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The role of the immune system in the immunopathogenesis of autoimmune diseases and the therapeutic modulation of autoimmune reaction by tolerogenic dendritic cells

Role imunitního systému v imunopatogenezi autoimunitních chorob a možnosti terapeutického ovlivnění autoimunitní reakce tolerogenními dendritickými buňkami

Doctoral thesis

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Abstract

Immunotherapy based on dendritic cells (DCs) was first tested in clinical trials for the treatment of cancer in the 1990s. Currently, the ability of DCs to modulate immune responses is also being tested in several clinical studies focusing on autoimmune disease treatment with the aim of suppressing the overactivated immune system and restoring immune tolerance. For this purpose, so-called tolerogenic DCs with considerable suppressive potential are used. Tolerogenic DCs can be generated *ex vivo* from monocytes using pharmacological agents, which in DCs induce a regulatory phenotype with low expression of activation markers, high expression of inhibitory markers and secretion of suppressive cytokines. In the first part of this study, we show that cultivation of human blood monocytes in the presence of glucocorticoid dexamethasone and 19-nor-1,25-dihydroxyvitamin D_2 (paricalcitol) enables *ex vivo* generation of tolerogenic DCs with a highly stable suppressive phenotype characterized by upregulated IL-10 production, inhibitory IL-T3 and PD-L1 molecule expression, the low stimulatory capacity and the ability to induce regulatory T cell development. Moreover, we show that metabolic changes and signaling through NF-κB, p38 MAPK, ERK1/2 molecules and the mTOR/STAT3 pathway play an important role in the maintenance of tolerogenic DC suppressive phenotype and function. In the next part of this study, we show that dexamethasone and vitamin D_2 can also be used to generate tolerogenic DCs of sufficient quality from patients with type 1 diabetes mellitus (T1D), despite their suffering from ongoing autoimmune processes. However, the patients' glycemic control has a crucial impact on the quality of the generated tolerogenic DCs. In fact, long-term hyperglycemia significantly influences not only the tolerogenic DC phenotype but also the possibility to induce stable antigen-specific T cell hyporesponsiveness and promote regulatory T cell differentiation in T1D patients. Thus, these findings provide important information for determination of a group of T1D patients who could benefit from the treatment with the tolerogenic DC-based therapy. In the last part of the study, we evaluate the possibility of generating tolerogenic DCs for the treatment of autoimmune diseases on a scale for clinical testing. We optimize the manufacturing protocol with respect to tolerogenic DC yield, purity, viability, phenotype and function. We also suggest assays that can be routinely used for control of the quality and suppressive capacity of tolerogenic DCs generated for a clinical study. The results summarized in this thesis represent important findings for the generation of tolerogenic DCs in patients with T1D and for the design of a potential clinical trial.

Keywords: tolerogenic dendritic cells, regulatory phenotype stability, type 1 diabetes mellitus, hyperglycemia, immunotherapy

Abstrakt

Imunoterapie založená na dendritických buňkách (DCs, z angl. dendritic cells) byla poprvé testována v klinických studiích zaměřených na léčbu nádorových onemocnění v 90. letech 20. století. V současné době se schopnost DCs modulovat imunitní odpovědi testuje také v několika klinických studiích zaměřených na léčbu autoimunitních chorob s cílem utlumit neadekvátně aktivovaný imunitní systém a obnovit imunologickou toleranci. K tomuto účelu se využívají takzvané tolerogenní DCs disponující výrazným supresivním potenciálem. Tolerogenní DCs se připravují *ex vivo* z monocytů zejména pomocí farmak, jež u DCs indukují regulační fenotyp s nízkou expresí aktivačních znaků, zvýšenou expresí inhibičních znaků a zvýšenou sekrecí tlumivých cytokinů. V první části této práce jsme ukázali, že kultivace lidských monocytů v přítomnosti glukokortikoidu dexamethasonu a 19-nor-1,25-dihydroxyvitaminu D_2 (paricalcitolu) umožňuje připravit tolerogenní DCs s vysoce stabilním supresivním fenotypem charakterizovaným vysokou produkcí IL-10, expresí inhibičních molekul IL-T3 a PD-L1, nízkou stimulační kapacitou a schopností indukovat regulační T buňky. Zároveň jsme také ukázali, že na udržení supresivního fenotypu a funkce tolerogenních DCs se podílejí metabolické změny a aktivace signálních drah zahrnujících NF-κB, p38 MAPK, ERK1/2 a mTOR/STAT3. V další části této práce jsme ověřili, že tolerogenní DCs mohou být pomocí dexamethasonu a vitaminu D_2 připraveny v dostatečné kvalitě také z krve pacientů s diabetem mellitem 1. typu (T1D, z angl. type 1 diabetes mellitus) navzdory tomu, že u nich probíhá autoimunitní proces. V tomto případě se však ukázalo, že významný vliv na kvalitu tolerogenních DCs má glykemická kontrola pacientů. Dlouhodobá hyperglykémie totiž významně ovlivňuje nejenom fenotyp tolerogenních DCs, ale také možnost u pacientů navodit stabilní antigenně specifickou T buněčnou neodpovídavost a vznik regulačních T buněk. Tyto poznatky tak představují důležité podklady pro určení skupiny pacientů s T1D, pro něž by byla terapie založená na tolerogenních DCs vhodná. V poslední části jsme poté testovali možnost vyrobit tolerogenní DCs pro léčbu autoimunitních chorob v rozsahu pro klinické testování. Výrobní protokol jsme optimalizovali s ohledem na zisk dostatečného počtu buněk, jejich čistotu, životnost, fenotyp a funkci. Zároveň jsme také navrhli testy, které mohou být použity pro rutinní kontrolu kvality a supresivní kapacity tolerogenních DCs vyráběných pro účely klinické studie. Výsledky prezentované v této práci přinášejí důležité poznatky pro výrobu tolerogenních DCs u pacientů s T1D a pro nastavení parametrů případné klinické studie.

Klíčová slova: tolerogenní dendritické buňky, stabilita regulačního fenotypu, diabetes mellitus 1. typu, hyperglykémie, imunoterapie

Table of contents

1 Introduction 13
2 Literature overview 15
2.1 Type 1 diabetes mellitus 15
2.1.1 Factors associated with breaking immune tolerance in T1D 15
2.1.1.1 Genetic factors 15
2.1.1.2 Environmental factors 16
2.1.2 Diagnosis and monitoring 18
2.1.3 Mechanisms of T1D pathogenesis 19
2.1.4 A proinflammatory environment in T1D patients 24
2.1.5 The treatment of T1D and clinical trials 25
2.1.5.1 Antigen-specific immunotherapies for T1D 26
2.1.5.2 Non-antigen-specific immunotherapies of T1D 27
2.1.5.3 Combinational therapies 28
2.1.5.4 Cell-based therapies 29
2.2 Tolerogenic dendritic cells 33
2.2.1 Generation of tolerogenic DCs 33
2.2.2 Requirements for tolerogenic DCs used in clinical trials 36
2.2.2.1 A semimature phenotype and regulatory function of tolerogenic DCs 36
2.2.2.2 The phenotypic and functional stability of tolerogenic DCs 40
2.2.2.3 The migratory capacity and route of tolerogenic DC application 40
2.2.2.4 Antigen-specific and non-specific modulation of the immune response 41
2.2.3 Clinical application 42
2.2.3.1 Type 1 diabetes 43
2.2.3.2 Rheumatoid arthritis 44
2.2.3.3 Crohn's disease 46
2.2.3.4 Multiple sclerosis 46
3 Aims of this thesis 50
4 Material and Methods 52
4.1 Monocyte separation 52
4.2 Human tolerogenic DC generation 52
4.3 Tolerogenic DC restimulation 53
4.4 Analysis of a tolerogenic DC phenotype by flow cytometry 53
4.5 Induction of T cell responses by tolerogenic DCs in allogeneic cultures 53

4.6 Suppression of T cell proliferation by tolerogenic DCs 53
4.7 Analysis of T cell responses by flow cytometry 54
4.8 Analysis of cytokines secreted by DCs using ELISA 54
4.9 Statistical analysis 54
5 Results 55
5.1 PART I: NF-κB, p38 MAPK, ERK1/2, mTOR, STAT3 and increased glycolysis regulate stability of paricalcitol/ dexamethasone-generated tolerogenic dendritic cells in the inflammatory environment 57
5.2 PART II: Tolerogenic dendritic cells from poorly compensated type 1 diabetes patients have decreased ability to induce stable antigen-specific T cell hyporesponsiveness and generation of suppressive regulatory T cells 76
5.3 PART III: Testing and optimization of a tolerogenic DC manufacturing protocol for potential application of the tolerogenic DC-based therapeutic product in a clinical trial 95
6 General discussion 110
7 Conclusions 118
8 References 119

Abbreviations

AGE	advanced glycation end product
AHST	autologous nonmyeloablative hematopoietic stem cell transplantation
AP-1	activator protein 1
APC	antigen-presenting cell
BB rat	biobreeding rat
CCR7	C-C chemokine receptor type 7
CD	Crohn's disease
CFSE	carboxyfluorescein succinimidyl ester
CTLA-4	cytotoxic T lymphocyte-associated protein 4
CXCL10	C-X-C motif chemokine ligand 10
CXCR3	C-X-C motif chemokine receptor type 3
DAMP	danger-associated molecular pattern
DAPI	2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride
DAS28	disease activity score 28
DC	dendritic cell
DiViD	Diabetes Virus Detection
DMARD	disease-modifying anti-rheumatic drug
DPT-1	Diabetes Prevention Trial of Type-1
ERK1/2	extracellular signal-regulated kinase 1/2
GAD65	glutamate decarboxylase 65
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	good manufacturing practice
GSK-3β	glycogen synthase kinase 3β
HbA_{1c}	glycated hemoglobin
HLA	human leukocyte antigen
HO-1	heme oxygenase-1
HSC	hematopoietic stem cell
Hsp60	heat shock protein 60
IA-2	islet cell antigen 512
ICA	islet cell antibody
ICOSL	signaling molecule inducible T cell costimulator ligand
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IL	interleukin
IL-2Rα/IL-2A/CD25	α-chain of IL-2 receptor
IL-T	immunoglobulin-like transcript
JAK	Janus kinase
JDRF	Juvenile Diabetes Research Foundation

LPS	lipopolysaccharide
MACS	magnetic activated cell sorting
MHC	major histocompatibility complex
MIP-1	macrophage inflammatory protein 1
MPLA	monophosphoryl lipid A
MS	multiple sclerosis
MSC	mesenchymal stem cell
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor kappa light chain enhancer of activated B cells
NK	natural killer
NOD mice	nonobese diabetic mice
nPOD	Network of Pancreatic Organ Donors with Diabetes
oGTT	oral glucose tolerance test
p38 MAPK	p38 mitogen-activated protein kinase
PBMC	peripheral blood mononuclear cell
PD-L1/2	programmed death-ligand 1/2
PI3K	phosphoinositide 3-kinase
PMA	phorbol-12-myristate-13-acetate
poly(I:C)	polyinosinic:polycytidylic acid
PRR	pattern recognition receptor
PTPN22	protein tyrosine phosphatase nonreceptor type 22
RA	rheumatoid arthritis
SC	stem cell
STAT3	signal transducer and activator of transcription 3
T1D	type 1 diabetes mellitus
TGF	transforming growth factor
Th	T helper
TLR	toll-like receptor
TNF	tumor necrosis factor
Tr1 cell	type 1 regulatory T cell
TRAIL	TNF-related apoptosis-inducing ligand
Treg	$FoxP3^+$ regulatory T cell
Ts	$CD8^+$ suppressive T cell
VDR	vitamin D receptor
VNTR	variable number of tandem repeat
ZnT8	zinc transporter 8

1 INTRODUCTION

Autoimmune diseases represent a group of heterogeneous and clinically distinct disorders, which are characterized by an excessive immune system reaction against normally tolerated components of one's own body (so-called autoantigens) [1]. In type 1 diabetes mellitus (T1D), the pathological immune response is directed against insulin-producing beta cells in the pancreas, and $CD8^+$ and $CD4^+$ T cells play the main role in beta cell mass destruction. Current treatment of T1D is based on the administration of exogenous insulin. The insulin supply prevents hyperglycemic states in patients, which are associated with severe complications such as nephropathy or retinopathy; however, it does not cure the underlying pathological reaction and thereby the basis of the autoimmune process. Recently, an increasing number of studies testing new therapeutic approaches have emerged, along with elucidation of the mechanisms of T1D development.

The tested immunotherapies have included antigen-specific approaches as well as approaches based on general suppression of the immune system. Nevertheless, the most promising results from preclinical testing on animal models of T1D and from initial small clinical trials have not yet been confirmed in subsequent phase III efficacy studies, potentially due to a therapy intervention period that is too short and, thus, unable to induce persistent immune suppression; an extremely robust immunopathological reaction, which is not easy to dampen; or a decline in beta cell mass below a critical threshold, from which the beta cells are unable to recover. However, the partial responses of T1D patients with certain disease parameters suggest the possibility of defining a group of patients who could be successfully treated [2].

Therefore, it is necessary to identify a new type of therapy with long-term effects on the immune system and, simultaneously, to carefully determine the inclusion criteria of patients for a given clinical trial. A promising approach in terms of long-lasting immunomodulation could be provided by cell-based therapies, which could initiate permanent changes in the patient immune system that lead to the attenuation of autoreactive cells and restoration of immune tolerance.

A possible cell-based therapy for autoimmune disorders is provided by so-called tolerogenic dendritic cells (DCs). Tolerogenic DCs can be generated *ex vivo* from the blood monocytes of the patient using various pharmacological or biological agents, pathogen products or methods of molecular biology. They are characterized by an immature or semimature phenotype with increased

expression of inhibitory molecules and secretion of suppressive cytokines. This tolerogenic phenotype enables them to exhibit regulatory functions: inhibition of proinflammatory immune responses and induction of immune tolerance. The ability of tolerogenic DCs to induce the differentiation of diverse subsets of suppressive cells and the modulation of immune responses towards suppression could be crucial for the establishment of long-term tolerance. The safety of tolerogenic DC-based therapies was verified in pilot clinical trials with patients suffering from T1D, rheumatoid arthritis (RA), Crohn's disease (CD) and multiple sclerosis (MS). However, their efficacy remains to be determined [3].

2 LITERATURE OVERVIEW

2.1 Type 1 diabetes mellitus

T1D is an autoimmune disorder characterized by a chronic high blood glucose level (hyperglycemia) that, if untreated, can result in serious health conditions, such as ketoacidosis, kidney failure, heart disease, stroke or blindness. The cause of hyperglycemia development is an immune-mediated destruction of beta cells in the islets of Langerhans in the pancreas, which subsequently leads to restricted production of a key hormone for glucose metabolism – insulin [4,5].

In healthy organisms, the blood glucose level is maintained in a very narrow range, usually between 3.3 and 8.3 mmol/l. Under fasting conditions, insulin secretion by beta cells is attenuated, and hepatic glycogenolysis and gluconeogenesis are activated to maintain a stable blood glucose concentration. In contrast, in postprandial periods, insulin released from beta cells inhibits glucose generation and enhances glucose utilization by certain organs, such as muscles, liver, gut and adipose tissue. Thus, correct insulin secretion and function are absolutely pivotal components in glucose homeostasis maintenance and, moreover, in lipid and protein metabolism; therefore, the lack of insulin gives rise to a complex metabolic disintegration [5].

T1D is typically diagnosed in children and adolescents, even though it can occur at any age. The incidence of T1D varies substantially across different countries. The regions with the highest incidence are Scandinavian countries, especially Finland (with more than 60 new cases per 100 000 people per year), the United Kingdom, Canada, Australia, Saudi Arabia and Kuwait (20–30 new cases per 100 000 people per year). China, India or Venezuela, with only 0.1 cases per 100 000 people each year, are notably on the opposite side of the spectrum. Such significant differences suggest that genetic predispositions as well as environmental factors and their mutual interactions play a role in T1D development [6-8].

2.1.1 Factors associated with breaking immune tolerance in T1D

2.1.1.1 Genetic factors

T1D is a polygenic disorder, with more than 50 loci contributing to disease susceptibility [9,10]. Among them, human leukocyte antigen (HLA) class II genes provide the strongest association with

the risk of TID development, accounting for approximately 50% of the genetic risk. DR4-DQ8 and DR3-DQ2 are high-risk haplotypes that are present in approximately 90% of children with T1D [11]. In contrast, the DR15-DQ6 haplotype is meant to be protective and is found in less than 1% of patients with T1D. HLA class I alleles also contribute to the risk of T1D development, but their impact is less prominent [11].

From the remaining loci, those encoding or regulating insulin, protein tyrosine phosphatase nonreceptor type 22 (PTPN22), α-chain of interleukin (IL)-2 receptor (IL-2Rα, IL-2A or CD25) and cytotoxic T lymphocyte-associated protein 4 (CTLA-4), have been found to significantly increase the risk of T1D development [12]. With regard to insulin, the polymorphism associated with T1D is situated in a promoter region of the gene. The risk for T1D development is determined by the size of variable number of tandem repeats (VNTRs) present in this section. Shorter VNTRs (26–63 repeats) are associated with increased risk, while longer ones (>140 repeats) are protective. It has been proposed that the size of VNTRs in the insulin promoter region influences the binding of a transcriptional factor AIRE and thereby drives the insulin expression level in the thymus: shorter repeats enable only weaker AIRE binding and therefore low insulin expression in the thymus. Low insulin expression might subsequently be reflected by insufficient elimination of autoreactive T cells during the process of negative selection and, in general, might lead to impaired development of peripheral tolerance to insulin [12-14].

PTPN22, CD25 and CTLA-4 are all involved in the regulation of T cell activation, and polymorphisms in the regions coding these proteins are not only associated with T1D but also with other autoimmune diseases. There are two possible mechanisms by which they can influence the establishment of self-tolerance and thereby support the development of autoimmune disorders. First, the weak activation of autoreactive T cells during negative selection in the thymus might cause inappropriate central tolerance development and the escape of autoreactive clones. Second, peripheral tolerance may be impaired as a consequence of insufficient activation of regulatory T cells and/or overactivation of autoreactive T cells through these molecules [12,15-17].

2.1.1.2 Environmental factors

Given that concordance in T1D development between monozygotic twins does not exceed 50%, it is very likely that environmental factors play an important role in T1D development [18]. To date, numerous different environmental factors have been suggested, comprising primarily viral

infections, the composition of the gut microbiota and the diet of the infant. However, none of them has been identified as a specific trigger with an unquestionable impact on the pathogenesis of T1D [19].

Introduction of cow's milk into the child's diet during the first few months of life has been described to increase the risk of T1D onset [20,21]. However, later human and animal studies provided ambiguous results, and recently, a randomized clinical study failed to confirm an association between the increased risk of T1D development and cow's milk [22,23]. Early exposures to wheat proteins, fruit, berries and root vegetables are also suspected to be predisposing agents for T1D [23-25]. In contrast, long-term breastfeeding and omega-3 fatty acid supplementation are thought to have a protective role [26,27]. Finally, vitamin D deficiency or polymorphisms in its receptor (vitamin D receptor, VDR) and enzymes involved in its metabolism may also contribute to T1D development [28].

