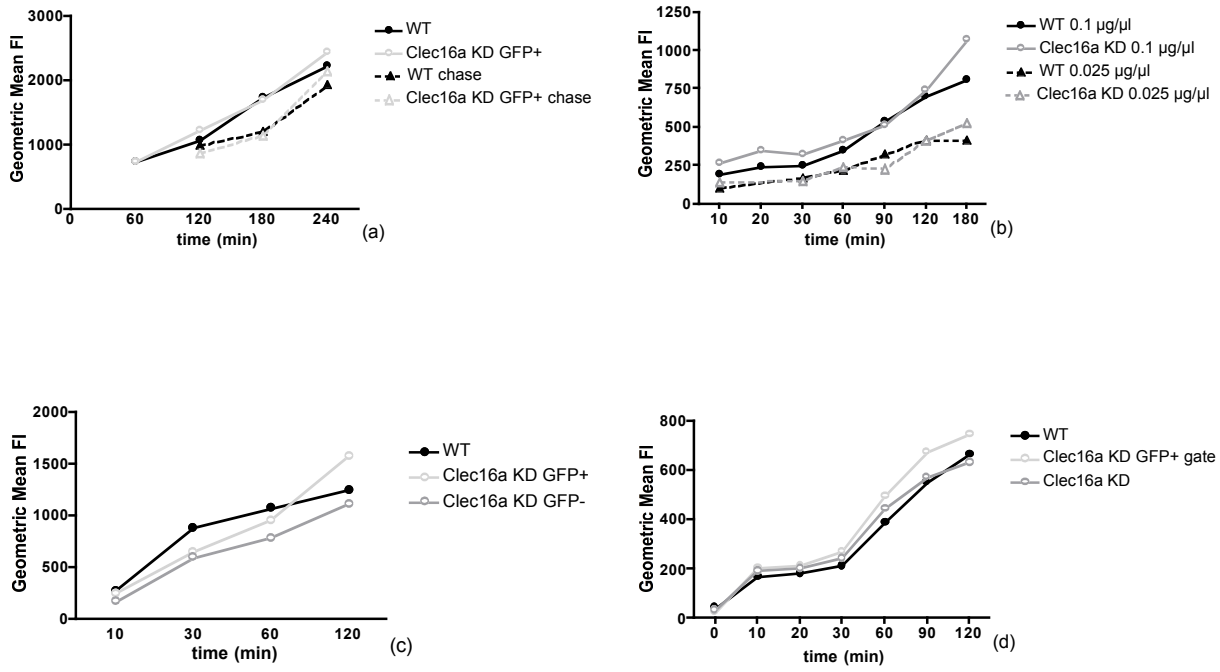

Figure 20 *Uptake and processing of phRodo™ labelled E.coli particles by peritoneal macrophages (a-c) or BMDC on Day 8 of differentiation (d); for (a) and (d) Clec16a KD cells were gated for GFP+ during FACS analysis, for (c) they were previously sorted with FACS for GFP+ and GFP- status, for (b) all Clec16a KD regardless of GFP status were analysed; phRodo™ was added in concentrations of 0.2 µg/µl (a), 0.025 µg/µl (b), 0.1 µg/µl (b-d) and left for the entire time except for the chase sub-experiment in (a) where particles were removed after 60 min; previous to FACS analysis cells were stained for viability with Annexin for (b-d); phRodo displayed as geometric mean fluorescence intensity (FI); results representative of three comparable experiments.*

5.3.4 Uptake and processing of Ovalbumin Peptide and Protein by APCs

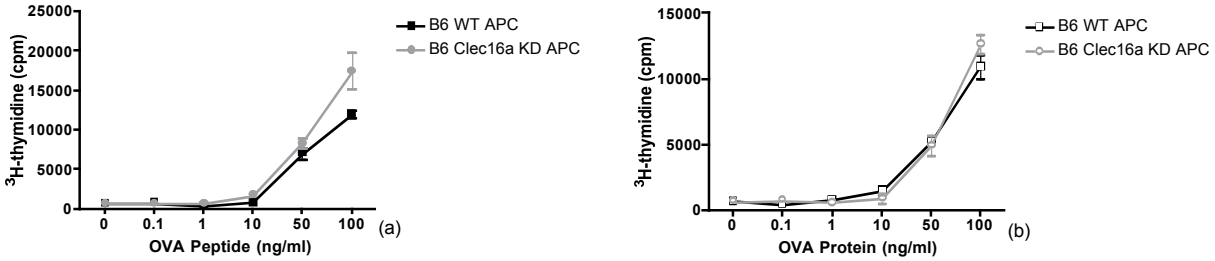

Figure 21 *^{3}H-Thymidin uptake of 2.5 x 10^4 OT II T cells after co-culture for 72 h with 3 x 10^5 irradiated APCs from OT II or Clec16a KD B6 mice and different concentrations of OVA Protein (a) or Peptide (b); results representative of two independent experiments;*