Recent studies have revealed an association between the pathogenesis of T1D and the composition of the gut microbiota. A significant reduction in diversity of the gut microbiota was observed in patients with diagnosed T1D compared with at-risk individuals [29]. Furthermore, the results from nonobese diabetic (NOD) mice revealed an important protective role of commensal microbes and the microbial balance in general [30]. Given that numerous bacterial strains have been described as potent modulators of both innate and adaptive immunity, it is likely that the imbalance of gut bacteria can potentiate the activation of immune cells that subsequently drive the pathogenic process [31-33]. Alternatively, the imbalance can result in impaired induction or function of regulatory cells, which are then unable to attenuate the pathogenic events [34,35]. Another mechanism of how gut bacteria can influence the pathogenesis of T1D is represented by the phenomenon of the so-called "leaky gut". The increased intestinal permeability before T1D onset may lead to unregulated, increased exposure of immune cells to antigens in the lamina propria and thereby an aberrant autoreactive response of the immune system [36-38].

Finally, viral infection is considered to be one of the most important environmental factors triggering the onset of T1D in predisposed individuals. The initial hypothesis linking T1D and viruses was strongly supported by the study from 1987 showing high levels of HLA class I molecule and interferon (IFN)-α, markers of viral infection, in pancreatic islets of recent-onset T1D patients [39]. The pathogenic mechanism of a viral infection can be based on the direct toxicity to

beta cells, the indirect effect of a local infection, antigen mimicry or modulation of general settings of the immune system. Various viruses have been tested to determine whether they could be responsible for T1D development, such as rotaviruses, Epstein-Barr virus, cytomegalovirus, parvovirus, encephalomyocarditis virus, congenital rubella virus or mumps [40,41]; however, most evidence has been gathered for enteroviruses, more specifically coxsackieviruses [42,43].

2.1.2 Diagnosis and monitoring

Determination of the blood glucose level (glycemia) is a basic method for T1D diagnosis. Glycemia is tested either after a period of fasting (lasting at least 8 hours) or anytime during the day or 2 hours after oral consumption of 75 g glucose (the oral glucose tolerance test, oGTT). Fasting glycemia higher than 7 mmol/l, glycemia during the day higher than 11.1 mmol/l and a level of 11.1 mmol/l in the oGTT confirm a diagnosis of T1D. A level of fasting blood glucose lower than 5.6 mmol/l excludes a T1D diagnosis, and a level between 5.6 and 6.99 mmol/l is considered a risk factor, in which case oGTT is used to confirm the presence of T1D. Other common clinical symptoms are polyphagia (excessive hunger), polydipsia (excessive thirst), polyuria (excessive urination volume) and fatigue. The diagnosis based on the presence of clinical symptoms and increased glycemia can be complemented by the measurement of characteristic T1D-related autoantibodies: islet cell antibodies (ICAs), autoantibodies against insulin, glutamate decarboxylase 65 (GAD65), tyrosine phosphatases (islet cell antigen 512, IA-2) and zinc transporter 8 (ZnT8) [44,45].

The next parameter that can be used for T1D diagnosis is the level of glycated hemoglobin (HbA_{1c}) found in red blood cells. HbA_{1c} is formed during the process of nonenzymatic glycation as a consequence of hemoglobin exposure to plasma glucose. Higher levels of plasma blood glucose are therefore reflected in higher levels of HbA_{1c}. Given that the lifespan of red blood cells is approximately 2–4 months, the level of HbA_{1c} corresponds to an average of glycemia over this previous period and therefore provides information about patients' long-term blood glucose concentration. Despite the advantage of fewer day-to-day perturbations in HbA_{1c} in comparison to blood glucose testing, in terms of disease diagnosis, HbA_{1c} does not reveal all the cases that are identified based on fasting glucose measurements [46,47].

HbA_{1c} examination is also used to monitor previously diagnosed patients to ascertain whether their T1D is well controlled. According to The International Diabetes Federation and the American

College of Endocrinology and American Diabetes Association, HbA_{1c} ≤6.5 or 7% (glycated hemoglobin of total hemoglobin), respectively, is considered an optimal level indicating well-controlled disease; higher levels indicate poor T1D control. The importance of glycemic control is shown by the observation that improving HbA_{1c} by 1% reduces the risk of microvascular complications by 25% and of some macrovascular events [45,48,49].

Treatment for T1D involves the lifelong application of exogenous insulin in combination with an individualized dietary plan, education in self-monitoring of blood glucose levels, recommendations regarding physical activities, and the treatment of associated complications, among others [50].

2.1.3 Mechanisms of T1D pathogenesis

George Eisenbarth first proposed the concept of T1D pathogenesis in 1986. He suggested that T1D development is based on autoreactive T cell-mediated beta cell destruction, which persists over a long period before the overt clinical manifestation of T1D [4]. According to his model (Figure 1), T1D development can be divided into six stages. The autoimmune process is initiated in genetically susceptible individuals (Stage I) by an unknown trigger (Stage II). Cellular and humoral immunity markers (activation molecules on T cells and the production of autoantibodies by B cells) appear as a consequence of immune activation (Stage III). However, metabolic markers appear only when a sufficient number of beta cells are damaged (Stage IV). Critically low numbers of insulin-producing beta cells result in overt hyperglycemia and manifestation of the clinical symptoms of T1D (Stage V). Finally, beta cells are completely destroyed (Stage VI). Although certain parts of this model have been challenged or specified over the years, the concept of T1D pathogenesis as described is still considered to be valid [4].

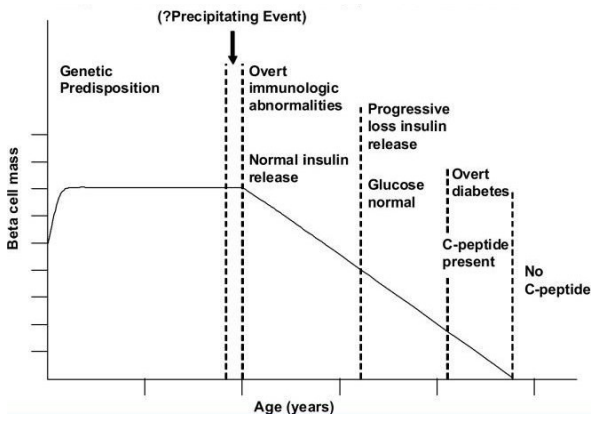

Figure 1. The original model of T1D development proposed by G. Eisenbarth. The individual stages of T1D development are listed according to the level of beta cell mass and patient age [4].

The idea of autoreactive T cells as key executors of the pathogenic process was based on observations from animal as well as human studies. Early experience with pancreas transplantation between identical twins showed that after transplantation of the pancreas to T1D patients from their healthy identical twin, loss of pancreatic function was associated with massive T cell infiltration. Moreover, the administration of T cell-suppressive agents, such as cyclosporin A, was shown to be beneficial for T1D patients [4].

Later, autopsies of new or recent-onset patients who died from ketoacidosis or shortly after diagnosis, corroborated the role of T cells in T1D development. Willy Gepts first discovered lymphocytic infiltration (termed insulitis) in the pancreas of 15 of 22 donors with recent-onset T1D in the 1960s. The subsequent larger study of Alan Foulis in 1986, in which nearly 200 individuals' samples were examined, confirmed Gepts' observations. More recently, original samples from Gepts and Foulis collections have been reanalyzed using modern methods. In this and other studies, attention has been focused on the composition of infiltrating leukocytes. A complex immune attack in the analyzed tissues was noted, and T cells were confirmed to be the main infiltrating cell type, with $CD8^+$ T cells being the most prominent, although $CD4^+$ T helper (Th) cells, $CD20^+$ B cells, natural killer (NK) cells and macrophages were also found. Nevertheless, the intensity or even the presence of immune cell infiltration was found to be very heterogeneous among individual patients, and different Langerhans islands varied in the extent to which they were affected by insulitis [51,52].

The significant drawback of the abovementioned studies is that they were based almost completely on data from patients who died after disease onset and often in a state of ketoacidosis, which represents a more fulminant version of T1D. Therefore, they may not reflect the situation in the majority of slower progressing patients. In this context, analysis of the immune infiltrate in the pancreas of living patients should be indisputably beneficial, as it can provide crucial information about the nature of T1D progression. However, such knowledge is very limited because the pancreas is very difficult to access or even to biopsy. Only two studies examining pathogenic immune processes in the pancreas of living T1D patients have been conducted to date: the Osaka study and the DiViD (Diabetes Virus Detection) study. These studies described findings mainly concerning the phenotype of autoreactive infiltrating T cells. Insulitis was detected in all examined recent-onset donors with 5–60% affected islets. The infiltrate consisted of $CD4^+$ cells, $CD8^+$ T

cells, B cells and macrophages skewed towards the proinflammatory phenotype. Infiltrating T cells produced IFN-γ and expressed the C-X-C motif chemokine receptor type 3 (CXCR3). Infiltrating antigen-presenting cells (APCs) produced tumor necrosis factor (TNF)-α. In addition, insulin-positive islet cells expressed increased levels of IFN-γ-induced C-X-C motif chemokine ligand 10 (CXCL10). In general, these observations strongly supported the role of IFN-γ and Th1 immune response in T1D pathogenesis [53].

In 2007, the Juvenile Diabetes Research Foundation (JDRF) launched a program called the Network of Pancreatic Organ Donors with Diabetes (nPOD). This program enables the collection and distribution of pancreatic and related tissues of cadaveric organ donors with prediabetes or ongoing T1D to researchers with the aim of completing the picture of T1D pathogenesis. The main novel findings emerging from those studies are as follows. Insulitis does not seem to be present in nondiabetic individuals who are positive for a single autoantibody. Additionally, insulitis and insulin-positive beta cells may persist for a long period of time after clinical onset, which underscores the chronic characteristics of T1D and the possibility that beta cells are not completely destroyed at the time of diagnosis; instead, they may be functionally incompetent, or they are able to regenerate (especially in the proinflammatory milieu). Interestingly, autoreactive $CD8^+$ T cells with antigen specificity for only one antigen have been observed in islets of patients within a short interval after diagnosis. In contrast, multiple antigen-specific T cells have been found in patients with long-standing T1D. Finally, a high variability in beta cell loss and intensity of insulitis have been shown not only for patients with short but also for patients with long disease durations [54].

In contrast to human studies, the possibility of directly analyzing leukocyte infiltration in the pancreas or other organs such as pancreatic or mesenteric lymph nodes is the main advantage of a rodent model of spontaneous T1D development – NOD mice or biobreeding (BB) rats. In the NOD mouse model (which shares many of the features of human T1D, such as disease risk conferred by major histocompatibility complex (MHC) genes, anti-insulin autoantibodies and insulitis), $CD8^+$ T cells were found to be responsible for beta cell killing [55]. Furthermore, knockouts or mutations of antigens (insulin or chromogranin A) recognized by autoreactive $CD4^+$ or $CD8^+$ T cells prevented T1D onset [56,57]. Finally, T cells from diabetic mice and rats, unlike autoantibodies, were able to transfer T1D into recipient animals [4]. Nevertheless, knowledge concerning the implications and antigen specificity of islet-infiltrating T cells that emerged from animal models

and resulted in subsequent successful therapy of T1D in those animals, has not yet been successfully translated to humans [53]. The discrepancies may be due to the observation that NOD mice differ from humans in some aspects of T1D. The course of T1D in NOD mice is, in general, more aggressive than in humans, as indicated by the observation that the insulitis in NOD mice is more severe than in humans. Furthermore, the honeymoon phase does not occur in NOD mice (the period after T1D diagnosis when insulin treatment is introduced, which probably enables the remaining beta cells to remain functional and to restart insulin production) [58]. Moreover, a robust ability of beta cells to proliferate was observed in NOD mice, while regeneration seems to occur at a lower rate in human beta cells (probably due to the absence of severe inflammation that is meant to induce beta cell proliferation) [59].

The processes at the cellular and molecular level, which are ongoing during T1D development, have not been completely examined thus far, mainly because the prediabetes stage progresses gradually over a long time in the pancreas, but the lack of accurate noninvasive imaging methods precludes the detection of immune changes as they occur. Nevertheless, findings to date have now given rise to the following model of how cellular and molecular mechanisms are implicated in T1D development (Figure 2). The initial phase is situated in the pancreas, where beta cells of genetically prone individual start to produce IFN-α and express MHC class I molecules to an increased degree, probably as a consequence of a viral infection or other environmental triggers. This phenomenon subsequently leads to beta cell killing by cytotoxic $CD8^+$ T cells and to the activation of APCs that engulf the released beta cell antigens. APCs then migrate into draining lymph nodes and prime autoreactive T cells. Both activated $CD4^+$ and $CD8^+$ autoreactive T cells are responsible for further massive beta cell destruction, which is caused by IFN-γ, perforin and granzyme or Fas-Fas ligand interactions in particular. APCs (DCs and macrophages) further enhance the T cell proinflammatory actions via secretion of the proinflammatory cytokine IL-12, and they also directly contribute to beta cell killing through the secretion of TNF-α, IL-1β and nitric oxide. In addition to T cells, NK cells may also induce beta cell death. B cells participate in antigen presentation and produce autoantibodies – an important detectable marker indicative of the immune deviation. The progressive proinflammatory response is probably enabled due to a break in the central and/or peripheral tolerance mechanism. In this regard, the role of suppressive T cells (mainly $FoxP3^+$ T regulatory cells, Tregs), important regulators of the immune system, is primarily discussed, which are probably present in low numbers or are functionally incompetent in T1D

patients. The next wave of beta cell destruction results in the release of other antigens. The massive immune reaction can subsequently lead to bystander immune system activation, which in general causes the activation of T cells with new antigen specificities. These T cells then induce subsequent and even more severe destruction of beta cells. The beta cell mass is finally depleted or functionally inhibited to such an extent that insulin secretion does not reflect demands [60].

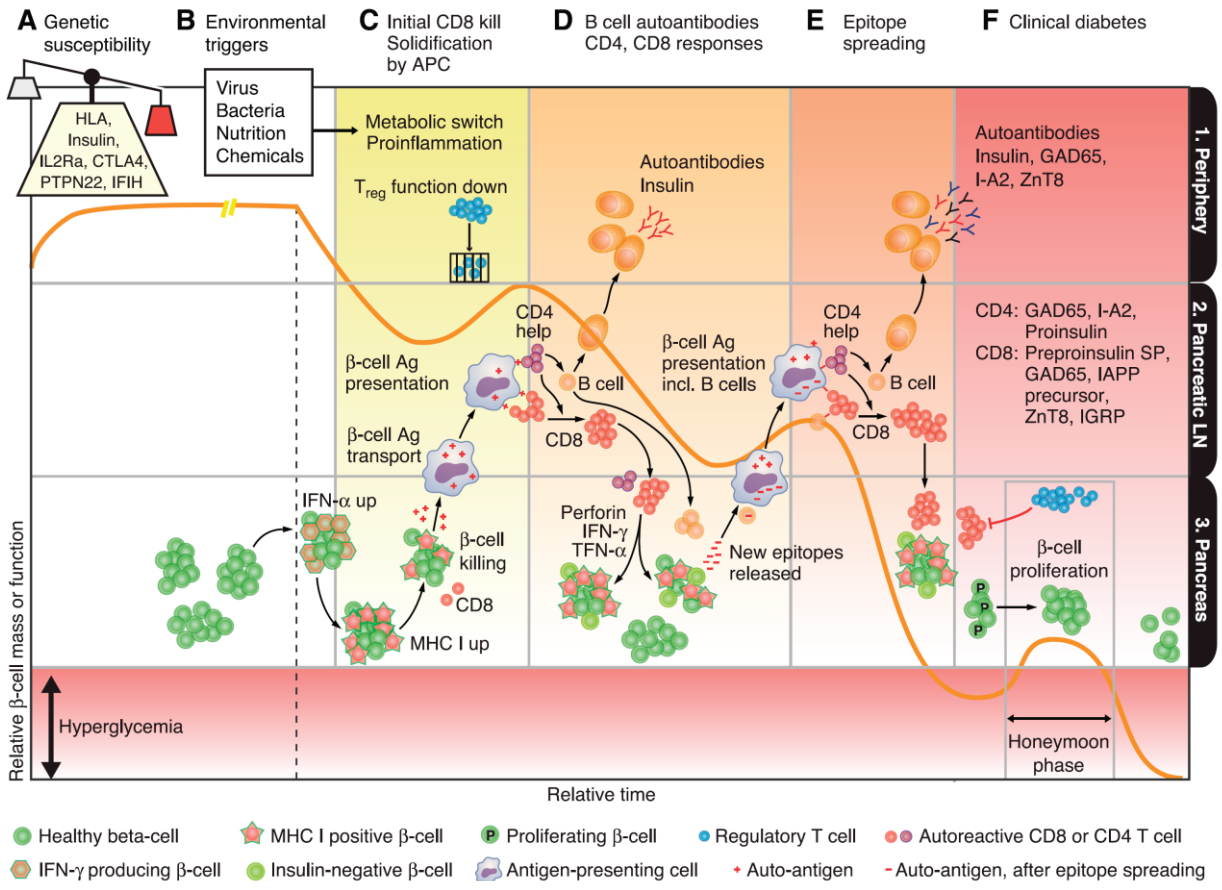

Figure 2. The cellular and molecular mechanisms implicated in T1D development. The immunological events associated with the immunological phases (columns A–F) of T1D development are shown in the context of time (x-axis) and the level of beta cell mass or function (y-axis, represented by the orange line). The events are depicted according to the anatomical site where they occur (rows 1–3). The red zone on the bottom represents a critically low beta cell mass or function, when the production of insulin is insufficient, and an individual is diagnosed with T1D. After T1D diagnosis and treatment introduction, beta cells are temporary reactivated and produce sufficient amounts of insulin (this period is termed the honeymoon phase). APC, antigen presenting cell; CTLA4, cytotoxic T lymphocyte-associated protein 4; GAD65, glutamate decarboxylase 65; HLA, human leukocyte antigen; I-A2, islet cell antigen 512; IAPP, islet amyloid polypeptide; IFIH, interferon-induced helicase; IFN, interferon; IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein; IL2Ra, α-chain of IL-2 receptor; MHC, major histocompatibility complex; PTPN22, protein tyrosine phosphatase nonreceptor type 22; SP, signal peptide; TNF, tumor necrosis factor; Treg, regulatory T cells; ZnT8, zinc transporter 8 [61].

2.1.4 A proinflammatory environment in T1D patients

Given the problems with biopsy access and limited data obtained from cadavers, additional important findings about the immune deregulation associated with T1D is obtained from studies analyzing peripheral blood immune markers.

T1D is mediated by Th1 cells, and IFN-γ plays the major role in pancreatic beta cell destruction during T1D development. However, recent findings have shown that IL-17-secreting cells also participate in T1D pathogenesis. IL-17 neutralization delays the development of T1D in NOD mice [62], and *in vitro* experiments have confirmed that IL-17 in combination with IL-1β and IFN-γ enhance apoptosis of human beta cells [63]. In line with these data, monocytes isolated from T1D patients more potently induce IL-17 production in healthy control memory T cells compared with monocytes isolated from control subjects. In addition, increased numbers of IL-17-secreting T cells have been reported in patients with long-term disease as well as in new-onset patients in comparison to healthy controls [63-65]. Moreover, the secretion of cytokines, such as IL-22 and IL-9, which are implicated in IL-17 immunity, is increased in T1D patients. Their production as well as IL-17 itself seems to be, in contrast to IFN-γ, driven by a high blood glucose level [66,67]. Interestingly, despite the indisputable role of Th1 cells in T1D pathogenic processes in the pancreas, the number of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells is rather reduced in the peripheral blood of newly diagnosed patients. Additionally, higher as well as lower secretion of IFN-γ has been found in cells of recent-onset patients compared with healthy subjects or patients with long-term T1D [68-71].

Given that central and/or peripheral tolerance is supposed to be broken in T1D, the number and functionality of suppressive T cells have been extensively investigated in T1D patients. Significant deficiencies in Treg numbers have been described for newly diagnosed as well as long-term T1D patients compared with healthy controls [72]. However, later analyses have shown no alterations (one study even showed an increase) in the frequency of Tregs in patients with T1D when compared to healthy controls. Rather than variability in Treg numbers, these studies pointed to impaired suppressive capacities of patient Tregs [73-76]. Some studies have also suggested that the reduced Treg suppression capacity is caused by the increased resistance of patients' $CD4^+CD25^-$ responder T cells to such suppression, while the suppressive capacity of Tregs themselves is either variable or rather comparable to those from healthy subjects [77-79]. The observed inconsistencies are

probably caused by the variability in Treg definition. While pilot studies defined Tregs as $CD4^{+}CD25^{+/high}$, later studies included FoxP3 expression as a critical marker, and other molecules were suggested as additional definition markers. Impaired function of Tregs has also been reported in NOD mice, in which defective IL-2 production and signaling are believed to be a cause. Indeed, many studies have shown that exogenous IL-2 supplementation protects NOD mice from T1D development, although crucial factors, such as the dosage, timing or potential combination partners, need to be finely adjusted to achieve the proper effect of IL-2 [17].

Finally, altered proportions of other immune cell populations (conventional DCs, plasmacytoid DCs, monocytes as well as Th cells or cytotoxic T cells) have been reported in T1D patients in comparison to healthy individuals [80-82]. Along with a proinflammatory phenotype of immune cells, elevated serum levels of proinflammatory mediators have also been found in children with T1D compared with healthy controls. Those mediators are the cytokines IL-1α, IL-6, TNF-α, IFN-α, and IFN-γ and the chemokines CXCL10 and macrophage inflammatory protein (MIP-1). Interestingly, no differences in IL-17 and IL-23 levels were found between diabetic patients and healthy controls, and the level of transforming growth factor (TGF)-β was lower in patient than in control serum. Results from the examination of prediabetic patients have revealed a nonsignificant trend towards higher concentrations of proinflammatory cytokines in patient compared with control serum [68,83-86].

Nevertheless, it should be noted that immune responses might differ among individual studies and patients with respect to the T1D characteristics of individual patients, such as disease duration, patient age, patient age at diagnosis, course of T1D, number of autoantibodies, and metabolic state of patients, among others. Overall, the abovementioned observations indicate that the breaking of central and/or peripheral tolerance in T1D patients translates into overreactive effectors, an incompetence of regulatory cells and the proinflammatory setting of the immune system in general.

2.1.5 The treatment of T1D and clinical trials

The current treatment of T1D patients is based on insulin therapy. Life-long insulin replacement, however, treats symptoms but does not cure the disease itself. The increasing incidence of T1D worldwide demands the introduction of new effective therapies. The goal of such newly developed treatments is to preserve beta cells and revive insulin secretion via permanent restriction of

autoreactive cells accompanied by the re-establishment of long-lasting immune tolerance. However, to date, no therapy has been found to meet these requirements in clinical testing.

2.1.5.1 Antigen-specific immunotherapies for T1D

Antigen-specific immunotherapies represent an ideal approach for autoimmune disease treatment, as they should induce specific self-tolerance without compromising the immune system for defense against infections and tumors due to the systemic immunosuppression.

To renew tolerance to the central diabetogenic autoantigen, insulin, its subcutaneous, oral and intranasal administration was tested in clinical trials in individuals with predispositions to T1D. Oral insulin administration was tested in recent-onset T1D. None of those insulin applications resulted in T1D prevention or delayed onset in predisposed individuals or delayed T1D progression in T1D patients. However, an *ad hoc* analysis of data from the phase III Diabetes Prevention Trial of Type-1 (DPT-1) revealed that a subgroup of patients with high levels of anti-insulin autoantibodies benefited from oral insulin administration, as demonstrated by delayed T1D development. This observation led to the follow-up phase III TrialNet Oral Insulin study, which was undertaken to replicate those findings [87-90]. The results obtained for antibody-positive relatives of patients with T1D, however, did not confirm the effect of oral insulin administration on the delay or prevention of T1D development [91]. Nevertheless, different studies based on insulin administration are still ongoing.

In addition to insulin, GAD65, another important autoantigen in T1D, has also been tested for the induction of antigen-specific tolerance. In the first trial consisting of subcutaneous application of GAD65 in the form of GAD-alum (an autoantigen GAD65 and aluminum hydroxide), preservation of residual insulin secretion was observed in a subgroup of patients with a shorter T1D duration. However, in subsequent studies, GAD-alum did not improve the clinical outcomes of recent-onset T1D patients during the 1-year follow-up phase in two (phase II and III) independent studies. The third (phase III) study was terminated. GAD-alum was also ineffective for the prevention of T1D onset in prediabetic individuals [92-94].

Additionally, it has been reported that subcutaneous administration of a part of the human heat shock protein 60 (Hsp60), called DiaPep277, leads to the preservation of beta function and improvement of glycemic control in patients with T1D in phase III clinical trial. However, those

articles were subsequently retracted due to serious misconduct and data analysis manipulation [95,96]. Finally, intradermal administration of a mixture of peptides from islet antigens (called MultiPepT1de) is another approach for the establishment of immune tolerance in T1D patients. The vaccine seems to be well tolerated; however, results regarding efficacy have not yet been published [97].

2.1.5.2 Non-antigen-specific immunotherapies of T1D

Given the indisputable pathogenic role of T cells in T1D pathogenesis, anti-CD3 antibodies (teplizumab and otelixizumab) have been intensively tested in clinical trials in patients with previously diagnosed T1D. The encouraging results from initial clinical trials with both teplizumab and otelixizumab led to initiation of large phase III trials. Although the phase III Protégé study with teplizumab did not meet its primary endpoints, subsequent data analysis revealed greater C-peptide preservation, which was especially evident in a group of responders (younger patients, patients with a short disease duration or better T1D control at study entry) over 2 years of follow-up [98,99]. Analogously, in two other phase II trials with teplizumab, a subgroup of responders with a greater effect on preservation of beta cell function over the 1-year and 2-year follow-up periods was observed. The responders were defined as younger individuals and those who had better metabolic control of T1D at the time of study enrollment (HbA_{1c} level and insulin use). The effect of teplizumab is now being tested in T1D-prone subjects [100,101]. Given that transient adverse events were reported in the initial study with otelixizumab (especially Epstein-Barr virus reactivation), the two subsequent phase III studies tested lower doses of otelixizumab to avoid the side effects [102]. However, the reduced doses were not efficacious and did not achieve the preservation of C-peptide levels or other markers of metabolic control. Therefore, increasing concentrations of otelixizumab are currently being tested in another study of previously diagnosed patients [103,104].

Different testing approaches targeting T cells include anti-thymocyte globulin (T cell depletion), cyclosporine (inhibition of T cell activation), azathioprine and mycophenolate mofetil (T cell inhibition), alefacept (memory T cell depletion), and CTLA-Ig/abatacept (selective modulation of T cell activation). Most of the mentioned treatments have demonstrated at least partial benefits: transient remission in cyclosporine- and azathioprine-treated patients [105,106]; preserved C-peptide secretion and reduced insulin use in alefacept-treated patients [107]; a decrease in HbA_{1c}

and slower beta cell decline in abatacept-treated patients [108,109]. These data together with data from trials with anti-CD3 antibodies have confirmed the possibility of inducing certain metabolic changes in patients with T1D using a treatment that is based on modulation of the T cell response. However, the findings did not confirm the potential to permanently reverse the autoimmune process and induce insulin independence.

Rituximab (anti-CD20 antibody) has been tested in clinical trials to eliminate the pathogenic effect of B cells. Rituximab-treated patients have lower HbA_{1c} levels, reduced loss of C-peptide and reduced insulin requirements. Nevertheless, differences in these parameters were not observed between rituximab- and placebo-treated patients after 2 years of follow-up [110,111].

Other clinical studies have focused on blocking proinflammatory cytokine signaling to attenuate autoreactive immune responses. This approach has been shown to be effective in the treatment of other autoimmune conditions such as RA or CD. While anti-IL-1 therapy (a recombinant, nonglycosylated human IL-1 receptor antagonist, anakinra or a human anti-IL-1β monoclonal antibody, canakinumab) is ineffective [112], anti-TNF therapy (a soluble recombinant TNF receptor fusion protein, etanercept) results in the preservation of beta cell function in recent-onset T1D patients [113]. Additionally, the effect of tocilizumab (a humanized monoclonal antibody against interleukin-6 receptor) is currently being tested [114]. Recently, α1-antitrypsin (an anti-inflammatory agent) has shown promising results in initial studies; however, larger efficacy placebo-controlled studies are needed [115-117].

2.1.5.3 Combinational therapies

In an effort to modulate immune system activity more effectively, combination therapies have been tested. The application of IL-2 in combination with rapamycin is based on the assumption that it would enhance Treg function while inhibiting effector Th1 and Th17 cells. However, regardless of the increased number of Tregs in patients receiving the combination, they suffered from transiently reduced beta cell function, manifested by lower C-peptide levels [118]. More promising results were obtained by testing the combination of anti-thymocyte globulin and pegylated granulocyte colony-stimulating factor (G-CSF), both of which have diverse effects on various components of the immune system. Patients treated with this combination tend to exhibit preserved beta cell function and a transient increase in the proportion of Tregs [119,120]. Finally, GAD-alum, which failed as a single therapy, has been tested in combination with orally administered vitamin D.

A pilot study has reported promising results, with preserved beta cell function and improved immunological markers in the treated group (increased Th2 response, decreased Th1 response and upregulated signs of Treg suppressive functions) [121]. In addition, subsequent studies testing GAD-alum in combination with vitamin D plus ibuprofen, vitamin D plus etanercept or gamma-amino butyric acid, are currently ongoing [122].

In conclusion, although the results of some clinical studies of T1D patients have resulted in at least a partial improvement of the disease course during the treatment application, the beneficial effect always vanished upon treatment withdrawal. The tested therapies probably did not fundamentally alter the underlying pathophysiology of the disease. Thus, it is necessary to focus on the improvement of existing approaches or on the development of novel therapies that will provide long-term tolerance for the successful treatment of T1D. Moreover, careful determination of a group of patients who are suitable for the given therapy is a key issue, as most studies revealed a group of patients for whom the therapeutic approach was more effective.

2.1.5.4 Cell-based therapies

Cell-based therapies that could meet expectations for the induction of long-term tolerance seem to be a new promising therapeutic approach in the field of autoimmune diseases. The utilization of different types of stem cells (SCs), autologous polyclonal Tregs and tolerogenic DCs has been tested for T1D in this regard. Table 1 (p. 32) summarizes completed clinical studies for the treatment of T1D based on cells with regulatory properties.

Autologous nonmyeloablative hematopoietic stem cell transplantation (AHST) is supposed to improve the T1D course through the elimination of autoreactive T cells. Upon this process, patients first undergo mobilization and collection of hematopoietic stem cells (HSCs). Subsequently, patients are treated with chemotherapy for induction of nonmyeloablation of the immune system. Finally, the collected HSCs are transplanted back into patients [123]. According to the results of studies, AHST stabilizes or increases C-peptide levels and, moreover, induces insulin independence in some T1D patients. The subsequent analysis of immune cell populations in the peripheral blood has revealed that the better clinical outcome was associated with higher frequencies of Tregs after transplantation, while the frequencies of autoreactive islet-specific T cells pre- and posttransplantation did not change. However, patients with lower basal numbers of autoreactive T cells demonstrated higher C-peptide levels and a longer insulin-free period after

ASHT. The disadvantage of the AHST approach is transient immune system ablation, which imposes a substantial risk for patients [124-127].

A broad therapeutic potential is the main advantage of mesenchymal stem cells (MSCs). MSCs can differentiate into various cell types; therefore, they can potentially be used for the replacement of patients' destroyed insulin-producing cells. In addition, MSCs have a great capacity to secrete bioactive molecules that support tissue regeneration and affect immune system reactions [128]. They have been tested in several clinical studies focused on the treatment of T1D, alone or in combination with beta cell replacement strategies. The results of these studies have revealed the potential efficacy of MSCs for preserving beta cell function in T1D patients, as demonstrated by higher C-peptide levels in MSC-treated patients in comparison to patients in the control arm during the follow-up period [129-131]. The application of insulin-producing cells differentiated from autologous adipose tissue-derived MSCs together with bone marrow-derived HSCs led to increased C-peptide levels, decreased insulin doses and improved HbA_{1c} levels in treated participants over the 2–3-year follow-up period. Whether this phenomenon is due to the generation and successful implementation of MSC-derived insulin producing cells or to the immunomodulatory properties of SCs requires further evaluation [132,133].

Tregs are an important subtype of T cells with the ability to downregulate immune responses. Thus far, two studies using *ex vivo* expanded autologous Tregs have been completed, and subsequent studies are currently ongoing. In both completed studies, Tregs ($CD4^+CD25^+CD127^{low}$ isolated from the blood of patients) were polyclonally expanded *ex vivo* using anti-CD3/28 plus IL-2 stimulation and subsequently applied to patients. In those studies, neither severe adverse effects nor suppression of immune responses to infectious agents were reported during the follow-up period. In the study reported by Bluestone *et al*., patients with established T1D (disease duration ranging from 14–104 weeks) received a single infusion of $0.05–26\times10^8$ polyclonal Tregs. During the 2-year follow-up, 7 of 14 enrolled patients tended to have stabilized beta cell function, as demonstrated by unaltered C-peptide levels (predominantly in the low-dose group). Approximately 25% of the injected Tregs were present in the circulation after 1 year. Moreover, analysis of the transferred Tregs found in the circulation after this period revealed that they retained a regulatory phenotype and did not differentiate into effector T cells. Despite promising results, larger studies are necessary to confirm these observations. The same group has initiated a study with autologous

polyclonal Tregs in combination with IL-2 administration, which should support the Treg expansion [134]. In the second study reported by Marek-Trzonkowska *et al*., patients with recent-onset T1D (disease duration of no more than 2 months) received a single infusion (10 or 20×10^6/kg b.w.) or two infusions (30×10^6/kg b.w.) of Tregs. Interestingly, an increase in C-peptide levels and a decrease in exogenous insulin requirements were detected in the majority (8 out of 12) of Treg-treated patients. Moreover, two patients remained insulin independent after 1 year, which is in sharp contrast to the nontreated control group, in which none of the patients were insulin independent and only 2 out of 10 patients remained in remission during the follow-up period. The therapy seemed to be beneficial mainly for patients with a short disease duration and high fasting C-peptide levels. The best metabolic outcomes were recorded in patients who received two doses of Tregs (30×10^6/kg b.w.). Taken together, the application of Tregs likely prolongs the survival of beta cells in newly diagnosed T1D patients [135].

Tolerogenic DCs are DCs that are manipulated *in vitro* towards increased suppressive potential. They promote the differentiation of regulatory T cells and B cells and inhibit effector T cell functions. One study examining the application of tolerogenic DCs in T1D patients has been completed to date. Immunosuppressive DCs were generated *ex vivo* from monocytes and modified using antisense oligonucleotides targeting primary transcripts of the costimulatory molecules CD40, CD80 and CD86 to reduce their surface expression, thus decreasing the stimulatory capacity of DCs. Patients received a total of 4 doses administered as a single injection with 1×10^7 DCs every second week. No adverse effects or systemic immunosuppression was detected. Interestingly, in some patients (4 out of 7), the C-peptide level became detectable despite its nondetectability before treatment, and the C-peptide even reached a physiological level in one patient. This observation is encouraging, especially given that patients enrolled in the study suffered from T1D for a long time (5–26 years) and, as observed in the abovementioned study with Tregs, the best efficacy of cell-based therapies could probably be achieved in patients with a short T1D duration. The analysis of immune cell populations did not reveal any changes except an upregulation of the level of $B220^+CD11c^-$ B cells, which were subsequently shown to have suppressive abilities. Three other phase I, II and I/II trials with tolerogenic DCs in T1D patients are currently registered (www.trialregister.nl and www.clinicaltrials.gov, current as of November 2018) [136,137].

Table 1. Clinical studies (completed and with published results) for T1D treatment based on cells with regulatory properties including Tregs, tolerogenic DCs and some examples of SCs.

	Tregs		DCs	SCs				
Trial ID	NCT01210664	ISRCTN06128462	NCT00445913	NCT01068951	NCT01374854	NCT00305344	NCT01350219	
Cell definition	$CD4^+CD127^{lo/-}CD25^+$ Polyclonal Tregs	$CD4^+CD25^{high}CD127^-$ Tregs	Immunoregulatory DCs	Autologous MSCs	Allogeneic UC-MSCs plus autologous BM-MNCs	Autologous Umbilical Cord Blood Transfusion	Cord blood-derived multipotent SCs	Adipose tissue-derived MSC-differentiated insulin-secreting cells plus BM-derived HSCs
Method of generation	Autologous Tregs isolated from the peripheral blood, expanded with anti-CD3/anti-CD28 beads in the presence of IL-2 and AB serum for 14 days	Autologous Tregs isolated from the peripheral blood, expanded with anti-CD3 and anti-CD28 antibodies, IL-2 and autologous serum for 7–14 days	Autologous DCs generated *ex vivo* from monocytes, modified using antisense oligonucleotides targeting primary transcripts of costimulatory molecules CD40, CD80 and CD86	MSCs aspirated from iliac crests and generated in growth media supplemented with lysed human platelets	Umbilical cord Wharton's jelly-derived MSCs generated in growth media supplemented with lysed human platelets; BM-MNCs aspirated from iliac crests	Umbilical cord blood as a source of immunomodulatory cells	Lymphocytes separated from a patient's blood are briefly co-cultured with adherent CB-SCs in the Stem Cell Educator and then returned to the patient	MSCs generated from adipose tissue, cultured for 10 days and further differentiate into insulin-secreting cells for 3 days; HSCs generated from BM
Application route	Intravenously	Intravenously	Intradermal (peri-umbilical region)	Intravenously	Infusion through pancreatic artery	Intravenously	Intravenously	Infused into portal circulation, thymus and into subcutaneous tissue
Cell number	0.05×10^8, 0.4×10^8, 3.2×10^8 or 26×10^8	10 or 20×10^6/kg b.w. or 30×10^6/kg b.w.	10×10^6	$2.1–3.6\times10^6$ autologous cells/kg b.w.	1×10^6/kg b.w. UC-MSCs plus 106.8×10^6/kg b.w. MNCs	-	-	$0.38–6.6\times10^4$/kg b.w. insulin-secreting cells plus $17.4–149\times10^6$/kg b.w. HSCs
Treatment application	1	1 or 2 (6–9 months apart)	4 (2 weeks apart)	1	1	1	1 or 2 (3 months apart)	1
Results	No significant changes in C-peptide levels (stable C-peptide levels in 7/14 patients), HbA_{1c} levels and insulin use after 2-year follow-up; transiently ↑Tregs	↑C-peptide levels (8/12 and 4/6 patients after the first and the second dose, respectively), ↓insulin requirements (8/12, 2 patients insulin-independent) after 1-year follow-up and ↓insulin requirements (4/12) after 2-year follow-up; transiently ↑Tregs, ↓serum IL-1 and TNF-α	Partial ↑C-peptide levels (4/7); transiently $\uparrow B220^+CD11c^-$ regulatory B cells over 1-year follow-up	Preserved or even increased C-peptide AUC (after meal tolerance test) over 1-year follow-up	↑C-peptide AUC (105.7%), ↑insulin AUC (49.3%) ↓fasting glycemia (24.4%), ↓HbA_{1c} (12.6%), ↓insulin requirements (29.2%) over 1-year follow-up	No metabolic improvement (C-peptide levels, HbA_{1c} levels, insulin requirements); ↑Tregs over 2-year follow-up	↑C-peptide levels (fasting as well as after meal tolerance test), ↓HbA_{1c}, ↓insulin requirements (25–38%); ↑Tregs, ↑serum TGF-β over 40-week follow-up after 1 application; residual beta cell function preserved; ↑naïve CD4+ T cells and $CD4^+$ T_{CM} cells, ↓$CD4^+/8^+$ T_{EM} cells over 1-year follow-up after 2 applications (only patients with some residual beta cell function)	↑C-peptide levels, ↓HbA_{1c} levels and insulin requirements (all patients); ↓serum GAD antibody levels
References	[134]	[135,138,139]	[136]	[129]	[140]	[141,142]	[143]	[133]

↑ increase; ↓ decrease; AUC, area under curve; BM, bone marrow; BM-MNCs, bone marrow mononuclear cells; DCs, dendritic cells; GAD, glutamic acid decarboxylase; HbA_{1c}, glycated hemoglobin; HSCs, hematopoietic stem cells; IL, interleukin; MSCs mesenchymal stem cells; SCs, stem cells; T_{CM}, central memory T cells; T_{EM}, effector memory T cells; TGF, tumor growth factor; TNF, tumor necrosis factor; Tregs, regulatory T cells; UC-MSCs, umbilical cord mesenchymal stromal cells. Results may be present only temporarily at specific time points during the study. Information available on www.clinicaltrials.gov (current as of November 2018).