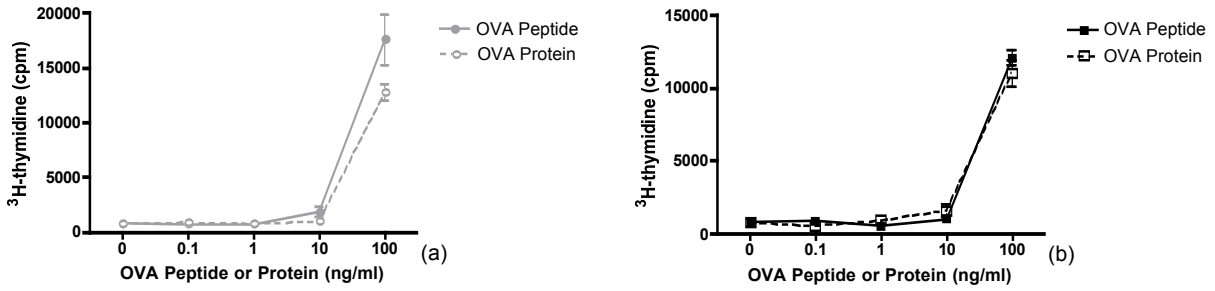

Figure 22 *^{3}H-Thymidin uptake of 2.5 x 10^4 OT II T cells after co-culture for 72 h with different concentrations of OVA Peptide or Protein and 3 x 10^5 irradiated APCs from Clec16a KD B6 mice (a) or WT B6 mice (b); results representative of two independent experiments;*

Peptide processing is an important task of APCs. Since a shortcoming in peptide processing and the consequent deficient presentation to T cells could also result in reduced T cell proliferation we chose to further explore peptide processing and presentation of Clec16a KD APCs. Peptide processing can be tested using $CD4^+$ T cells from OT-II transgenic B6 mice. These cells express transgenic OVA-specific αβ-T cell receptors that recognise ovalbumin (OVA) peptide as presented by APCs[362]. If APCs adequately process and present OVA protein or peptide, OT-II B6 T cells are stimulated, which is reflected by their subsequent proliferation.

Irradiated APCs (i.e. splenocytes) from B6 WT (with known unaltered peptide processing) or Clec16a KD B6 mice were co-cultured with OVA recognising OT-II B6 T

cells and either OVA peptide or protein in different concentrations. OT-II B6 T cell proliferative response was then measured to detect whether Clec16a KD B6 APCs were equally able to process OVA protein and peptide.

OT II T cells responded with equal proliferation to presentation of protein (peptide) by OT II as well as Clec16a KD B6 APCs (Figure 21a (b)). Furthermore both protein and peptide presentation of B6 WT and B6 Clecl6a KD APCs triggered a comparable response by OT II cells (Figure 22). These results suggest that Clec16a KD B6 APCs are able to process and present protein and peptide in a regular manner.

5.3.5 Characterisation of thymic epithelial cells

This and the following experiment were mainly carried out by Kay Gerold. They are mentioned here to provide a comprehensive overview of findings concerning Clec16a KD mice. A more detailed description of experiments can be found in Kay Gerold's PhD thesis[363].

All previous experiments had shown no apparent defect in the function of peripheral APCs. Under the hypothesis that the observed reduction of proliferative capacity in T cells could have been brought about by alterations in T cell selection, investigating those APCs with a key role in the early development of T cells, the so called thymic epithelial cells (TECs) appeared to be a logical next step. Within these essential antigen presenting cells, it is possible to distinguish two subgroups with different responsibilities: positive selection is accomplished by cortical thymic epithelial cells (cTECs), while negative selection is the domain of medullary thymic epithelial cells (mTECs)[249].

After collagenase digestion of WT and Clec16a KD NOD thymi, a Percoll gradient was used to isolate TECs from other thymocytes. Cells were stained for either Ly51- (mTECs) or CD45- EPCAM+MHCII+Ly51+ (cTECs) (cf. Gerold, 2011[363] for gating strategy) and further markers of activation (CD80, CD86).