2.2 Tolerogenic dendritic cells

DCs are a central regulator of immune processes with the ability to initiate and modulate adaptive immune responses. DCs reside in peripheral tissues, and their main function is to sample and present antigen. When an infection occurs in the body, DCs become activated in response to stimulation with proinflammatory mediators. The activation process consists of the upregulation of MHC class II and costimulatory molecule expression and upregulation of proinflammatory cytokine secretion, especially IL-12. DCs also increase expression of the C-C chemokine receptor type 7 (CCR7), which enables them to migrate into lymphoid structures where they contact naïve T cells and, due to their activation phenotype, provide them with sufficient signals for activation and expansion.

In case of the acquisition and presentation of antigens in the absence of inflammatory signals, DCs remain in an immature state without increased expression of MHC class II and costimulatory molecules or secretion of proinflammatory cytokines. Therefore, immature DCs provide unsatisfactory signals to T cells recognizing such antigens, which results in antigen-specific unresponsiveness (anergy) or even apoptosis of those T cells. The induction of T cell anergy or apoptosis represents an important mechanism for the maintenance of peripheral tolerance. Another mechanism by which DCs can secure peripheral tolerance to self-antigens is the modulation of the immune response towards suppression. Indeed, DCs can promote the expansion or differentiation of suppressive T and B cells.

Immature DCs, however, still have the capacity to mature and transform into immunogenic DCs, which represents the main obstacle for their application as an immunosuppressive cell-based therapy for the treatment of inflammatory or autoimmune conditions. Therefore, numerous efforts have been focused on the development of so-called tolerogenic DCs with a stable semimature phenotype that is resistant to further activation and that mediates the suppressive function of tolerogenic DCs [144-146].

2.2.1 Generation of tolerogenic DCs

To date, various protocols for tolerogenic DC manufacturing have been described. The process of DC generation is based on the isolation of monocytes from donor peripheral blood and their *ex vivo* differentiation into monocyte-derived DCs using IL-4 and granulocyte-macrophage colony-

stimulating factor (GM-CSF). To induce suppressive abilities in DCs, tolerizing agents are added or immunomodulatory approaches are introduced either during the process of DC differentiation or during the subsequent process of DC maturation. Based on the tolerance-induction strategy, tolerogenic DCs vary in the regulatory mechanisms that they employ to manipulate immune responses.

Several pharmacological or biological agents or pathogen products have been found to induce suppressive properties in DCs. The most used pharmacological agents are, notably, vitamin D, glucocorticoid dexamethasone and the macrolide immunosuppressant rapamycin. However, a variety of other immunosuppressive drugs can be used for tolerogenic DC generation [147], for example, the immunosuppressant mycophenolate mofetil and anti-inflammatory agents such as acetylsalicylic acid, butyric acid, N-acetyl-l-cysteine and aspirin [148,149]. The mechanism by which glucocorticoid dexamethasone drives induction of the tolerogenic phenotype in DCs lies in the regulation of gene transcription. Dexamethasone binds to the glucocorticoid receptor that is then transported from the cell cytoplasm to the nucleus, where it can bind to regulatory DNA sequences of target genes and regulate their transcription. On the one hand, it can promote the transcription of anti-inflammatory genes such as phospholipase A2, which then drives the production of prostaglandins, leukotrienes or an inhibitor of the nuclear factor kappa light chain enhancer of activated B cells (NF-κB), playing the pivotal role in DC maturation. On the other hand, activated glucocorticoid receptor can repress expression of multiple inflammatory cytokines, chemokines and adhesion molecules. Finally, activated glucocorticoid receptor can directly interact with the transcription factors NF-κB and activator protein 1 (AP-1) and thus block expression of their target genes encoding various proinflammatory mediators [150]. In addition to low expression of costimulatory molecules, dexamethasone-conditioned tolerogenic DCs are characterized by upregulated expression of immunoglobulin-like transcript (IL-T)2/3 inhibitory molecules, secretion of high amounts of IL-10 and increased capacity to promote Treg differentiation [151].

The tolerizing effect of vitamin D and its analogues is mediated via its receptor VDR, which acts as a transcription factor. After vitamin D binding, activated VDR receptor forms a heterodimer with the retinoid X receptor and activates the expression of indoleamine 2,3-dioxygenase (IDO), chemokines, IL-10, TGF-β and other molecules involved in inhibitory immune responses. Alternatively, VDR has been shown to interact with phosphoinositide 3-kinase (PI3K) and activate

its downstream pathway. The PI3K-driven pathway controls the activation of transcription factors such as NF-κB and glycogen synthase kinase 3β (GSK-3β), which impact the expression of costimulatory molecules and secretion of cytokines. Moreover, the pathway including PI3K, Akt and the mammalian target of rapamycin (mTOR) is also crucial for induction and maintenance of the tolerogenic phenotype in DCs due to its role in the regulation of DC metabolism. Glucose availability and glycolysis activation regulated by PI3K/Akt/mTOR axis are essential for reprogramming DCs to a tolerogenic phenotype. Once reprogrammed, the tolerogenic phenotype is stable and independent of the glycemic or oxygen conditions [152]. Tolerogenic DCs prepared using vitamin D typically express inhibitory molecules such as IL-T3 and programmed death-ligand (PD-L)1, secrete enhanced levels of IL-10 and promote IL-10-producing type 1 regulatory T cells (Tr1) cells [151]. Given that vitamin D and dexamethasone use distinctive pathways for tolerance induction, their combination might strengthen the suppressive potential of DCs. Indeed, the capacity to suppress allogeneic T cell activation is higher the in case of dexamethasone plus vitamin D-treated DCs compared with DC conditioned with either dexamethasone or vitamin D alone [149,153]. Interestingly, dexamethasone has been found to enhance VDR expression and thereby vitamin D/VDR-mediated suppressive effects, which supports the idea of utilizing vitamin D and dexamethasone in combination for tolerogenic DC generation [154].

Among the biological molecules, the suppressive cytokines IL-10 and TGF-β are the most studied [146]. The tolerogenic effect of IL-10 is mediated via IL-10 receptor and downstream activation of Janus kinase (JAK) and signal transducer and activator of transcription 3 (STAT3) axis. IL-10 stabilizes DCs in an immature state with impaired expression of costimulatory molecules, augmented expression of IL-T molecules and other inhibitory markers and low production of IL-12. IL-10 also stimulates DCs to further produce IL-10 and thereby activates a positive feedback loop, which strengthens the regulatory phenotype in DCs. Tolerogenic DCs generated using IL-10 exhibit significant regulatory potential and preferentially promote the differentiation of IL-10-producing Tr1 cells rather than $FoxP3^+$ Tregs [151,155].

The application of pathogen products for tolerogenic DC *in vitro* generation takes advantage of the ability of pathogens to modulate the host immune system towards a suppressive immune response. Many components of various pathogens, including viruses, bacteria as well as parasites, have been recognized as tolerogenic DC inducers, such as cholera toxin of Vibrio cholerae, lysophosphatidyl

serine of Schistosoma mansoni, lysophosphatidic acid of Lactobacillus acidophilus and many others [148].

Genetic manipulation of DCs represents another strategy for the induction of a tolerogenic phenotype in DCs. One applied approach is the utilization of RNA interference to silence the expression of costimulatory molecules, IL-12 cytokine or NF-κB transcription factor. Alternatively, DCs can be manipulated towards constitutively high expression of molecules that mediate suppressive signals to T cells, e.g., IL-10, IL-4, CTLA-4, IDO and PD-L1, or that mediate apoptosis-inducing signals, e.g., Fas (CD95) and TNF-related apoptosis-inducing ligand (TRAIL). Finally, transfection of DCs with microRNAs that normally control DC functions can be used, e.g., miR-155, miR-34, miR-21 or miR-23-b [146,156].

A different approach for tolerogenic DC generation is the cultivation of DCs with other cell types, such as SCs, or with apoptotic bodies from dying cells, which induce the suppressive phenotype in DCs [148].

2.2.2 Requirements for tolerogenic DCs used in clinical trials

As mentioned above, specific agents used for tolerogenic DC generation give raise to tolerogenic DCs with a specific phenotype and distinct suppressive functions. However, some general requirements and phenotypic or functional markers that can be used to define clinically applicable tolerogenic DCs are as follows.

2.2.2.1 A semimature phenotype and regulatory function of tolerogenic DCs

First, tolerogenic DCs typically have an immature or semimature phenotype, which enables them to exhibit their two basic functions: inhibit proinflammatory immune responses (induction of anergy or apoptosis in effector T cells) and promote immune tolerance (*de novo* induction of diverse subsets of suppressive cells or modulation of immune responses towards suppression). The immature or semimature phenotype is characterized by reduced or unchanged expression of MHC class II molecules and low or intermediate expression of the costimulatory molecules CD80, CD83, CD86 and CD40. A level of costimulatory molecule expression then influences mechanisms employed in mediating tolerogenic DC functions. Low surface exposure of costimulatory molecules is associated with the induction of anergy in T cells that recognize antigens presented on such DCs. This effect is primarily mediated through the lack of sufficient CD80/86 stimulation

of CD28, the key coreceptor on T cells essential for T cell activation. Moreover, low or no signal through the CD28 coreceptor serves as a prerequisite for induction of FoxP3$^+$ Treg differentiation. The intermediate level of CD80/86 expression is, by contrast, necessary for CD28-dependent maintenance of FoxP3$^+$ Tregs and for the generation of IL-10-producing Tr1 cells from naïve precursors. Tr1 cells are, in addition to FoxP3$^+$ Tregs, another T cell subset with suppressive functions. Finally, sufficient expression of CD80/86 is important for the differentiation of Th2 secreting IL-10 plus IL-4 and for the induction of IL-10 production in other Th subsets. In this case, CD80/86 signaling is mediated via the T cell high-affinity receptor CTLA-4 [157].

In contrast to low expression of costimulatory molecules, tolerogenic DCs typically upregulate the expression of inhibitory markers, including PD-L1 and PD-L2, molecules from the IL-T family (IL-T2, IL-T3, IL-T4) or their ligand (HLA-G), and galectins. PD-L1 and PD-L2 belong to a group of surface molecules that are upregulated upon DC maturation and act as ligands of the PD-1 molecule expressed on activated T cells, providing them with inhibitory signals. Indeed, the interaction between PD-L1/2 and PD-1 can block T cell effector functions, proliferation or induce T cell apoptosis [158]. PD-L1 expression on tolerogenic DCs has been shown to be crucial for the induction of a regulatory phenotype in CD4$^+$ T cells. Blockade of PD-L1 on tolerogenic DCs during T cell priming drives T cells towards the proinflammatory Th1 phenotype with limited suppressive potential [149,159,160]. Moreover, PD-L1 is involved in both the induction as well as the maintenance of FoxP3$^+$ Tregs [161]. Overexpression of IL-T3 and IL-T4 on tolerogenic DCs is associated with their decreased capacity to stimulate T cells, which is manifested by inhibited proliferation and production of IFN-γ and IL-17, as well as, in contrast, an enhanced capacity to induce differentiation of FoxP3$^+$ Tregs, IL-10-producing Tr1 cells and CD8$^+$ suppressive T cells (Ts) [162-164]. Additionally, expression of IL-T molecules on the tolerogenic DC surface guarantees the activation-resistant phenotype, since IL-T3$^+$ or IL-T4$^+$ DCs display impaired NF-κB-dependent transcription of costimulatory molecules and decreased toll-like receptor (TLR)-mediated production of proinflammatory cytokines [165]. Among the galectin family, gal-1 and gal-3 have been identified as regulatory receptors that are expressed endogenously or exogenously by tolerogenic DCs. Gal-1/3 molecules modulate the immunogenic potential of DCs, favoring the induction of IL-10-producing T cells while downregulating the polarization of T cells into Th1 or Th17 [166-168].

IDO is another molecule that is typically expressed by tolerogenic DCs. IDO has two distinct functions, signaling and enzymatic, both of which contribute to its suppressive effect. Regarding IDO enzymatic function, IDO catalyzes transformation of L-tryptophan to N-formylkynurenine. Depletion of tryptophan, an important energy source for activated T cells, together with the accumulation of kynurenines causes proinflammatory Th1 cell apoptosis and inhibits T cell proliferation [169]. In contrast, a low tryptophan concentration and tryptophan degradation products promote the differentiation of naïve T cells into Tregs. This effect may be driven by IDO-dependent expression of IL-T3/4 by DCs [162,170]. Signaling-mediated suppression via IDO is associated with the induction of TGF-β production by DCs [171]. Finally, among various pattern recognition receptors (PRRs), the expression of TLR2 is associated with the tolerogenic phenotype of DCs. Signaling through this molecule induces IL-10 production in DCs and favors the induction of Tregs [172].

Complementary to the surface expression of inhibitory molecules, tolerogenic DCs secrete cytokines with a regulatory effect. TGF-β and IL-10 production seems to be essential for the capacity of tolerogenic DCs to induce differentiation of suppressive subsets of T cells, $FoxP3^+$ Tregs and Tr1 cells, respectively [157]. Additionally, IL-10 and TGF-β suppress T cell responses in general and, moreover, further strengthen the immunosuppressive capacity of tolerogenic DCs via autocrine signaling or via a suppressive T cell-mediated positive feedback loop. Production of IL-27 polarizes the DC phenotype towards a tolerogenic one with decreased secretion of proinflammatory cytokines, high production of IL-10 and inhibitory molecule expression. IL-27 also reduces the polarization of T cells into Th1 or Th17 and promotes the differentiation of $FoxP3^+$ Tregs or Tr1 cells [173]. In line with the secretion of mediators with an anti-inflammatory effect, tolerogenic DCs do not secrete proinflammatory cytokines. Especially, the absence of IL-12 secretion is crucial because IL-12 is a key driver of the proinflammatory Th1 response, which is thereby prevented. Alternatively, tolerogenic DCs may secrete IL-4, which also blocks the expansion of Th1 cells and instead promotes the Th2 response [146].

To summarize, tolerogenic DCs are characterized by the specific combination of expressed surface molecules and produced cytokines according to the applied approach for their generation. Their tolerogenic phenotype determines the mechanisms employed for the manipulation of immune responses (Figure 3). In addition to the abovementioned molecules, many others (signaling

molecule inducible T cell costimulator ligand (ICOSL), death-inducing molecule CD95L or heme oxygenase-1 (HO-1) etc.) can contribute to the suppressive capacity of tolerogenic DCs and drive the final fate of T cells [156]. For clinical application, the specific combination of molecules and cytokines that are present or absent can be used as a marker for tolerogenic DC quality control and as a marker of their suppressive potential [174].

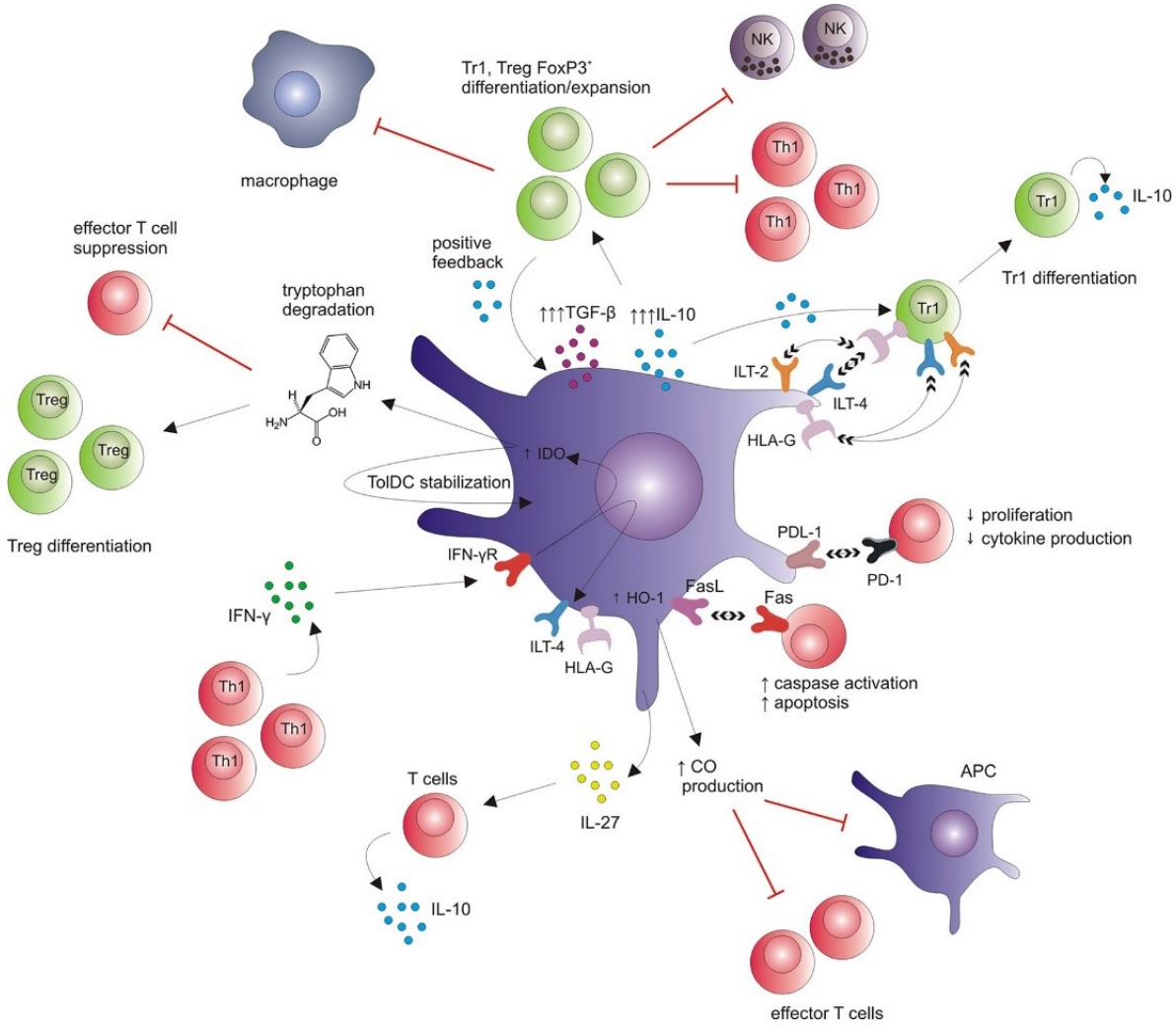

Figure 3. Tolerogenic DC markers and mechanisms of immune response modulation. In accordance with the specific spectrum of surface-expressed molecules, secreted soluble factors and intracellular enzymes, tolerogenic DCs employ different mechanisms to modulate immune responses towards immune suppression and restoration of immune tolerance. These mechanisms include the inhibition of effector T cells, inhibition of antigen-presenting cells, induction of apoptosis in effector T cells, skewing of T cells towards a suppressive phenotype and induction of different regulatory T cell populations. APC, antigen-presenting cell; CO, carbon monoxide; FasL, Fas ligand; HLA-G, human leukocyte antigen-G; HO-1, heme oxygenase-1; IDO, indoleamine-2,3-dioxygenase; IFN-γR, interferon-γ receptor; IL, interleukin; ILT, immunoglobulin-like transcript family; NK, natural killer; PDL-1, programmed death-ligand 1; TGF-β, transforming growth factor β; Th1, T helper cells type; Tr1, type 1 regulatory T cell; Treg, FoxP3+ regulatory T cell [175].