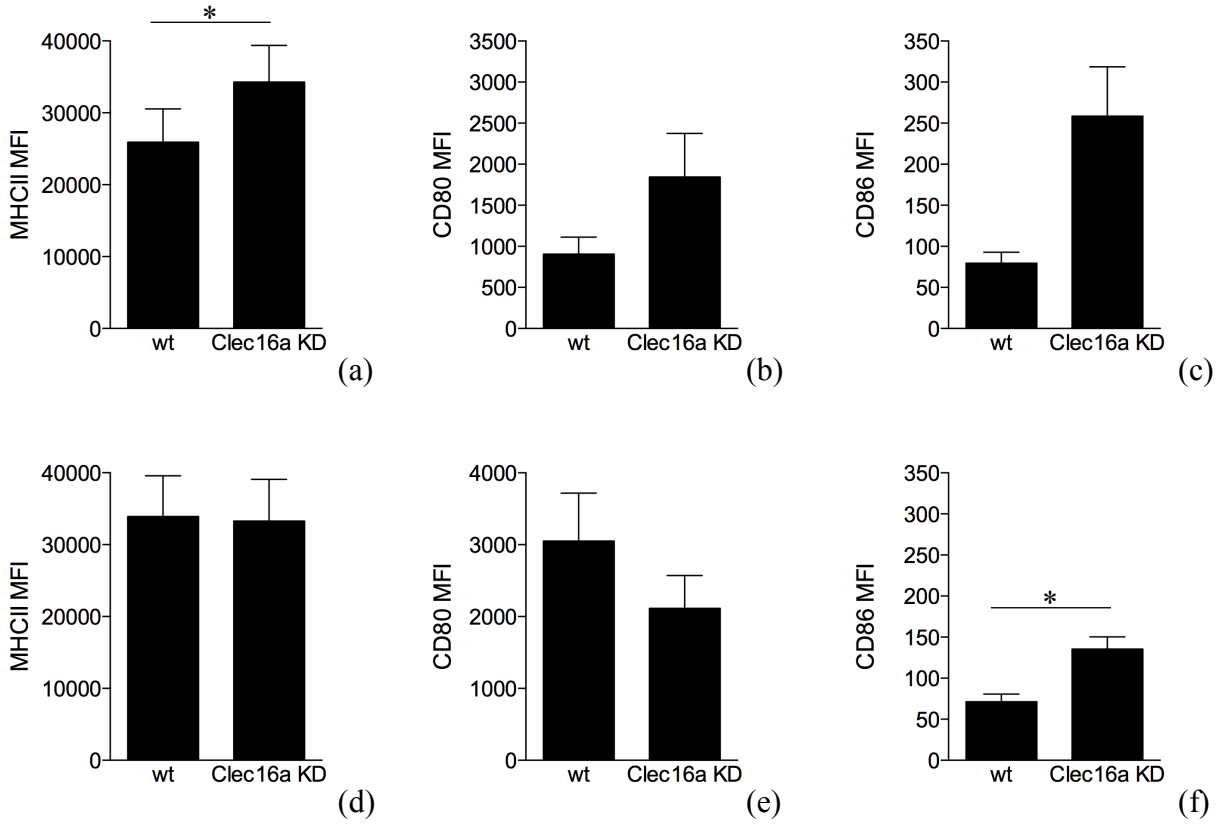

Figure 23 *Expression of surface markers on cTECs (a-c) and mTECs (d-f); MHCII level of cTECs, p=0.0207 (a); CD80 level of cTECs, not significant (n.s.) (b); CD86 levels of cTECs, n.s (c); MHCII level of mTECs, n.s (d); CD80 level of cTECs, n.s. (e); CD86 levels of cTECs, p=0.0247 (f); Geometric mean fluorescence intensities (MFI), graphs summarize five independent experiments; data provided by Kay Gerold.*

Clec16a KD cTEC also featured significantly higher levels of extracellular MHC II and a trend to increased CD80/86 surface markers (Figure 23a-c). As to Clec16a KD mTECs, while MHC II and CD80 levels were comparable to WT, a significant rise of CD86 could be observed (Figure 23d-f).

5.3.6 Analysis of the TCR repertoire

TCR specificities significantly depend on positive selection and consequently on changes in antigen processing capacities of cTECs. In view of the above mentioned alterations of the cTEC compartment, closer examination of the TCR repertoire of Clec16a KD NOD mice seemed justified. Hence FACS analysis was employed to contrast levels of ten different Vβ chains in thymocytes and splenocytes of both genotypes.

No significant difference in the distribution of Vβ chains between Clec16a KD mice and NOD WT for neither thymus nor spleen was found (Figure 24 and data not shown).

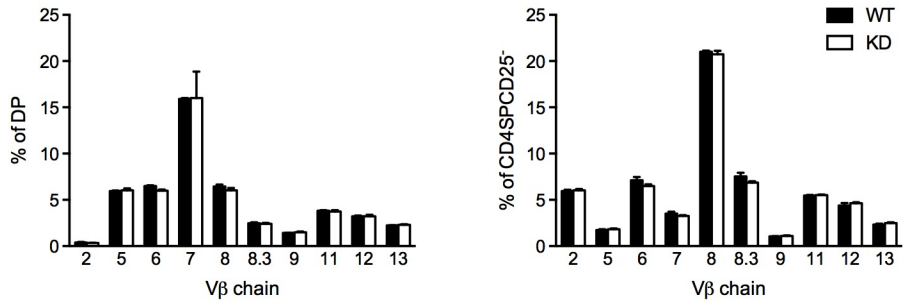

Figure 24 *Frequency of DP or CD4SPCD25⁻ thymocytes expressing different TCR Vβ chains in the thymus of WT or Clec16a KD mice; graphs summarize 6 different mice per genotype; Vβ5 antibody recognizes both Vβ 5.1 and Vβ 5.2; Vβ8 antibody recognizes both Vβ8.1 and Vβ8.2; data provided by Kay Gerold and Stephan Kissler.*

Summing up, silencing of Clec16a in the NOD strain was shown to result in strong protection against type 1 diabetes. This protection was conferred by T cells, more specifically an *in vitro* T cell hyporeactivity could be identified. However, the origin of this T cell hyporeactivity seems to be T cell extrinsic. While peripheral antigen presenting cells appeared unaffected by Clec16a KD we identified changes in the thymic epithelial cell compartment. Alterations in thymic T cell development could hence be the driving force underlying the increased observed protection from autoimmunity in Clec16a KD mice.

6 Discussion and Outlook

Clec16a variations have been found to be associated with several auto-immune diseases including T1D[18, 19, 124-147] and the gene appears to be expressed selectively in immune cells such as DCs, NK cells and B cells[136, 148, 150].The C-type lectin[17,] and the ITAM motif[154] predicted by bioinformatical analysis of the gene are structural domains known to be engaged in ligand recognition and signalling within immune cells[156]. All this hints at a role of Clec16a in immune regulation, yet no evidence on its physiological and molecular function in mammals had been published at the moment this dissertation project was started. The only findings available related to Clec16a's Drosophila orthologue *ema,* which seemed to be involved in endosomal and autophagosomal processes[159, 160].

Targeting Clec16a by means of RNA interference we aimed to investigate the role of this gene within the immune system and auto-immune diseases such as T1D. A NOD background was chosen for this knock-down since the NOD strain provides a genetic repertoire highly susceptible to auto-immunity and is the best studied animal model in T1D so far[179].

As I entered the project, Kay Gerold and Stephan Kissler had already generated Clec16a KD NOD mice and validated the knock-down. 70% of lymphocytes, and more than 85% of B cells and granulocytes were shown to be enhanced GFP (EGFP) positive, i.e. expressed the transgene comprising EGFP and the shRNA. Such differences in levels of EGFP and hence transgene expression are not unusual for RNAi using lentiviral vectors. They are the result of random integration sites which may be subject to epigenetic changes or even silencing of the transgene[195, 219]. The knock-down was further confirmed by qPCR, which revealed a significant but small reduction of mRNA expression levels of about 25% in lymphoid organs of male and female Clec16a KD NOD mice. Western blot analysis further confirmed reduced expression of Clec16a protein in thymus and spleen cells.

General development of Clec16a KD NOD mice was normal, and no alterations of mendelian frequency could be observed. However, compared to NOD WT, Clec16a KD mice were strongly protected from developing diabetes. The incidence of spontaneous diabetes in Clec16a KD mice, measured by 200-day follow up of urine glucose levels,

proved to be significantly lower than in NOD WT mice (Figure 4). Equally experimental induction of diabetes by administration of CY was significantly lower in Clec16a KD mice than in NOD WT mice (Figure 5). CY reduces the number of proliferating peripheral lymphocytes, particularly B cells and $CD4^+CD25^+$ T regulatory cells in a rapid but temporary manner (peak decrease on day 4, cell levels restored on day 10)[357]. CY further inhibits the suppressive function of $CD4^+CD25^+$ T regulatory cells and hence their capacity to control the emerging auto-reactive T cell response, which leads to an accelerated diabetes onset 2-3 weeks after CY treatment[355, 357, 358]. Since Clec16a KD mice proved resistant to CY-induced diabetes, it seems unlikely that the protective effect of Clec16a silencing is conveyed by a mechanism involving B cells or $CD4^+CD25^+$ T regulatory cells.

To verify that the lower diabetes incidence observed in Clec16a KD mice originated in the immune system we resorted to adoptive transfer experiments. Immuno-deficient NOD.SCID were reconstituted with splenocytes from either NOD WT or Clec16a KD donor mice. Protection against CY-mediated diabetes was confined to mice who had received Clec16a KD splenocytes, whereas NOD.SCID mice receiving WT splenocytes developed diabetes upon CY administration as expected (Figure 6). A second experiment was however necessary to finally rule out any pancreatic islet cell involvement. WT splenocytes transferred into NOD.SCID Clec16a KD mice successfully induce diabetes upon CY administration, which implies that potential changes in pancreatic islets due to the Clec16a silencing are not able to prevent diabetes (data not shown). These two experiments confirm that immune-system specific changes and not β cell intrinsic effects induced by Clec16a silencing prevent diabetes onset in transgenic mice. This seems consistent with the previously mentioned evidence that situates the function of Clec16a within the immune system. It is however at odds with the position taken by Soleimanpour et al.(2014). Based on findings from selective deletion of Clec16a in the endocrine fraction of the pancreas, these authors claim that Clec16a modulates insulin secretion, and that it is this role within β cells that underlies the association between the gene and type 1 diabetes[161]. This hypothesis appears difficult to maintain considering the numerous immune-related diseases for which Clec16a has been identified as susceptibility gene. If the primary function of Clec16a lies in insulin secretion in the pancreas, how is it possible to explain the association with diseases such as multiple sclerosis or

juvenile idiopathic arthritis? In contrast to this, a function within the immune system, as indicated by our experiments, seems much easier to reconcile with the multiple types of organs involved and the likely common origin in immune-system failures.