2.2.2.2 The phenotypic and functional stability of tolerogenic DCs

The stability of the tolerogenic DC suppressive phenotype is the second key criterion of tolerogenic DCs that needs to be achieved. In fact, resistance to a further maturation stimulus is a prerequisite for tolerogenic DC clinical applications, since a potential transformation of immature or semimature DCs into fully mature DCs would lead to acquisition of the capacity to promote immunogenic instead of protective immune responses and thereby exacerbation of the patient's autoimmune condition [176]. This resistance must be established against microbial, viral or other pathogen-associated molecules that cause strong DC activation during an infection. Moreover, tolerogenic DCs must remain refractory to danger-associated molecular patterns (DAMPs) and inflammatory conditions, which are present in patients with autoimmune diseases and are able to induce DC maturation [177]. A comprehensive study by Boks *et al*. showed that tolerogenic DCs prepared according to different protocols remained refractory to treatment with TLR7/8 and TLR2 agonists added together with IFN-γ, in contrast to immature DCs. Indeed, tolerogenic DCs did not largely upregulate costimulatory molecules and did not secrete increased amounts of proinflammatory cytokines upon restimulation [155]. Similarly, a study by Naranjo-Gómez *et al*. showed a stable anti-inflammatory cytokine profile in rapamycin, dexamethasone or vitamin D_3-conditioned tolerogenic DCs after TLR4 agonist treatment [178]. The phenotypic stability of tolerogenic DCs prepared with the combination of dexamethasone and vitamin D_3 was confirmed upon restimulation with proinflammatory cytokines, lipopolysaccharide (LPS) and peptidoglycan [179]. Taken together, these studies support the stability of the tolerogenic DC phenotype.

2.2.2.3 The migratory capacity and route of tolerogenic DC application

The requirements for the semimature regulatory phenotype and its stability can be extended by other properties that could improve the therapeutic potential of tolerogenic DCs, such as a sufficient migratory capacity. Given that expression of the chemokine receptor CCR7 is low on immature DCs and increases during maturation to enable DCs to migrate into secondary lymphatic organs where they potentiate T cell responses, it is not unexpected that immature or semimature tolerogenic DCs display a poor migratory capability. A very low migratory efficacy (approximately 1%) of intravenously, intradermally or subcutaneously injected DCs has been confirmed in mice, monkeys and humans. These studies also demonstrated that the selected route of administration affects the final site of DC accumulation and their potency to modulate the immune response [180-183]. Therefore, it is necessary to evaluate different routes of tolerogenic DC application and a

number of applied DCs in terms of their homing efficacy and consequent capacity to influence T cell responses in the given region. The optimal setting can also differ for different types of tolerogenic DCs and for different indications. In human clinical studies with tolerogenic DCs, various administration routes are currently used: intraperitoneal application in CD, intradermal application in T1D and RA and subcutaneous or direct intraarticular application also in RA [184]. Other approaches for improvement of the migratory capacity of tolerogenic DCs have also been tested. One possibility is genetic modification of the generated tolerogenic DCs to constantly express CCR7 receptor. Indeed, tolerogenic DCs manipulated in this way show an improved capacity to migrate into lymph nodes and provide prolonged heart allograft survival in a mouse model [185]. A similar observation of improved CCR7-dependent DC migration has also been reported in DC-based vaccines for cancer therapy, which enabled the application of a smaller number of DCs for sufficient induction of T cell activation and *in vivo* efficacy [186]. Another possibility is direct intralymphatic injection of DCs [187]. Alternatively, in the case of some organ-specific autoimmune diseases, the application of tolerogenic DCs *in situ* may be feasible, as it has been tested for RA [184].

2.2.2.4 Antigen-specific and non-specific modulation of the immune response

Analogously to the question of the most appropriate route for tolerogenic DC administration, there is no consensus regarding the use of antigen-loaded or unloaded tolerogenic DCs. The antigen-specific approach should generate immune suppression only to the required, disease-related antigen and thereby avoid the induction of systemic immunosuppression and disruption of immune surveillance, which could result in the development of severe infections or malignancies.

In contrast, autoreactive T cells are often present in autoimmune diseases, with more than one antigen specificity due to the antigen spreading phenomenon or neo-epitope development. Therefore, the induction of tolerance to only one of them could not be sufficient, and in some autoimmune diseases (for example, inflammatory bowel disease), autoantigens have not even been identified [156,188,189]. In addition, some studies have reported that the utilization of antigen-unloaded tolerogenic DCs prevents disease manifestation, while the utilization of autoantigen-loaded tolerogenic DCs leads to disease acceleration [190-192].

The application of antigen-loaded tolerogenic DCs seems to be beneficial, particularly in the case of MS and RA, as repeatedly demonstrated in relevant mouse models [191-194]. In line with

observations from animal studies, in all clinical trials with patients suffering from RA and MS, tolerogenic DCs pulsed with antigens have been used. Interestingly, in addition to known myelin peptides in the case of MS and RA-related autoantigens (mainly citrullinated proteins or peptides) used in most clinical studies, autologous synovial fluid has been used for DC antigen-loading in one RA clinical trial, which provides a broad spectrum of patient-specific autoantigens to which immune tolerance may be established [184]. The antigen-unloaded manner was effective notably in the prevention of T1D in mouse models and is currently being tested in clinical trials focused on tolerogenic DCs for T1D treatment in humans [184,190]. One possibility for how antigen-non-specific suppression can be mediated is that tolerogenic DCs engulf disease-related antigens after their administration to the patient and subsequently induce at least partial antigen-specific tolerance [195]. However, there is a risk of induction of suppression to unwanted antigens. The other possibility is that the induction of suppression in an antigen-unspecific fashion may be based on a general reversal of an immunostimulatory environment – for example, by IL-10 secretion. This phenomenon should prevent disease-related T cell activation, further T cell recruitment, cytokine production and tissue destruction, all of which contribute to the deterioration of a disease course. This approach seems to be advantageous mainly for organ-specific autoimmune diseases or for tolerance induction in the case of transplantation [189].

2.2.3 Clinical application

The first application of *ex vivo* generated autologous DCs to healthy humans was performed by the group of Ralph Steinman in 1999. Their study demonstrated the possibility of inducing a T cell antigen-specific response *in vivo* by the injection of activated antigen-pulsed DCs [196]. Since then, the efficacy of DC-based vaccine to expand anti-cancer T cell immunity has been widely tested in numerous clinical studies. In 2001, the same group also conducted the pioneering application of immature DCs. In that study, they showed that the application of *ex vivo* generated antigen-pulsed immature DCs led to the induction of long-lasting (more than 6 months) antigen-specific suppression of $CD8^+$ T cells, confirming the rationale for the utilization of DC-based therapies not only for boosting the immune system but also for situations in which the induction of immune system suppression is desired [197]. However, progress in the field of tolerogenic DCs with the potential to attenuate the overactivated immune system has been slower than in the case of immunogenic DCs. The first clinical trial with tolerogenic DCs was conducted in 2011 with

patients suffering from autoimmune T1D [136], and tolerogenic DCs are currently being tested in clinical studies for the treatment of T1D, RA, MS and CD [156].

Apart from autoimmune diseases, inflammatory and allergic diseases as well as transplant rejections after organ transplantations represent the main indications where tolerogenic DCs can be used as a treatment. The necessity to identify new therapeutic approaches for these disorders is underscored by the fact that current therapies, such as immunosuppressive drugs, do not solve the causation of diseases or transplant rejections, and thus, patients are reliant on the administration of life-long treatments. Moreover, current therapies are often accompanied by severe side effects. Therefore, in comparison to standard treatments, the main advantages of the utilization of tolerogenic DCs is a complex modulation of the immune system and the possibility to restore long-lasting immune tolerance. However, to achieve the best possible results, it is important to carefully determine all the parameters in terms of tolerogenic DC application. The most important are as follows: dose, route and frequency of tolerogenic DC administration and timing of application with respect to the course of disease in individual patients [184]. Table 2 summarizes (p. 49) clinical studies for the treatment of autoimmune diseases using tolerogenic DCs that have been completed to date or are currently ongoing, based on data available at www.clinicaltrials.gov, current as of November 2018.

2.2.3.1 Type 1 diabetes

T1D was the first disorder in which the application of monocyte-derived tolerogenic DCs was tested. A phase I clinical study of 10 patients (7 treated subjects and 3 controls that obtained DCs prepared without *ex vivo* tolerogenic manipulation, so-called control DCs) suffering from long-term T1D (5–26 years after diagnosis) demonstrated that the application of *ex vivo* generated tolerogenic DCs is safe and well-tolerated in patients.

Monocytes used for the generation of DCs were separated by elutriation from leukocytes obtained via leukapheresis and subsequently cultured in the presence of IL-4 and GM-CSF for 6 days. For induction of immunosuppressive abilities in DCs, the harvested DCs were treated with the mix of antisense oligonucleotides, which impaired expression of the costimulatory molecules CD40, CD80 and CD86. Finally, the DCs were tested for their viability, sterility, endotoxin level and mycoplasma presence. They were then divided into aliquots, and the first aliquot of freshly generated DCs was injected into patients; the remaining aliquots were cryopreserved until further

application. The schedule of treatment comprised four injections with 1×10^7 DCs administered every second week. Every dose of each treatment was divided among 4 intradermal injections applied close to the expected anatomical location of the pancreas. This application arrangement was introduced with the aim of achieving the best DC migration into the pancreatic and peri-pancreatic lymph nodes.

Promising results, mentioned in the chapter 3.1.5.3, supported further testing of *ex vivo* engineered tolerogenic DCs in subsequent studies. These studies should evaluate the efficacy of tolerogenic DCs to improve glycemic control (C-peptide level) in new/recent-onset patients. The first phase II study is planned for 24 patients suffering from T1D for a maximum of 100 days, and another phase I/II study is planned for 90 T1D patients suffering from T1D for a maximum of 6 months [156]. Finally, another phase I study with higher doses of tolerogenic DCs (5×10^6, 10×10^6 and 20×10^6) with patients suffering from T1D for at least 18 months is currently recruiting.

2.2.3.2 Rheumatoid arthritis

RA is an autoimmune disorder characterized by chronic joint inflammation. The inflammation is associated with immune system cell infiltration and results in cartilage destruction, bone erosion and joint deformity in general. As the disease progresses, the nonjoint structures are affected, which can cause severe cardiovascular and respiratory complications.

The first-in-human study for the treatment of RA with tolerogenic DCs (the final product was named Rheumavax) has been completed, and the results were published in 2015. Only patients (18 treated subjects, 16 controls) with a confirmed HLA risk genotype and positivity for autoantibodies against citrullinated peptide antigens and a disease duration of at least 3 months but no longer than 1 year were enrolled in the trial. Tolerogenic DCs were generated from purified monocytes for 2 days using the NF-κB inhibitor Bay 11-7082, which disrupts the expression of DC maturation markers. To induce antigen-specific immune system suppression, tolerogenic DCs were exposed to four RA-associated citrullinated peptides of vimentin, collagen type II and fibrinogen α and β chain. Rheumavax was applied in patients as a single intradermal injection containing $0.5–1\times10^6$ or $2–4.5\times10^6$ tolerogenic DCs. Rheumavax was well tolerated, did not cause disease deterioration in patients with minimal disease activity and demonstrated biological activity in patients with active disease. Indeed, patients with active disease who received Rheumavax showed improvement in DAS28 (disease activity score 28, a system used to assess the severity of RA). Moreover, a

decreased level of effector T cells, increased ratio of regulatory-to-effector T cells and reduced serum levels of proinflammatory cytokines, chemokines and C-reactive peptide were found in patients one month after treatment, indicating an anti-inflammatory effect of the tolerogenic DC-based treatment [198].

Another clinical trial with tolerogenic DCs for RA treatment was the Autologous Tolerogenic Dendritic Cells for Rheumatoid and Inflammatory Arthritis (AuToDeCRA) study, which included participants (9 treated, 3 controls) suffering from RA for 2–43 years who were unresponsive to at least one currently used anti-RA drug (disease-modifying anti-rheumatic drug, DMARD). Monocytes were separated using density centrifugation followed by anti-CD14 magnetic separation with the CliniMACS system (magnetic activated cell sorting, MACS). For the generation of tolerogenic DCs, dexamethasone and vitamin D_3 were used. They were added during the process of DC generation: dexamethasone on day 3 and 6, vitamin D_3 on day 6. On day 6, DCs were activated with monophosphoryl lipid A (MPLA) and cultivated with autologous synovial fluid obtained from affected joints, which allowed the preparation of therapeutic DCs with a range of relevant autoantigens specific to the particular patient. After 20 hours, tolerogenic DCs were tested for viability, sterility, surface marker expression and cytokine production. Tolerogenic DCs were not cryopreserved prior to administration. Participants received 1×10^6, 3×10^6 or 10×10^6 tolerogenic DCs. Intraarticular application was chosen since rapid disease worsening could be easily observed, potentially irrigated and locally treated with corticoids. At 7 days after administration, synovitis of the target knee or systemic synovitis developed in some patients who received 1×10^6 or 3×10^6 tolerogenic DCs. Additionally, all the patients from those cohorts as well as all three controls (received a single injection of saline) had to be treated with local corticoids for arthroscopic synovitis two weeks post-treatment. However, given that 2 out of 3 patients who received the highest dose of tolerogenic DCs did not require corticoid treatment, the problems observed in patients treated with lower DC doses might have been due to the suboptimal therapeutic dose. Moreover, the adverse effects of knee synovitis could alternately reflect the invasive intervention. Nevertheless, in one patient, severe adverse effects were recorded. Therefore, the safety of this treatment cannot be definitely confirmed. Regarding treatment efficacy, intra-articular application of tolerogenic DCs led to neither systemic immune system modulation, since no effect on T cells or serum cytokines was detected, nor to a consistent improvement in the DAS28 score. Stabilized disease symptoms were observed in two patients from the 10×10^6 cohort. A

subsequent study utilizing higher doses of tolerogenic DCs is therefore planned for the future [199]. According to www.clinicaltrails.gov, another study with intraarticular application of tolerogenic DCs is currently ongoing, in which tolerogenic DCs are prepared by the treatment of monocytes with IFN-α and GM-CSF during differentiation into DCs and tolerized with dexamethasone. The starting number of applied tolerogenic DCs is 1×10^6, and depending on safety/tolerability outcomes, the dose can be gradually increased to 3×10^6, 5×10^6, 8×10^6 and 10×10^6 DCs. The first results are expected in the second half of 2019.

2.2.3.3 Crohn's disease

CD is a chronic inflammatory disorder that can affect any part of the gastrointestinal tract. An aberrant immune reaction to harmless antigens is particularly mediated by Th1 and Th17 T cell subsets. The first-in-human study with tolerogenic DCs for the treatment of CD was completed in 2015. Nine patients suffering from CD for at least 6 months enrolled in this study were divided into 6 cohorts that received different amounts of tolerogenic DCs. The first three cohorts were treated with a single dose of 2×10^6, 5×10^6 and 10×10^6 DCs, and the second three cohorts were treated with 3 doses of 2×10^6, 5×10^6 and 10×10^6 DCs applied every second week. Tolerogenic DCs were generated from monocytes using dexamethasone and vitamin A, both of which were added on day 3. On day 6, DCs were activated with a cytokine mix containing IL-1β, IL-6, TNF-α, and prostaglandin E2. Tolerogenic DCs were applied intraperitoneally to support DC migration into mesenteric lymph nodes. The application of tolerogenic DCs was not associated with any adverse effects. Clinical response was observed in two patients. One patient demonstrated clinical remission during the study. These results were encouraging, mainly with respect to the knowledge that patients participating in this study were refractory to conventional therapy [200].

The next study (TolDecCDintra) with 20 participants with refractory CD was launched in November 2015. To support the suppressive capacity of tolerogenic DCs, doses of 10×10^6 or 100×10^6 of tolerogenic DCs were suggested. The study should be completed in the second half of 2019.

2.2.3.4 Multiple sclerosis

MS is an autoimmune disorder in which immune cells attack the central nervous system. As a consequence, loss of the myelin sheath insulating the nerves (demyelination) occurs.

Currently, three phase I clinical trials utilizing tolerogenic DCs for MS treatment are ongoing; however, the complete results have not yet been published. The first study recruited patients suffering from MS or neuromyelitis optica for more than a year. DCs were generated from monocytes and tolerized using dexamethasone. Moreover, tolerogenic DCs were loaded with myelin peptides during the tolerizing process to induce antigen-specific suppression in patients. A total of three doses of undisclosed escalating numbers of tolerogenic DCs was applied intravenously once per two weeks. Preliminary results showed that treatment with tolerogenic DCs did not cause side effects in patients, and analysis of the immune profile revealed a switch towards the Th2 immune response and an elevation of IL-10 production with decreasing IFN-γ production [3]. The study should be completed in the second half of 2019.

The tolerogenic DCs in the second study (TOLERVIT-MS) were generated using vitamin D_3, and they were also pulsed with a pool of myelin peptides. The increasing number (5×10^6, 10×10^6 or 15×10^6) of tolerogenic DCs will be applied intranodally into cervical lymph nodes to determine the best dose. The last cohort will receive the best-selected dose in combination with IFN-β. The first four doses of tolerogenic DCs will be administered every second week, and the last two doses then every four weeks. The end of the study is planned by September 2019.

The third study is examining tolerogenic DCs pulsed with myelin peptides administered to patients by intradermal injections applied into five sites close to cervical lymph nodes. The planned doses of tolerogenic DCs are 5×10^6, 10×10^6 or 15×10^6. Results are expected in December 2020.