Clec16aKD splenocytes appearing to be the likely cause of the lower diabetes incidence in transgenic mice, we conducted further adoptive transfer experiments to closer identify the responsible cell population. After reconstitution of NOD.SCID mice with all four possible combinations of B and T cells from Clec16a KD and WT donors, only mice that had received T cells from Clec16a KD mice remained resistant to CY-mediated diabetes (Figure 7). In contrast to this, the origin of co-transferred B cells had no impact on the fate of mice. This is consistent with our results from CY diabetes induction experiments, which already made a relevant role of B cells appear quite unlikely. In conclusion, *in vivo* experiments suggest that silencing Clec16a impairs the ability of T cells to trigger diabetes.

Having discerned this *in vivo* deficiency of Clec16a KD T cells to induce diabetes we next sought to improve our understanding of the underlying mechanism through *in vitro* experiments.

Examination of the *in vitro* proliferative response of NOD WT and Clec16a KD $CD4^+CD25^-$ T effector cells to antigen-receptor stimulation by soluble anti-CD3 revealed a significant proliferative impairment of transgenic T effector cells. This hyporeactivity proved independent of the genotype of co-stimulating irradiated APCs (Figure 11) and could be equally observed under APC-independent stimulation via anti-CD3/anti-CD28 coated beads (Figure 17). This general APC-independent $CD4^+CD25^-$ T effector cell hyporeactivity found *in vitro* further corroborates findings from the above mentioned *in vivo* adoptive transfer experiments that discard a major role of B cells for the diabetes protection of Clec16a KD mice. This result is also consistent with Zouk et al. (2014) who test the effect of a Clec16a knock-down in a human lymphoblastoid cell line. Such a knock-down does not seem to compromise the ability of these cells to stimulate and activate T-cells[364].

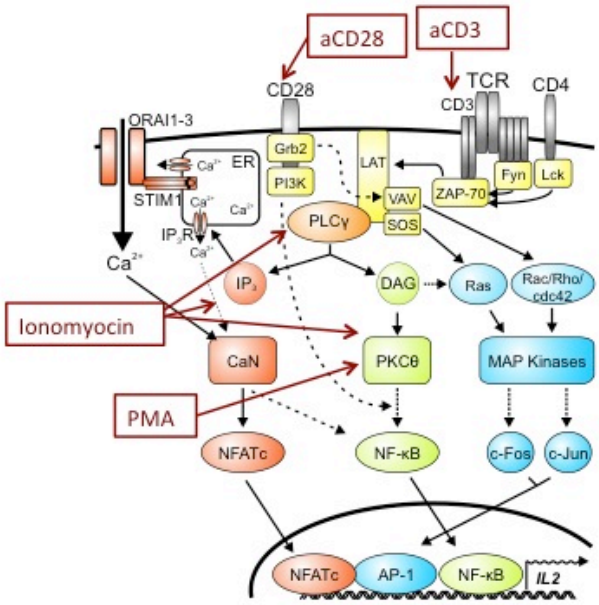

Figure 25 *TCR signalling pathways and in vitro activation mechanisms (adapted from Sieber 2009[365])* **.**

Proliferative impairment of Clec16a KD T effector cells could not be overcome by increasing the strength of the antigen-receptor stimulation through higher concentrations of soluble anti-CD3 (Figure 12). Transgenic T effector cells increased proliferation significantly in response to co-stimulation of CD28 (Figure 13). However this increase was lower for Clec16a KD T effector cells than for NOD WT cells, further widening the proliferative gap between transgenic and WT cells. In contrast to this hyporeactivity to antigen-receptor stimulation, Clec16a KD $CD4^{+}CD25^{-}$ T effector cells responded similar to WT when confronted with mitogenic stimuli circumventing the proximal T cell receptor (TCR) and instead targeting Protein Kinase C θ (through PMA) or Ca^{2+} release (through Ionomycin) (Figure 17). Taken together, this suggests that silencing Clec16a impacts proximal TCR signalling, but leaves intact the pathway distal of PKCθ (Figure 25). So while T cell proliferation in Clec16a KD mice in principle is possible, it is much more difficult to elicit such a proliferative response via the TCR. Furthermore co-stimulation of CD28 is able to trigger increased proliferation among transgenic cells, albeit in a slightly weaker manner than among WT cells. This means that at least to a certain extent CD28 co-signals are able to successfully enhance TCR induced Clecl6a KD T effector cell activation. Since CD28 co-signals engage in TCR pathways in mul-

tiple manners and the interplay between CD28 co-signals and TCR signalling is far from being fully understood[360], any further inferences drawn from these results would be speculative. Yet, it seems feasible to conclude that T cell hyporeactivity of Clec16a KD mice is likely to be brought about by specific alterations of proximal TCR receptor signalling and not by a general T cell dysfunction.

General dysfunction of T cells also appears unlikely considering that in Mixed Lymphocyte Reactions (an experiment that only activates the 5-10% share of T cells that respond to allogeneic MHC) WT and Clec16a KD cells behaved in a comparable manner (Figure 16). This might indicate that allo-reactive WT and Clec16a KD $CD4^+$ cells respond similar to allogeneic MHC. Given that within this experiment the full set of splenocytes was used, an undistorted reactivity of Clec16a KD $CD8^+$ T cells could provide an alternative explanation for this result. Repeating the same experiment with $CD4^+$ cells only or generally assessing the response of $CD8^+$ T cells (e.g. to anti-CD3) could shed further light on this question. (In fact later experiments by Kissler et al. have shown that both $CD8^+$ T cells of WT and Clec16a KD mice respond similarly to stimulation with anti-CD3 providing some support for the latter explanation.)

Conservation of general T cell function is also suggested by normal *in vitro* suppressive capacity of Clec16a KD $CD4^+CD25^+$ T regulatory cells (Figure 9). As already indicated by in vivo CY induction experiments, Clec16a silencing does not seem to impair the function of T regulatory cells.

Differences in the level of expression of the lentiviral transgene imply that this transgene and the associated GFP are only expressed by 70-75% of T lymphocytes in Clec16a KD mice. The T cell activation experiments just discussed had been performed on Clec16a KD $CD4^+CD25^-$ T cells obtained through magnetic separation, i.e. without discriminating between GFP^+ and GFP^- T cells. In order to identify whether the identified T cell hyporeactivity was attributable to T cell intrinsic effects or rather external changes we tested activation of GFP^+ and GFP^- Clec16a KD T cells (sorted by flow cytometry) separately. Experiments revealed comparable levels of proliferative impairment among both GFP^+ and GFP^- Clec16a KD T Cells (Figure 15). This renders it relatively unlikely that T cell hyporeactivity is a consequence of gene silencing within T cells but rather points towards a cause external to T cells, for instance within thymic

selection. Further *in vivo* experiments, such as the generation of bone marrow chimeric mice, could serve to confirm this hypothesis, If irradiated NOD mice reconstituted with Clec16a KD bone marrow are protected from T1D, but irradiated NOD mice reconstituted with Clec16a KD bone marrow are susceptible, it would appear even more unlikely that the cause of T1D protection lies in silencing of Clec16a within hematopoietic cells.

The just discussed findings suggest a T cell extrinsic source of T cell hyporeactivity. Experimental evidence from the Clec16a Drosophila orthologue *ema* indicates a role of this gene in endo- or autophagosomal processes. Further T cell reactivity is likely to be influenced by the way APCs present pMHC complexes. We hence investigated whether Clec16a silencing impaired the function of central and peripheral antigen presenting cells. No abnormalities in peripheral antigen presenting cells could be detected: peripheral lymph nodes and spleens in WT and Clec16a KD mice contained similar shares of B cells, dendritic cells and macrophages, and these cells exhibited comparable expression levels of surface markers such as IgM and FcgRII/III (B cells) or CD54, PD-L1/2 (=CD274/CD273), GITR-L and CD40 (all three types of cells)(Figure 18). Furthermore upon activation with different concentrations of anti-CD40 or LPS B cells of both genotypes responded with comparable changes in the expression of CD86, extracellular and intracellular MHC throughout the whole time course (Figure 19). The same applied for bone marrow-derived dendritic cells.

Since the Clec16a orthologue *ema* in Drosophila melanogaster appears to have a crucial role in endosomal maturation and trafficking we were particularly interested in assessing the endosomal function of macrophages and bone marrow-derived dendritic cells. We thus tested the ability of Macrophages and BMDCs to uptake and process fluorescently labelled E.coli particles (pHrodo™). Neither qualitative (light microscopy, data not shown) nor quantitative (FACS, Figure 20) differences between WT and Clec16a KD cells could be detected throughout the whole time course (max. of 240 min) and for different concentrations of E.coli particles. Due to the above-mentioned differences in expression of the lentiviral transgene we also separately assessed the endocytic capacity of Clec16a KD GFP^+ Macrophages and BMDCs: again we were not able to detect any deviations from WT or Clec16a KD GFP^- cell behaviour (Figure 20).

This suggests that silencing of Clec16a does not importantly compromise endosomal function in Macrophages and BMDC. Proliferation experiments with OT II transgenic T cells provide further support of unaltered peripheral antigen processing and presentation in Clec16a KD mice. APCs from B6 WT and B6 Clec16a KD co-cultured with OVA protein or peptide were able to activate OT II transgenic T cells in a comparable manner (Figure 21). Due to a genetically modified T cell receptor OT II transgenic cells recognise APCs presenting OVA peptide. This finding implies that Clec16a KD does not constrain the ability of APCs to uptake, process and present OVA peptide. Furthermore whether Clec16a KD APCs hade been administered OVA protein or OVA peptide did not significantly affect the resulting activation levels of OT II transgenic T cells (Figure 22). Protein uptake and processing of APC thus does not appear to be compromised significantly by the Clec16a KD.