In summary, the results from clinical studies evaluating the possibility of using tolerogenic DCs for the treatment of various autoimmune diseases have provided some important information. First, the application of tolerogenic DCs seems to be safe and well-tolerated by patients. Second, even though the completed trials were phase I studies and thus did not evaluated the efficacy of the tested therapies as the primary end point, it seems that the treatment of patients suffering from autoimmune diseases with tolerogenic DCs is beneficial at least in some patients with a particular therapeutic regime. Regarding the setting of tolerogenic DC application, different doses, routes of administration and frequency of administrations have been tested. In current trials, higher doses of tolerogenic DCs (up to 15×10^6 DCs) are being used, since they have displayed more promising biological efficacy along with encouraging safety data. In line with the increasing numbers of DCs per dose, longer treatment with more doses is also being tested. Such study designs should support

the capacity of tolerogenic DCs to reset immune tolerance and to induce a long-term effect on the disease course. The following studies focused on the efficacy of tolerogenic DC-based treatment and a longer follow-up period are therefore necessary to determine whether these optimizations will have such an impact.

Table 2. A comparison of completed and ongoing clinical trials with tolerogenic DCs for the treatment of autoimmune diseases.

Disease	Type 1 diabetes				Rheumatoid arthritis			Crohn's disease		Multiple sclerosis		
Title acronym			D-sense		Rheumavax	AuToDeCRA	TolDCfoRA		TolDecCDintra	TolDec-EM-NMO	TOLERVIT-MS	MS-tolDC
Trial ID	NCT00445913	NCT02354911		NCT01947569		NCT01352858	NCT03337165		NCT02622763	NCT02283671	NCT02903537	NCT02618902
Phase	I	II	I	I/II	I	I	I	I	I	I	I	I
Status	Completed	Not yet recruiting	Unknown	Unknown	Completed	Unknown	Recruiting	Completed	Recruiting	Recruiting	Not yet recruiting	Recruiting
Patient number (treated/controls)	10 (7/3)	24 (12/12)	3 per group	90	34 (18/16)	12 (9/3)	15	9	20	22	16	9
Disease duration	>5 years (5–26 years)	<100 days	>18 months	<6 months	3–12 years	>6 months (2–43 years)	>6 months	>6 months	>6 months	>1 year	<15 years	>6 months, <15 years
Method of tolerogenic DC generation	Anti-sense oligonucleotides anti CD40, CD80, CD86	Anti-sense oligonucleotides anti CD40, CD80, CD86	Vitamin D3	*Ex vivo*-engineered	NF-κB inhibitor, Bay 11-7082	Dex Vitamin D3 MPLA activated	Dex (DCs differentiated in the presence of IFN-α/GM-CSF)	Dex Vitamin A Activated with cytokine mix (IL-1β, IL-6, TNF-α, and prostaglandin E2)	Unpublished	Dex	Vitamin D3	Unpublished
Antigen	No	No	Unspecified beta cell protein	Unpublished	Citrullinated peptides of vimentin, collagen type II and fibrinogen α and β chain	Autologous synovial fluid	Unpublished	No	Unpublished	Myelin peptides	Myelin peptides	Myelin peptides
DC number	10×10^6	10×10^6	5×10^6, 10×10^6 or 20×10^6	Unpublished	$0.5–1\times10^6$ or $2–4.5\times10^6$	1×10^6, 3×10^6 or 10×10^6	1×10^6, 3×10^6, 5×10^6, 8×10^6 or 10×10^6	A: 2×10^6, 5×10^6 or 10×10^6 B: 2×10^6, 5×10^6, 10×10^6	10×10^6 or 100×10^6	Unpublished	5×10^6, 10×10^6 or 15×10^6	5×10^6, 10×10^6 or 15×10^6
Injection number	4	4	2	Unpublished	1	1	1	A: 1, B: 3	1	3	6	1
Application schedule	2 weeks apart	2 weeks apart	28 days apart	Unpublished	-	-	-	A: - B: 2 weeks apart	-	2 weeks apart	4×2 weeks apart then 4×4 weeks apart	-
Application route	Intradermal (peri-umbilical region)	Intradermal (peri-umbilical region)	Intradermal	Unpublished	Intradermal	Intraarticular	Intraarticular	Intraperitoneal	Intralesional	Intravenous	Intranodal (cervical lymph nodes)	Intradermal (subclavicular region)
Results	Tolerated				Tolerated	Tolerated		Tolerated		Tolerated		
Research outcomes	Partial ↑C-peptide (4/7) ↑$B220^+$ $CD11c^-$ regulatory B cells				DAS28 improvement ↑Treg/Teff ratio ↓Proinflammatory markers	Partial disease stabilization (2/9)		Partial clinical response (2/9) or clinical remission (1/9) ↓IFN-γ production ↑circulating Tregs		Switch to Th2 responses ↑IL-10↓IFN-γ production		
References	[136]				[198]	[199]		[200]		[3]		

↑ increase; ↓ decrease; DAS28, disease activity score 28; DCs, dendritic cells; Dex, dexamethasone; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; MPLA, monophosphoryl lipid A; NF-κB, nuclear factor kappa light chain enhancer of activated B cells; Teff, effector T cells; Th2, T helper cells type 2; TNF, tumor necrosis factor; Tregs, regulatory T cells. Research outcomes may be present only temporarily at specific time points during the study. Information available on www.clinicaltrials.gov (current as of November 2018).

3 AIMS OF THIS THESIS

The aim of the first part of this project was to establish a protocol for tolerogenic DC generation, to evaluated their phenotype and function and comprehensively test their phenotypic and functional stability in the proinflammatory environment. The aim of the second part was to generate tolerogenic DCs according to the established protocol from the blood of patients suffering from autoimmune T1D and to examine their phenotype and suppressive functions in order to assess the possibility to use tolerogenic DCs as a cell-based therapy for T1D. The partial goal of the second part was to define clinical factors influencing functional characteristics of tolerogenic DCs in T1D patients. The aim of the third part was to test and optimize the tolerogenic DC manufacturing protocol for the generation of a clinical-scale tolerogenic DC-based product that can be applied to patients in a potential clinical trial. The next goal of the third part was to develop assays for tolerogenic DC quality testing and assays for testing of tolerogenic DC suppressive function.

The specific aims of the thesis were:

PART I:

1. Preparation of a good manufacturing practice (GMP)-compliant protocol for tolerogenic DC generation using paricalcitol (19-nor-1,25-dihydroxyvitamin D_2), a synthetic analogue of active form of vitamin D_2, the immunosuppressive drug dexamethasone and MPLA, a non-toxic alternative of LPS

2. Testing of the stability of the DC regulatory phenotype and function upon restimulation with various proinflammatory stimuli

3. Examination of signaling pathways that control the stability of the regulatory profile of tolerogenic DCs upon their repetitive *in vitro* stimulation

PART II:

4. Examination of the phenotype and function of tolerogenic DCs prepared from patients with T1D with the focus on the ability to induce antigen-specific T cell hyporesponsiveness and regulatory immune responses

5. Examination of blood hyperglycemia as a key factor influencing functional characteristics of tolerogenic DCs prepared from T1D patients

PART III:

6. Optimization of the tolerogenic DC manufacturing process in order to improve parameters of a tolerogenic DC-based product
7. Development and optimization of assays that could be used for tolerogenic DC quality control and for evaluation of suppressive capacity of tolerogenic DCs

4 MATERIAL AND METHODS

Material and methods in this part describe the material and methods used for the generation of extended unpublished results presented separately in the chapter 5.3 (PART III). Material and methods used in presented publications are described in given publications.

4.1 Monocyte separation

Monocytes used for DC generation were obtained from leukapheresis products from healthy donors a day before start of the processing. Monocytes were separated by adherence to plastic in triple flasks (TripleFlask™ cell culture flask, Nunc) for 2 hours or using magnetic separation based on CD14 expression by the CliniMACS Prodigy® cell separator (Miltenyi Biotec). $CD14^+$ separated monocytes were differentiated into DCs in triple flasks (Nunc) or in culture bags (MACS GMP Cell Differentiation Bag, Miltenyi Biotec).

4.2 Human tolerogenic DC generation

To generate monocyte-derived DCs, monocytes were cultivated in serum-free GMP-compliant CellGro media (CellGenix) in the presence of GM-CSF (500 IU/ml; Gentaur) and IL-4 (20 ng/ml; CellGenix) for 6 days, fresh media with cytokines were replenish after 3 days of cultivation. On day 6, generated DCs were harvested, counted and seeded in Nunclon Sphera 6F-well plates (Thermo Fisher Scientific) at 1×10^6 DCs/ml in fresh media with cytokines. To induce tolerogenic properties, dexamethasone (1 μM; Medochemie) was added with cytokines on day 3 and dexamethasone (1 μM) plus vitamin D (1.5 ng/ml; Zemplar, Abbott Laboratories) were added on day 6 after DC collection. Finally, immature antigen-unpulsed tolerogenic DCs were activated with vacci-grade MPLA (1 or 2 μg/ml; Cayla-InvivoGen) for 24 hours. As a control, we used control DCs that were generated simultaneously with tolerogenic DCs using the same protocol but without any tolerogenic agents. Subsequently, after final activation, DCs were frozen and stored at liquid nitrogen. After defrosting, tolerogenic and control DCs were analyzed for the yield, viability, surface marker expression and cytokine secretion or they were restimulated in order to test the stability of their phenotype.

4.3 Tolerogenic DC restimulation

To prove the stability of the tolerogenic phenotype of defrosted tolerogenic DCs, tolerogenic and control DCs were washed and cultivated in the absence of tolerogenic agents in complete RPMI 1640 media (Gibco) with 5% human AB serum (Invitrogen) for 24 hours. DCs were left in media without any stimulation or LPS (1 μg/ml; Sigma-Aldrich) was added. Subsequently, culture supernatants were collected and stored at -80°C until analysis and the phenotype and viability of tolerogenic DCs and control DCs were analyzed.

4.4 Analysis of a tolerogenic DC phenotype by flow cytometry

DCs were stained with following fluorochrome-conjugated antibodies: anti-CD86-FITC (2231 FUN-1) purchased from BD Biosciences; CD80-FITC (MAB104) and CD83-PerCP-Cy5.5 (HB15a) purchased from Beckman Coulter; TLR2-FITC (T2.1) purchased from BioLegend; CD14-PE-DL594 (MEM-15), CD11c-APC (BU15) purchased from Exbio; CD85k (IL-T3)-PE (293623) purchased from R&D Systems. Data were acquired by LSR Fortessa cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star). DCs were gated according to the forward scatter, side scatter and CD11c parameter. Dead cells were excluded from the analysis based on 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) staining.

4.5 Induction of T cell responses by tolerogenic DCs in allogeneic cultures

Allogeneic enriched $CD3^+$ T cells (2×10^5), obtained using The EasySep™ Human T Cell Enrichment Kit (STEMCELL Technologies) were stained with carboxyfluorescein succinimidyl ester (CFSE; 1 μM; CellTrace CFSE Cell Proliferation Kit; Thermo Fisher Scientific) and stimulated with unpulsed tolerogenic or control DCs (2×10^4) for 4 days. IL-2 (20 U/ml, PeproTech) was added on day 2. Allogeneic DC/T cell cultures were carried out in complete RPMI 1640 containing 5% human AB serum. Proliferation and cytokine production of $CD3^+$ T cells were determined by flow cytometry.

4.6 Suppression of T cell proliferation by tolerogenic DCs

Allogeneic enriched $CD3^+$ T cells (1×10^5), obtained using The EasySep™ Human T Cell Enrichment Kit (STEMCELL Technologies), were stained with CFSE and activated with

Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher Scientific) at a bead-to-cell ratio of 1:1. To examine the tolerogenic DC suppressive capacity various numbers of tolerogenic or control DCs were added (8, 4 or 2 × 10^4 reflecting DC-to-T cell ratio of 0.8:1, 0.4:1 or 0.2:1, respectively) to cultures for 3 days. The cell cultures were carried out in complete RPMI 1640 containing 5% human AB serum. Proliferation of $CD3^+$ T cells was determined by flow cytometry and cytokine production was analyzed by ELISA.

4.7 Analysis of T cell responses by flow cytometry

T cells were stained with following fluorochrome-conjugated antibodies: anti-CD3-PerCP-Cy5.5 (OKT3) and CD4-PE-Cy7 (RPA-T4) purchased from eBioscience; CD8-V500 (RPA-T8) and Ki-67-A700 (B56) purchased from BD Biosciences; IFN-γ-BV421 (4S.B3) purchased from BioLegend. Intracellular Ki-67 and IFN-γ detection was assessed using fixation/permeabilization buffer kit (eBioscience). Data were acquired by LSR Fortessa and analyzed using FlowJo software. Only $CD3^+$ T cells were included into analysis. For intracellular detection of IFN-γ on day 4, T cells were restimulated with phorbol-12-myristate-13-acetate (PMA, 50 ng/ml, Sigma-Aldrich) plus ionomycin (1 µg/ml, Sigma-Aldrich) for 4 hours in the presence of Brefeldin A (5 µg/ml, BioLegend) prior staining.

4.8 Analysis of cytokines secreted by DCs using ELISA

Determination of cytokine concentrations in cell culture supernatants was performed using ELISA assay (DuoSet ELISA Kit, R&D systems) according to manufacturer's instruction. The data were acquired using Sunrise™ absorbance microplate reader (Tecan).

4.9 Statistical analysis

Paired or unpaired tests were applied for data analysis using GraphPad Prism 6. A value of $p \leq 0.05$ was considered statistically significant.

5 RESULTS

The list of publications with direct connection to presented thesis:

Dáňová K, Klapetková A, Kayserová J, Šedivá A, Špíšek R, Palová-Jelínková L. NF-κB, p38 MAPK, ERK1/2, mTOR, STAT3 and increased glycolysis regulate stability of paricalcitol/dexamethasone-generated tolerogenic dendritic cells in the inflammatory environment. Oncotarget. 2015 Jun 10;6(16):14123-38; **IF$_{2015}$: 5.008**; *awarded Milan Pospíšil & Mario Campa Prize for the best publication on natural and anti-tumor immunity in 2015 by the Czech Immunological Society*

Dáňová K, Grohová A, Strnadová P, Funda DP, Šumník Z, Lebl J, Cinek O, Průhová Š, Koloušková S, Obermannová B, Petruželková L, Šedivá A, Fundová P, Buschard K, Špíšek R, Palová-Jelínková L. Tolerogenic Dendritic Cells from Poorly Compensated Type 1 Diabetes Patients Have Decreased Ability To Induce Stable Antigen-Specific T Cell Hyporesponsiveness and Generation of Suppressive Regulatory T Cells. J Immunol. 2017 Jan 15;198(2):729-740; **IF$_{2017}$: 4.539**; *awarded Dr. Josef Liška Prize for the best publication in 2017 by Czech Society of Allergology and Clinical Immunology*

The list of patent applications with direct connection to presented thesis:

Palová-Jelínková L, **Dáňová K**, Špíšek R. "Tolerogenic Dendritic Cells, Methods of Producing Same and Uses Thereof", international application number PCT/EP2015/074536, international publication number WO 2016/062827

Palová-Jelínková L, **Dáňová K**, Špíšek R. "Tolerogenic Dendritic Cells", international application number PCT/EP2017/079990, international publication number WO 2018/095938

The list of reviews with direct connection to presented thesis:

Grohová A**, Dáňová K**, Špíšek R, Palová-Jelínková L. Cell based therapy for type 1 diabetes: should we take hyperglycemia into account? Front Immunol. 2019 Feb 5;10:79; **IF$_{2018}$: 5.511**; Review

Grohová A, **Dáňová K**, Palová-Jelínková L. Tolerogenic dendritic cells and their application in immunopathological processes. (Tolerogenní dendritické buňky a jejich využití v léčbě imunopatologických stavů, přehledový článek; Article in Czech language). Čes-slov Pediat. 2017;72(4):256-262; Review

Palová-Jelínková L, **Dáňová K**. Immune cell crosstalk in development and prevention of type 1 diabetes. (Vzájemná kooperace mezi imunitními buňkami při vzniku a prevenci diabetu 1. typu; Article in Czech language). Alergie. 2019 Accepted; Review

The list of other publications:

Paračková Z, Kayserová J, **Dáňová K**, Šišmová K, Dudková E, Šumník Z, Koloušková S, Lebl J, Štechová K, Šedivá A. T regulatory lymphocytes in type 1 diabetes: Impaired CD25 expression and IL-2 induced STAT5 phosphorylation in pediatric patients. Autoimmunity. 2016 Dec;49(8):523-531; **IF_{2016}: 2.629**

Palová-Jelínková L, **Dáňová K**, Drašarová H, Dvořák M, Funda DP, Fundová P, Kotrbová-Kozak A, Černá M, Kamanová J, Martin SF, Freudenberg M, Tučková L. Pepsin digest of wheat gliadin fraction increases production of IL-1β via TLR4/MyD88/TRIF/MAPK/NF-κB signaling pathway and an NLRP3 inflammasome activation. PLoS One. 2013 Apr 29;8(4):e62426; **IF_{2013}: 3.534**

5.1 PART I: NF-κB, p38 MAPK, ERK1/2, mTOR, STAT3 and increased glycolysis regulate stability of paricalcitol/ dexamethasone-generated tolerogenic dendritic cells in the inflammatory environment

A tolerizing agent used for the generation of tolerogenic DCs fundamentally influences the DC phenotype and mechanisms that they employ for suppression of overactivated effector T cells and restoration of immune tolerance. Based on data of Sochorová *et al*., we used paricalcitol (19-nor-1,25-dihydroxyvitamin D_2), for tolerogenic DC generation, as it showed the comparable immunomodulatory capacity as vitamin D_3 [201]. Moreover, we applied vitamin D_2 in combination with dexamethasone because recent studies have revealed that administration of vitamin D_3 and its analogues in combination with glucocorticoid dexamethasone strengthens tolerogenic DC potential to suppress proinflammatory T cell responses. In fact, dexamethasone potentiates the regulatory effect of vitamin D via upregulation of VDR. In addition, both agents induce distinctive molecular pathways for tolerance induction, which results in a more robust tolerogenic phenotype [149,153,154]. We used MPLA as a final activation signal, since MPLA augments IL-10 production and does not induce IL-12 secretion compared with LPS. Moreover, MPLA supports the stable semimature phenotype of tolerogenic DCs [179].

In this study, we showed that monocyte-derived tolerogenic DCs prepared according to GMP using serum-free CellGro media, vitamin D_2 plus dexamethasone and MPLA (referred as Dex/VitD_2 tolerogenic DCs) acquired the stable regulatory phenotype and function. In comparison to control DCs generated without tolerizing agents (referred as control DCs), Dex/VitD_2 tolerogenic DCs were characterized by lower expression of activation markers CD86, CD80, CD83, CD40, increased expression of inhibitory markers TLR2, CD14, TIM-3, IL-T3 and unaltered expression of HLA-DR, IL-T4, PD-L1 and PD-L2. The regulatory phenotype of surface markers was accompanied with augmented production of the suppressive cytokines IL-10 and TGF-β and very low levels of the proinflammatory cytokines TNF-α and IL-12. We next reported that, in comparison to control DCs, Dex/VitD_2 tolerogenic DCs induced lower proliferation in allogeneic T cells and lower production of IFN-γ and IL-17A, the key mediators of Th1 and Th17 responses, which are responsible for pathogenic processes and autoimmune beta cell destruction in T1D. In

contrast, Dex/VitD$_2$ tolerogenic DCs displayed a greater ability to induce suppressive IL-10-producing T cells compared with control DCs. Finally, repetitive stimulation of naïve T cells by Dex/VitD$_2$ tolerogenic DCs promoted IL-10-producing Tregs that were able to inhibit responder T cell proliferation and production of IFN-γ and IL-17A.