With peripheral antigen processing and presentation largely unaffected by the Clec16a KD this still left the possibility that intra-thymic changes in central antigen presentation and processing could be responsible for the observed T cell hyporeactivity in Clec16aKD mice. Given the crucial role of thymic epithelial cells (TECs) in T cell selection[250] we hypothesized that Clec16a KD could affect the capacity of TECs to interact with thymocytes. Previous evidence suggested an involvement of the Drosophila ortholog *ema* in autophagy[160] and high levels of constitutive autophagy in TECs[262]. It has further been shown that TEC autophagy has an important function for the generation of MHC-peptide complexes, the presentation of self-antigen to developing thymocytes and immune tolerance[262, 270, 366]. We thus investigated thymic epithelial cells more closely. FACS analysis of surface molecules on antibody stained cortical and medullar TEC (cTEC and mTEC respectively) subsets showed an increased expression of MHCII on Clec16a KD cTECs (Figure 23); this difference was not found for mTECs who instead featured a higher expression of the CD86 co-stimulatory molecule. Having observed these changes in the cTEC compartment and being aware of the contribution of these cells for positive selection and TCR repertoire we decided to compare the distribution of TCRVβ chains in thymocytes and splenocytes of NOD WT and Clec16a KD mice. We were not able to identify any differences for the ten TCRVβ chains examined (Figure 24), this however cannot rule out that any of the other TCRVβ chains are affected.

These findings could indicate that Clec16a KD results in alterations of the function of thymic epithelial cells, which could in turn affect positive and/or negative selection and thus be responsible for the observed T cell hyporeactivity. However, more experiments are warranted to clarify and confirm this notion. Looking at the distribution of Clec16a KD thymocytes throughout the maturation process in the thymus, e.g. analysing of the expression of surface receptors (TCR, CD69), could reveal whether the development of thymocytes is affected by Clec16a KD. *In vitro* differentiation could then serve to confirm whether such maturation defects are is attributable to alterations in the thymic epithelium or the thymocytes themselves. It would also be important to elucidate at which stage of the process the observed hyporeactivity emerges (e.g. earlier during positive or later during negative selection): this could be done testing the *in vitro* reactivity of Clec16a KD thymocytes at varying stages of differentiation, i.e. when $CD4^+CD8^+$ double positive, $CD4^+$ single positive, etc.

Closer examination of the functioning of thymic epithelial cells from Clec16a KD mice and more specifically their autophagy machinery may also be insightful. Not only because of the just discussed link between TEC autophagy and T cell selection, but because evidence by Soleimanpour et al.(2014) suggests that Clec16a is a membrane associated endosomal protein involved in late stage autophagy: it participates in the endo-lysosomal trafficking that engenders fusion of autophagosomes with the lysosomal compartment[161]. For instance immunohistochemical staining of autophagosome-associated proteins could serve to both qualitatively and quantitatively assess autophagy.

Finally, to verify that the findings of this thesis are actually attributable to Clec16a silencing and not the mere result of off-target and insertional effects, it will be necessary to generate a second NOD Clec16a KD line (based on an independent shRNA) on which experiments can then be confirmed[vi].

Summing up, to study the function of Clec16a in an environment susceptible to autoimmune disorders and to shed light on the association between Clec16a and type 1 diabetes, a Clec16a deficient NOD mouse strain was generated. Clec16a KD mice proved

[vi] At the moment of finalisation of this manuscript a second Clec16a KD mouse line based on a different shRNA had already been generated and initial experiments had confirmed the here reported findings.

to be strongly protected against developing type 1 diabetes. Evidence from *in vivo* and *in vitro* experiments revealed that this protection originates from a T cell hyporeactivity. This T cell hyporeactivity appears to result from an impairment of proximal TCR signalling and its cause is highly probable to be external to T cells. Given evidence on the involvement of the Clec16a ortholog *ema* in endo- and autophagosomal processes, alterations in peripheral and/or central antigen presenting cells appeared to be potential reasons for the observed T cell hyporeactivity. While we were not able to identify any changes in quantity and quality of peripheral antigen presenting cells due to Clec16a silencing, surface receptors of thymic epithelial cells in Clec16a KD mice deviated from NOD WT. While this may be indicative of a function of Clec16a for thymic T cell development, further investigations are needed to better understand the role of Clec16a in this process.

7 Summary

Genome wide association studies (GWAS) have identified Clec16a as disease susceptibility gene for numerous auto-immune disorders in particular type 1 diabetes. In spite of this strong genetic link, the role of Clec16a for immune regulation continues to be largely unknown. To study the function of Clec16a in an environment susceptible to autoimmune diseases a Clec16a deficient non obese diabetic (NOD) mouse strain was generated by means of lentiviral RNA interference. Clec16a knock down (KD) mice prove to be strongly protected against developing type 1 diabetes, an effect that is mediated by hyporeactive T effector cells. T cell hyporeactivity seems to result from an impairment of proximal TCR signalling and its cause is likely to be external to T cells. Given evidence on the involvement of the Clec16a Drosophila ortholog *ema* in endo- and autophagosomal processes, alterations in peripheral and/or central antigen presenting cells appeared to be potential reasons for the observed T cell hyporeactivity. While we are not able to identify any changes in quantity and quality of peripheral antigen presenting cells due to Clec16a silencing activation status of thymic epithelial cells in Clec16a KD mice deviates from NOD WT. The findings presented here suggest that thymic T cell development is affected by Clec16a variation. Such a relationship could explain the genetic association between Clec16a variations in humans and susceptibility to immune-mediated diseases, yet further investigations are needed to confirm this notion.