Furthermore, we evaluated the stability of the regulatory phenotype and function of Dex/VitD$_2$ tolerogenic DCs after their transfer into media containing human serum and after treatment with a mix of proinflammatory cytokines (IL-1β, TNF-α, IL-6 and IFN-γ), LPS, polyinosinic:polycytidylic acid (poly(I:C)) or CD40 ligand, which mimicked a proinflammatory environment in patients with autoimmune disorders, the presence of bacterial or viral infection or immune system activation in general. We confirmed that, despite repetitive activation, Dex/VitD$_2$ tolerogenic DCs were able to retain their tolerogenic phenotype. More importantly, we also showed that Dex/VitD$_2$ tolerogenic DCs maintained the stable, low T cell stimulatory capacity. Therefore, our data extended the previously described phenotypic stability of tolerogenic DCs generated with different tolerizing agents, where stable anti-inflammatory cytokine production and surface marker expression in response to restimulation with TLR2, TLR7/8 agonists plus IFN-γ, LPS, proinflammatory cytokines and peptidoglycan were demonstrated [155,178].

Since previous studies mostly examined molecular mechanisms implicated in the establishment of a regulatory phenotype in tolerogenic DCs [153], we wanted to address molecular pathways important for its maintenance. We showed the key role of NF-κB, p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal-regulated kinase 1/2 (ERK1/2) molecules and the mTOR/STAT3 pathway in the preservation of upregulated IL-10 production, surface expression of IL-T3 and PD-L1 and a low stimulatory capacity of Dex/VitD$_2$ tolerogenic DCs. Moreover, we found that, compared with control DCs, enhanced glycolysis characterizes Dex/VitD$_2$ tolerogenic DCs and is necessary for the tolerogenic phenotype maintenance.

My contribution: experimental design, generation of tolerogenic and control DCs, restimulation of DCs, analysis of DC yield, viability and phenotype (flow cytometry and following analysis using FlowJo software; ELISA; Luminex), DC and T cell cultures, analysis of proliferation and cytokine production by T cells, analysis of Treg differentiation from naïve T cells (flow cytometry and following analysis using FlowJo software), analysis of activated signaling pathways (whole cell lysate preparation), data analysis and interpretation, manuscript and figure preparation.

NF-κB, p38 MAPK, ERK1/2, mTOR, STAT3 and increased glycolysis regulate stability of paricalcitol/dexamethasone-generated tolerogenic dendritic cells in the inflammatory environment

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ABSTRACT

Tolerogenic dendritic cells (tDCs) may offer an intervention therapy in autoimmune diseases or transplantation. Stable immaturity and tolerogenic function of tDCs after encountering inflammatory environment are prerequisite for positive outcome of immunotherapy. However, the signaling pathways regulating their stable tolerogenic properties are largely unknown. In this study, we demonstrated that human monocyte-derived tDCs established by using paricalcitol (analogue of vitamin D2), dexamethasone and monophosphoryl lipid A exposed for 24h to LPS, cytokine cocktail, polyI:C or CD40L preserved reduced expression of co-stimulatory molecules, increased levels of inhibitory molecules ILT-3, PDL-1 and TIM-3, increased TLR-2, increased secretion of IL-10 and TGF-β, reduced IL-12 and TNF-α secretion and reduced T cell stimulatory capacity. tDCs further induced IL-10-producing T regulatory cells that suppressed the proliferation of responder T cells. In the inflammatory environment, tDCs maintained up-regulated indoleamine 2, 3 dioxygenase but abrogated IκB-α phosphorylation and reduced transcriptional activity of p65/RelA, RelB and c-Rel NF-κB subunits except p50. Mechanistically, p38 MAPK, ERK1/2, mTOR, STAT3 and mTOR-dependent glycolysis regulated expression of ILT-3, PDL-1 and CD86, secretion of IL-10 and T cell stimulatory capacity of tDCs in the inflammatory environment. Stability of tDCs in the inflammatory environment is thus regulated by multiple signaling pathways.

INTRODUCTION

Dendritic cells (DCs) are specialized antigen presenting cells that, depending on their activation status, can induce tolerance or immunity [1]. Tolerogenic DCs (tDCs) can be generated from precursor cells *in vitro* and represent potentially promising tool for inducing or restoring immune tolerance in the context of transplantation and autoimmune diseases [2]. Tolerogenic DCs are usually defined by low or intermediate expression of co-stimulatory molecules CD80, CD86 and CD40 in contrast to high levels of inhibitory factors such as immunoglobulin-like transcript (ILT) molecules 2, 3, 4, and/or programmed death ligand (PDL)-molecules. Additionally, tDCs secrete low amounts of pro-inflammatory cytokines and high quantities of anti-inflammatory cytokines, such IL-10. This results in the attenuation of T cell stimulatory capacity and/or induction and expansion of T regulatory cells (Tregs). Different approaches targeting DCs differentiation and function have been shown to establish tDCs *in vitro* [3-5]. Notably, dexamethasone (Dex) and/or vitamin D (VitD) receptor agonists ($1,25(OH)_2D3$ and its analogues) have been described to generate tDCs through the suppression of NF-κB-dependent DCs maturation [6, 7]. Such Dex/VitD conditioned tDCs have been shown to acquire a robust

immunoregulatory phenotype and are currently tested in early stage clinical trial in patients with rheumatoid arthritis [8].

One of the major concerns associated with *in vivo* administration of *in vitro* established tDCs is their functional stability. Once injected into patients with chronic inflammation, such as autoimmune disease, tDCs should remain stable and retain their tolerogenic properties in the absence of tolerogenic agents. Furthermore, there is a potential risk that *ex vivo* differentiated tDC might switch to an activated phenotype when encountering pro-inflammatory signals *in vivo* and contribute to the further expansion of the autoimmune reaction.

The pro-inflammatory DC maturation initiated by pathogen associated molecular patterns or by inflammatory cytokines is connected with the activation of numerous signaling pathways including transcription factor NF-κB and p38 mitogen-activated protein kinase (MAPK) pathway [9, 10]. Recently, the mammalian target of rapamycin (mTOR) signaling pathway has been reported to coordinate the production of pro- versus anti-inflammatory cytokines in human monocytes and DCs through regulating NF-κB and signal transducer and activator of transcription 3 (STAT3) activity [11, 12]. The pattern of activated signaling events triggered in tolerogenic DC maturation is profoundly different and is associated namely with activation of extracellular-signal-regulated kinase (ERK) 1/2 and non-canonical NF-κB pathway [13-15]. However, little is known about the signaling pathways triggered in tDCs after encountering inflammatory environment and their role in preserving tolerogenic properties of tDCs.

In this study, we established a good manufacturing practice (GMP)-compliant protocol for the human tDCs differentiation using paricalcitol (19-nor-1, 25-dihydroxyvitamin D2), synthetic analogue of active form of VitD2 that retains significant immunomodulatory activity [16] and immunosuppressive drug dexamethasone (Dex). Finally, VitD2/Dex-generated tDCs (Dex/VitD2 tDCs) were activated with monophosphoryl-lipid A (MPLA), a non-toxic alternative of lipopolysaccharide (LPS), to obtain so-called "alternatively activated tDCs" with improved tolerogenic properties as reported previously [17]. We comprehensively tested their phenotypic and functional stability after mimicking inflammatory environment by using LPS, cocktail of pro-inflammatory cytokines (CC), polyinosinic:polycytidylic acid (polyI:C) and CD40L. In our study, we addressed for the first time a detailed analysis of molecular mechanisms responsible for the maintenance of stable tolerogenic properties of tDCs in the inflammatory environment.

RESULTS

tDCs preserved semimature tolerogenic phenotype after restimulation with LPS, CC, polyI:C and CD40L

To study the functional properties and stability of tDCs, we cultured freshly isolated human monocytes in GMP–compliant medium Cell Gro in the presence of GM-CSF, IL-4, and tolerogenic factors Dex and VitD2. Control DCs (cDCs) were cultured without Dex and VitD2. Finally, DCs were activated with MPLA.

As shown in Figure 1A and Supplementary Figure 1, tDCs cultured in Cell Gro exhibited tolerogenic phenotype with significantly lower surface levels of CD86, CD83, CD80 and CD40 but higher levels of Toll-like receptor (TLR)-2, CD14 and inhibitory molecules TIM-3 and ILT-3 in comparison to cDCs. The levels of CD1a, CD11c, HLA-DR and inhibitory molecules ILT-4, PDL-1 and PDL-2 were comparable in tDCs and cDCs. To study the stability of DCs, cDCs and tDCs generated in Cell Gro were recultured in complete RPMI without tolerising agents and subsequently stimulated with LPS, CC, polyI:C and CD40L for 24 h (Figure 1B). Restimulation led to a slight upregulation of CD86, CD83 and CD40 on tDCs, however, it remained significantly lower when compared to cDCs. Importantly, the expression of TLR2, CD14 and ILT-3 on tDCs remained high after restimulation when compared to cDCs. The expression of TIM-3 decreased approximately two-fold after CC, LPS and CD40L stimulation, however, it remained higher in comparison to cDCs. The expression of tolerogenic molecule PDL-1, that was low on tDCs from Cell Gro, dramatically increased after restimulation of tDCs with CC as well as LPS and slightly after polyI:C stimulation for 24 h.

In line with tolerogenic cell-surface phenotype, tDCs produced higher levels of IL-10 and TGF-β, low quantities of TNF-α and no IL-12p70 as compared to cDCs (Figure 1C). Subsequent restimulation of tDCs with CC, LPS, polyI:C or CD40L led to robust increase of IL-10 production, slight up-regulation of TGF-β, low production of TNF-α and minimal IL-12 production. (Figure 1D). Collectively, these data demonstrate that, in spite of the presence of maturation stimuli, Dex/VitD2 tDCs preserve non-proinflammatory profile with high expression of tolerogenic markers, high IL-10/IL-12p70 ratio and sustained TGF-β production.

Dex/VitD2 tDCs preserved reduced T cell stimulatory capacity after restimulation

TDCs or cDCs were cultured with allogeneic T cells at a ratio of 1:10. TDCs were weaker inducers of CD4+ as well as CD8+ T cell proliferation, even after

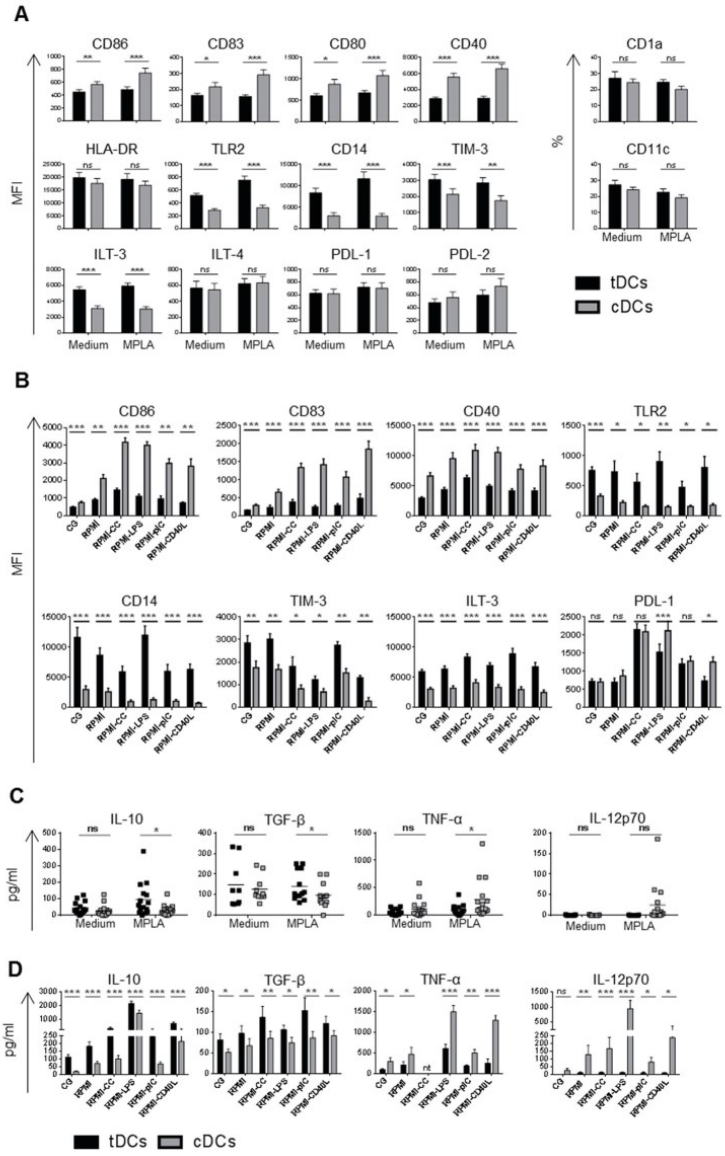

Figure 1: Dex/VitD2 tDCs exhibit a stable semimature phenotype and anti-inflammatory cytokine secretion profile. DCs were differentiated from monocytes in Cell Gro supplemented with GM-CSF and IL-4 in presence (tDCs, black bars) or absence of Dex and VitD2 (cDCs, grey bars) to obtain immature tDCs or immature cDCs (MEDIUM). Cells were finally activated with MPLA for 24 h (MPLA). **A.** Surface marker expression was analyzed by flow cytometry and **C.** cytokines released by DCs were analyzed from supernatants by Luminex (tDCs black squares, cDCs grey squares). After activation with MPLA for 24 h in Cell Gro (CG), cells were washed and recultured in complete RPMI without tolerising factors and treated with cytokine cocktail (CC) containing IL-1β, TNF-α, IL-6 (all 10 ng/ml) and IFN-γ (100 ng/ml) or LPS (1 μg/ml) or polyI:C (25 μg/ml) or CD40L (1000 ng/ml) or they were left unstimulated (RPMI). **B.** Bar graphs represent surface marker expression analyzed by flow cytometry and **D.** cytokines released by DCs analyzed by Luminex or ELISA after 24 h of restimulation. Data represent MFI ± SEM or percentages of positive cells (CD1a and CD11c expression) from at least 3 independent experiment and minimal 10 donors. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ (paired *t*-test), nt-not tested.

the restimulation, irrespective of the maturation agent when compared to cDCs (Figure 2A). In line with this, tDCs induced low IL-17A production by allogeneic T cells even after restimulation in contrast to cDCs that were potent inducers of IL-17A by T cells especially after CC and LPS stimulation (Figure 2B). Moreover, co-incubation of allogeneic T cells with tDCs cultured in Cell Gro skewed the T cell cytokine profile towards reduced IFN-γ and significantly increased IL-10 production by CD4+ as well as CD8+ T cells, in comparison to cDCs (Figure 2C, 2D). In addition, co-incubation of T cells with tDCs restimulated with CC, LPS, polyI:C and CD40L led to marked reduction of CD4+ IFN-γ producing T cells together with stable numbers of CD4+ IL-10 producing T cells. The percentage of CD8+ IFN-γ producing T cells remained stable or slightly decreased after CC and CD40L restimulation of tDCs, while the amount of CD8+ IL-10 producing T cells remained almost the same after restimulation of tDCs with LPS and slightly decreased after restimulation of tDCs with CC, polyI:C and CD40L.

Dex/VitD2 tDCs induced Tregs differentiation from naïve CD4+ T cells

Increased capacity to promote differentiation/induction of Tregs from naïve precursors seems to be one of the most important hallmarks of tDCs [19]. We showed that co-culturing of allogeneic T cells with Dex/VitD2 tDCs induces higher and stable levels of IL-10 producing CD4+ T cells when compared to cDCs. Previously, IL-10 producing CD4+ T cells generated by repetitive priming of CD4+ naïve T cells with immature DCs or tDCs generated by VitD3 were shown to display regulatory properties [20, 21]. Thus, to test whether IL-10 producing CD4+ T cells induced by Dex/VitD2 tDCs (referred to as Tregs) possess regulatory activity after being expanded by repetitive priming, we cultured naïve CD4+ T cells with allogeneic Dex/VitD2 tDCs for two rounds of stimulation. As shown in Figure 3A, Tregs expanded by Dex/VitD2 tDCs produced IL-10 but virtually no IFN-γ and IL-17A. IL-10 production was increased upon specific activation with cDCs. IFN-γ and IL-17 production was only slightly increased upon specific activation with cDCs. To analyze the suppressive function of Tregs expanded after two rounds of stimulation with Dex/VitD2 tDCs, Tregs were titrated into a MLR comprising allogeneic cDCs (from the same DCs donor as used in the original stimulation) and autologous responder T cells (from the same T cell donor as Tregs). As shown in Figure 3B, Tregs dose-dependently inhibited responder T cell proliferation. Moreover, adding of Tregs into MLR led to up-regulation of IL-10 and down-regulation of IFN-γ and IL-17A production in a dose-dependent manner (Figure 3C). Therefore, IL-10 producing Tregs induced by Dex/VitD2 tDCs are functional and suppress proliferation of responder T cells.

Dex/VitD2 tDCs used NF-κB, p38 MAPK and ERK1/2 to regulate their tolerogenic properties in the inflammatory environment

To decipher the molecular mechanisms that play a role in maintaining tolerogenic properties of tDCs, signaling pathways including p38 MAPK, c-Jun N-terminal kinases (JNK/SAPK), ERK1/2, NF-κB, indoleamine 2, 3 deoxygenase (IDO), mTOR, and STAT3, previously reported to affect DC maturation and orchestrate IL-10 and IL-12 production, were analyzed [9, 11, 13, 14, 22].

First, we checked whether MAPK, including p38 MAPK, JNK/SAPK and ERK1/2, are differentially regulated in tDCs and cDCs. As shown in Figure 4A, tDCs from Cell Gro expressed higher levels of activated JNK/SAPK, however, p38 MAPK and ERK1/2 were comparably activated in tDCs and cDCs. After re-exposing DCs to inflammatory stimuli, tDCs expressed higher level of activated JNK/SAPK, lower level of the activated p38 MAPK and markedly up-regulated ERK1/2 in contrast to cDCs.

Next, we found that tDCs expressed high level of immunoregulatory molecule IDO in all the stimulatory conditions tested. In contrast, IDO was absent or weakly expressed in cDCs. These results suggest that p38 MAPK, JNK/SAPK, ERK1/2 and IDO are differentially regulated in tDCs compared to cDCs, which might play a role in maintaining tolerogenic properties of tDCs after rechallenge.

Given that DC differentiation and maturation is associated with activation of NF-κB and Dex/VitD tDCs differentiation was shown to be mediated through the suppression of NF-κB pathway [6, 7], we tested whether LPS, polyI:C, CC or CD40L can reverse NF-κB suppression in the absence of tolerogenic factors. First, we documented that phosphorylation of IκB-α, a regulatory protein that inhibits NF-κB by complexing with and trapping it in the cytoplasm, was dramatically reduced in tDCs in all the stimulatory conditions tested. In contrast, IκB-α was phosphorylated in cDCs (Figure 4A). To quantify NF-κB activation, we analyzed DNA binding activity of NF-κB subunits p50, p65/RelA, RelB and c-Rel in the nucleus (Figure 4B). Dex/VitD2 tDCs from Cell Gro exhibited low binding activity of p65/RelA, and low binding activity of RelB, shown to reflect DCs maturation [23], and c-Rel, shown to be involved in IL-12 production [24], in nuclear extracts when compared to cDCs. DNA binding activities of p65/RelA, RelB and c-Rel in tDCs remained lower even after rechallenge in the absence of VitD2 and Dex. On the other hand, binding activity of NF-κB subunit p50, shown to create homodimers increasing production of IL-10 [25], was high in tDCs in all the conditions tested (Figure 4B).