8 Zusammenfassung

Genom-weite Assoziationsstudien haben Clec16a als Kandidaten-Gen für zahlreiche Autoimmunerkrankungen identifiziert, insbesondere für Diabetes Typ 1. Trotz dieser starken genetischen Assoziation ist die Rolle von Clec16a für die Regulierung des Immunsystems weitestgehend unbekannt. Um die Funktion von Clec16a in einer für Autoimmunerkrankungen prädisponierenden Umgebung zu untersuchen, wurde Clec16a im Mausmodell der non-obese diabetes (NOD) Maus mit Hilfe von lentiviraler RNA Interferenz herunterreguliert. Clec16a Knock down (KD) Mäuse zeigen eine deutlich reduzierte Inzidenz von Diabetes Typ 1, ein Effekt der durch hyporeaktive T Effektor Zellen vermittelt wird. Die verringerte Reaktivität der T Zellen ergibt sich vermutlich aus einer Beeinträchtigung des proximalen T Zell Rezeptor Signalweges. Die Ursache dafür scheint außerhalb der T-Zellen zu liegen. Studien die das Clec16a Drosophila Ortolog *ema* mit endo- und autophagosomalen Prozessen in Verbindung bringen, legen Veränderungen in peripheren und/ oder zentralen antigenpräsentierenden Zellen als mögliche Gründe für die beobachtete T Zell Hyporeaktivität nahe. Während infolge der Clec16a Herunterregulierung keine qualitativen und quantitativen Abweichungen in peripheren antigenpräsentierenden Zellen identifiziert werden konnten, zeigte sich ein veränderter Aktivierungsstatus bei Clec16a KD Thymusepithelzellen. Die hier vorgestellten Ergebnisse deuten an, dass die Entwicklung von T Zellen im Thymus durch das Niveau der Clec16a Expression beeinflusst wird. Solch eine Beziehung könnte die Assoziation zwischen Clec16a Varianten im Menschen und die Prädisposition für Autoimmunerkrankungen erklären. Jedoch sind weitere Untersuchungen notwendig, um diesen Zusammenhang zu bestätigen.

9 Abbreviations

AICD	activation induced cell death
AIRE	autoimmune regulator gene
APC	antigen presenting cell
B6	C57BL/6J
BCR	B cell receptor
BMDC	bone marrow-derived dendritic cells
CD	cluster of differentiation/designation
CIITA	major histocompatibility complex transactivator gene
Clec16A	C-type lectin domain family 16 member
cTEC	cortical thymic epithelial cell
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen 4
CY	cyclophosphamide
DC	dendritic cell
DEXI	dexamethasone induced gene
DMEM	Dulbecco's modified Eagle medium
DN	double negative
DNA	deoxyribonucleic acid
DP	double positive
ema	endosomal maturation defective
EGFP	enhanced green fluorescent protein
FACS	fluorescence activated cell sorting
Foxp3	forkhead box P3
GAD65	glutamic acid decarboxylase 65
GFP	green fluorescent protein
GITR	glucocorticoid-induced tumor necrosis factor receptor
GWA	genome-wide-association studies
HLA	human leukocyte antigen
Idd	insulin dependent diabetes
IDDM	insulin-dependent diabetes mellitus
IDO	indoleamine-2,3-dioxygenase
IFN	interferon
IL	interleukin

INS	insulin
ITAM	immunoreceptor tyrosine-based activation motif
kb	kilo base
KD	knockdown
KIAA0350	KI= “Kazusa DNA Research Institute”; AA= reference characters
KO	knockout
LPS	lipopolysaccharide
LTR	long terminal repeat
mAb	monoclonal Antibody
MACS	magnetic cell separation
Ci	Curie
MHC	major histocompatibility complex
miRNA	micro RNA
MS	multiple sclerosis
mTEC	medullary thymic epithelial cell
NKT cell	natural killer T cell
NOD	non obese diabetic
OVA	ovalbumin
PD	programmed death
PKCθ	Proteinkinase Cθ
PLN	peripheral lymph node
PMA	phorbol 12-myristate13-acetate peptide
pMHC	MHC complex
PTPN22	protein tyrosine phosphatase
rmGM-CSF	recombinant murine Granulocyte-Macrophage Colony-Stimulating Factor
RNA	ribonucleic acid
RNAi	RNA interference
RPMI-1640	Roswell Park Memorial Institute cell culture medium
RT	room temperature
SCID	severe combined immunodeficiency
shRNA	short hairpin RNA
siRNA	short interfering RNA
SNP	single nucleotide polymorphism
SOCS1	suppressor of cytokine signalling 1 gene
SP	single positive

T1D	type 1 diabetes
TEC	thymic epithelial cell
TCR	T cell receptor
T eff	effector T cell
Tg	transgene
TLR	Toll-like-receptor
TRA	tissue restricted antigen
T reg	regulatory T cell
WT	wildtype

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EDUCATION

PUBLICATIONS