Next, we determined how MAPK and NF-κB

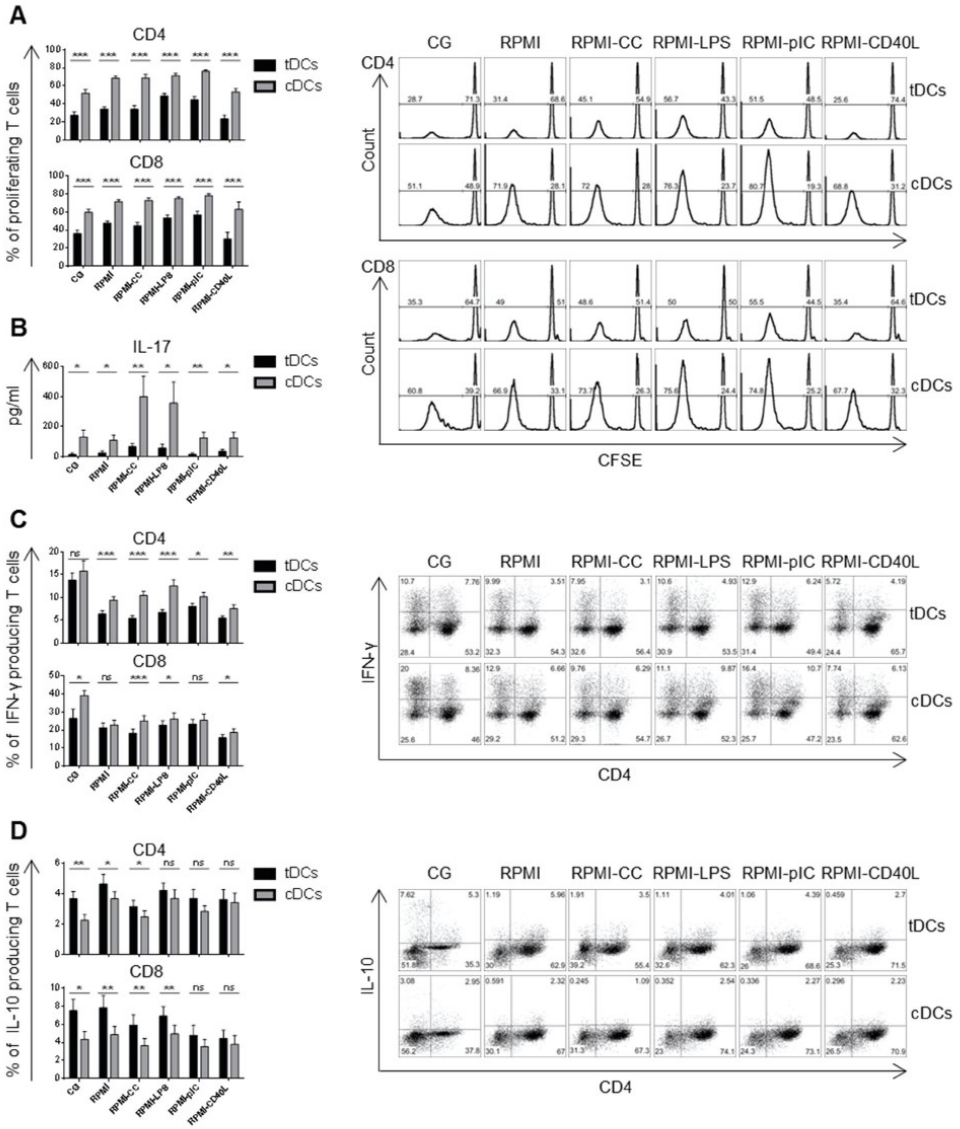

Figure 2: Dex/VitD tDCs maintain reduced T cell stimulatory capacity after restimulation. DCs were differentiated in Cell Gro in presence (tDCs) or absence of Dex and VitD2 (cDCs) and activated with MPLA (CG). Then, DCs were washed, recultured in complete RPMI without tolerising factors and treated with cytokine cocktail (CC) described in Figure 1B or LPS (1 μg/ml) or polyI:C (25 μg/ml) or CD40L (1000 ng/ml) or they were left unstimulated (RPMI). After 24 h, tDCs and cDCs were washed and incubated with allogeneic T cells at 1:10 ratio (DCs/T cells). **A.** Proliferation of T cells was assessed on day 6 by CFSE dilution method. Percentages of proliferating T cells and representative histograms are shown. **B.** Production of IL-17A in DCs/T cell co-cultures was analyzed by ELISA on day 6. **C.** Percentages of IFN-γ producing T and **D.** IL-10 producing T cells was assessed on day 6 or day 9, respectively. Representative dot plots are shown. Data represent mean ± SEM for at least 3 independent experiments and at least 10 donors. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ (paired *t*-test).

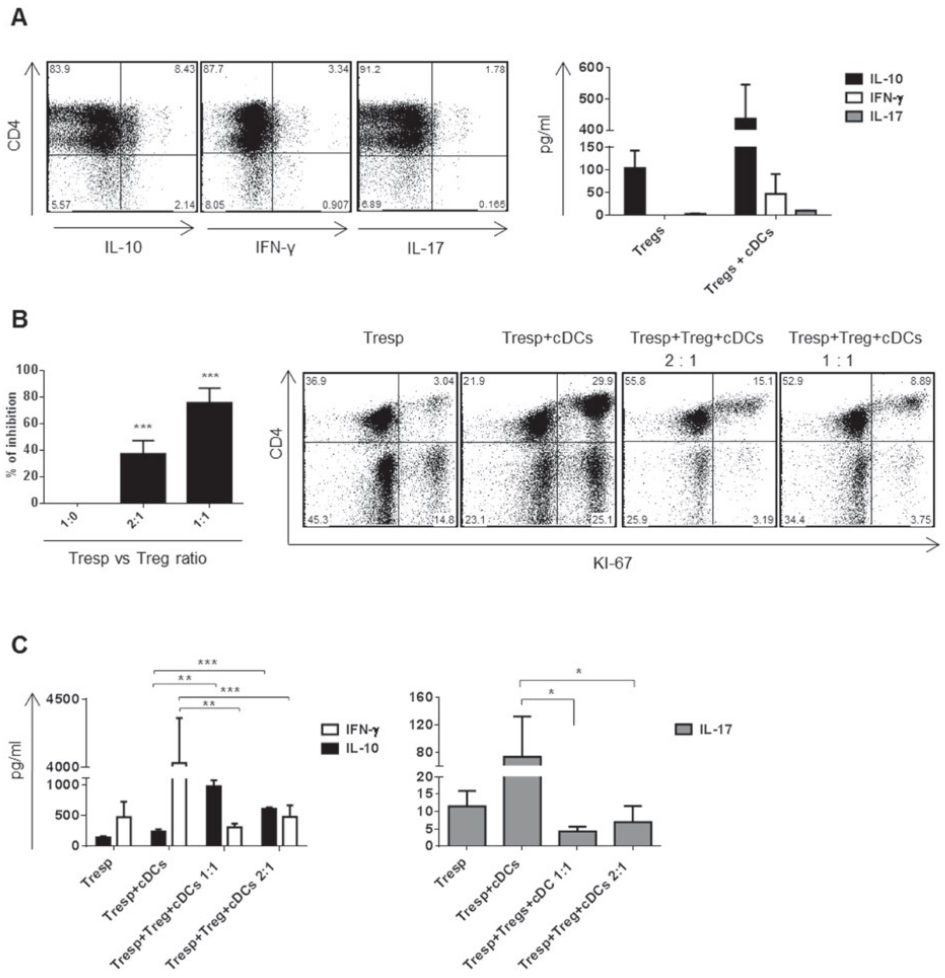

Figure 3: Dex/VitD2 tDCs induce IL-10 producing Tregs that are able to suppress proliferation of responder T cells. DCs (donor B) were differentiated in Cell Gro in presence (tDCs) or absence (cDCs) of Dex and VitD2 and activated with MPLA. Dex/VitD2 tDCs were incubated with allogeneic T cells (donor A) at 1:10 ratio (DCs/T cells) in RPMI (5% human AB serum) for two rounds of priming. Then, the cytokine production and suppressive capacity of induced Tregs was evaluated. **A.** Tregs (donor A) were co-cultured with specific cDCs (donor B) at 1:10 ratio (DCs/T cells). Representative dot plots from 3 independent donors show percentages of IL-10, IFN-γ and IL-17 producing T cells assessed on day 6. Production of IL-10, IFN-γ and IL-17 was analyzed in cell supernatants by ELISA on day 6. **B.** CD4+ Tregs were tested for suppressive capacity in MLR assay. CD4+ Tregs (donor A) were plated with responder T cells (donor A) and cDCs (donor B). cDCs were from the same donor as the Dex/VitD2 tDCs used to induce Tregs. Cells were plated in a Treg/Tresp/DCs ratio of 10:10:1 or 5:10:1. As additional controls, Tresp were cultured alone or with cDCs. After 6 d, cells were recovered and proliferation of responder cells was analyzed by measuring KI-67 by flow cytometry. The percent inhibition of responder T cell proliferation (black bars, mean ± SEM for 3 independent donors, each performed in triplicate) and one representative dot plot showing proliferation of responder T cells are depicted. **C.** Cell culture supernatants were recovered for IL-10, IFN-γ and IL-17A analysis. Data represent mean ± SEM for 3 independent donors (each performed in triplicate). *$p \leq 0.05$, **$p \leq 0.01$ (paired *t*-test).

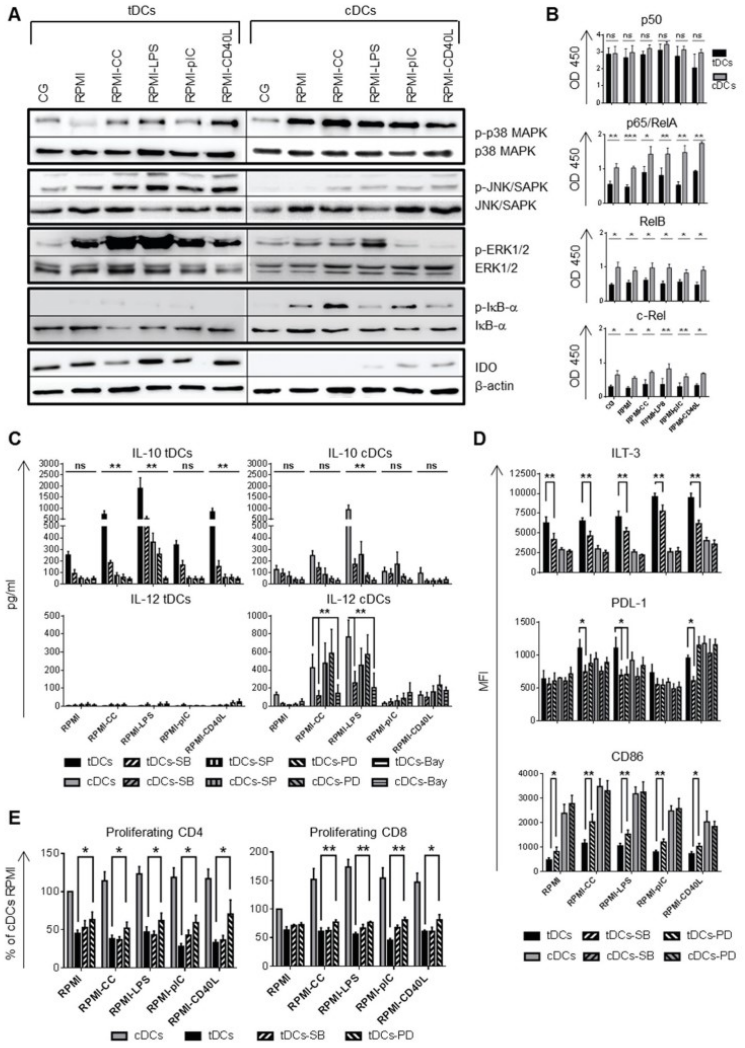

Figure 4: Different intracellular signaling pathways are triggered in tDCs and cDCs after mimicking *in vivo* DC activation. DCs were differentiated in Cell Gro in presence (tDCs) or absence of Dex and VitD2 (cDCs) and activated with MPLA (CG). Then, tDCs and cDCs were washed, recultured in complete RPMI without tolerising factors and treated with following stimuli: cytokine cocktail (CC) described in Figure 1B or LPS (1 μg/ml) or polyI:C (25 μg/ml) or CD40L (1000 ng/ml) or they were left unstimulated (RPMI). **A.** After 60 min of restimulation, the phosphorylation of p38 MAPK, JNK/SAPK, ERK1/2, IκB-α and the level of IDO was analyzed by western blot analysis. Total p38 MAPK, JNK/SAPK, ERK1/2, IκB-α or β-actin in each sample were used as the equal loading control. One of three experiments performed is shown. **B.** After 90 min of restimulation, DNA-binding activity of NF-κB subunits was analyzed by colorimetric assay. **C.** Production of IL-10 and IL-12 after restimulation of tDCs and cDCs with CC, LPS, polyI:C and CD40L for 24 h in the presence of p38 MAPK inhibitor SB203580 (SB), JNK/SAPK inhibitor SP600125 (SP), ERK1/2 inhibitor PD98059 (PD), NF-κB inhibitor Bay 11-7082 (Bay) was evaluated by ELISA. **D.** ILT-3, PDL-1 and CD86 expression on tDCs and cDCs after restimulation with CC, LPS, polyI:C and CD40L for 24 h in the presence of p38 MAPK inhibitor SB203580 (SB) and ERK1/2 inhibitor PD98059 (PD) was evaluated by flow cytometry. **E.** Before restimulation, tDCs were pretreated with p38 MAPK inhibitor SB203580 (SB) and ERK1/2 inhibitor PD98059 (PD) and stimulated for 24 h. tDCs were then cocultered with allogeneic T cells. Proliferation was measured on day 6. Data represent mean ± SEM from at least three independent experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ (paired *t*-test).

signaling pathway utilization contributes to inflammatory versus tolerogenic phenotype of DCs in response to LPS, CC, polyI:C and CD40L. Before stimulation, DCs were pretreated by p38 MAPK, JNK/SAPK, ERK1/2, and NF-κB inhibitor SB203580, SP600125, PD98059 and Bay 11-7082, respectively. Analyzing IL-10 and IL-12 production, we found that IL-10 production was significantly dependent on p38 MAPK, JNK/SAPK and ERK1/2 activation pathways after CC, LPS and CD40L restimulation in tDCs. Also Bay 11-7082 abrogated IL-10 production in tDCs. However, we observed the same situation only after LPS triggering in cDCs (Figure 4C). On the other hand, p38 MAPK and NF-κB inhibitor markedly down-regulated IL-12 production in cDCs after LPS and CC triggering, but did not affect IL-12 production in tDCs. Analyzing cell-surface molecules, we found that p38 MAPK inhibition down-regulated ILT-3 and PDL-1 expression in tDCs, in contrast to cDCs (Figure 4D). Moreover, ERK1/2 inhibitor down-regulated PDL-1 expression after LPS stimulation in tDCs but significantly up-regulated CD86 expression in tDCs in all the conditions tested (Figure 4D). Other inhibitors tested had no significant effect on ILT-3, PDL-1 and CD86 expression in tDCs (data not shown). The ability of p38 MAPK and ERK1/2 inhibitors to modulate IL-10 production and expression of costimulatory and inhibitory molecules in tDCs suggest an impact on subsequent T cell activation. By employing the allogeneic T cell activation model, we found that ERK1/2 inhibitor increased the ability of Dex/VitD tDCs to stimulate CD4+ as well as CD8+ T cell proliferation when compared to tDCs without ERK1/2 inhibitor (Figure 4E). Collectively, these data suggest the distinct pattern of activated signaling pathways in tDCs versus cDCs, with p38 MAPK, ERK1/2 and down-regulated NF-κB being important for maintaining down-regulated CD86 and up-regulated ILT-3 and PDL-1 expression, high IL-10 production and reduced allostimulatory potential of Dex/VitD tDCs.

mTOR and STAT3 regulate IL-10 production and ILT-3, PDL-1 and CD86 expression in tDCs after restimulation

Recently, mTOR was found to coordinate pro- versus anti-inflammatory events in human monocytes and DCs by attenuating NF-κB and up-regulating STAT3 activity [11, 12]. Western blot analysis revealed that tDCs strongly phosphorylated mTOR and STAT3 after re-exposing to inflammatory stimuli while the phosphorylation of mTOR is weaker and phosphorylated STAT3 is barely detectable in cDCs. mTOR phosphorylation led to the phosphorylation of p70S6K, mTOR dependent event, that was abrogated by the mTOR specific inhibitor rapamycin (Figure 5A). To further corroborate the link between mTOR and STAT3 activation and IL-10 and IL-12 production as well as CD86, ILT-3 and PDL-1 expression, we performed blocking experiments of mTOR and STAT3 using chemical inhibitors rapamycin and Stattic, respectively. Upon mTOR and STAT3 inhibition tDCs reduced IL-10 production (Figure 5B). Rapamycin and Stattic down-regulated IL-10 production after LPS restimulation in cDCs (Figure 5B). However, in contrast to cDCs, where rapamycin treatment markedly increased IL-12 production after CC and LPS treatment, rapamycin was not able to restore IL-12 production in tDCs irrespective of the stimulatory agent. IL-12 production was unaffected after Stattic treatment in both DCs tested (Figure 5B). Furthermore, mTOR and STAT3 inhibition markedly reduced expression of tolerogenic markers PDL-1 and ILT-3 but significantly increased CD86 expression in tDCs after CC and LPS trigger (mTOR inhibition) or in all the conditions tested (STAT3 inhibition), respectively (Figure 5C). This was paralleled by a partial restoration of the ability of tDCs to stimulate especially CD4+ T cell proliferation when compared to tDCs cultivated without Rapamycin or Stattic (Figure 5D). Altogether, these data suggest that mTOR and STAT3 controls not only IL-10 production and ILT-3, PDL-1 and CD86 expression in tDCs after restimulation but also play a role in their immunoregulatory function.

mTOR-dependent glycolysis regulate stable tolerogenic properties of tDCs after restimulation

TLR-induced proinflammatory maturation and activation of DCs was shown to be dependent upon PI3/Akt-mediated metabolic reprogramming, switching from oxidative phosphorylation (OXPHOS) to aerobic glycolysis [26]. mTOR is a downstream target of PI3/Akt and was shown to regulate glycolytic metabolism [27]. However, our data showed strong phosphorylation of mTOR in Dex/VitD tDCs after activation with TLR ligands, cytokine cocktail and CD40L which was not accompanied with tDCs maturation. In addition, mTOR inhibition down-regulated tolerogenic molecules ILT-3 and PDL-1 expression and IL-10 production in Dex/VitD tDCs. Therefore, we investigated whether mTOR activation in tDCs is accompanied with glycolytic activation and how glycolysis regulates stable tolerogenic profile of tDCs in the inflammatory environment.

To investigate the glycolytic activity, glucose consumption and lactate production were analyzed in tDCs and cDCs supernatants as an indicator for glycolytic activity. As shown in Figure 6A, Dex/VitD tDCs cultured in Cell Gro secreted similar levels of lactate as cDCs. However, the restimulation of tDCs led to robust accumulation of lactate in cell supernatants that was accompanied with more pronounced gradual decrease in the media glucose content when compared to cDCs. Consistent with the increased glucose consumption and

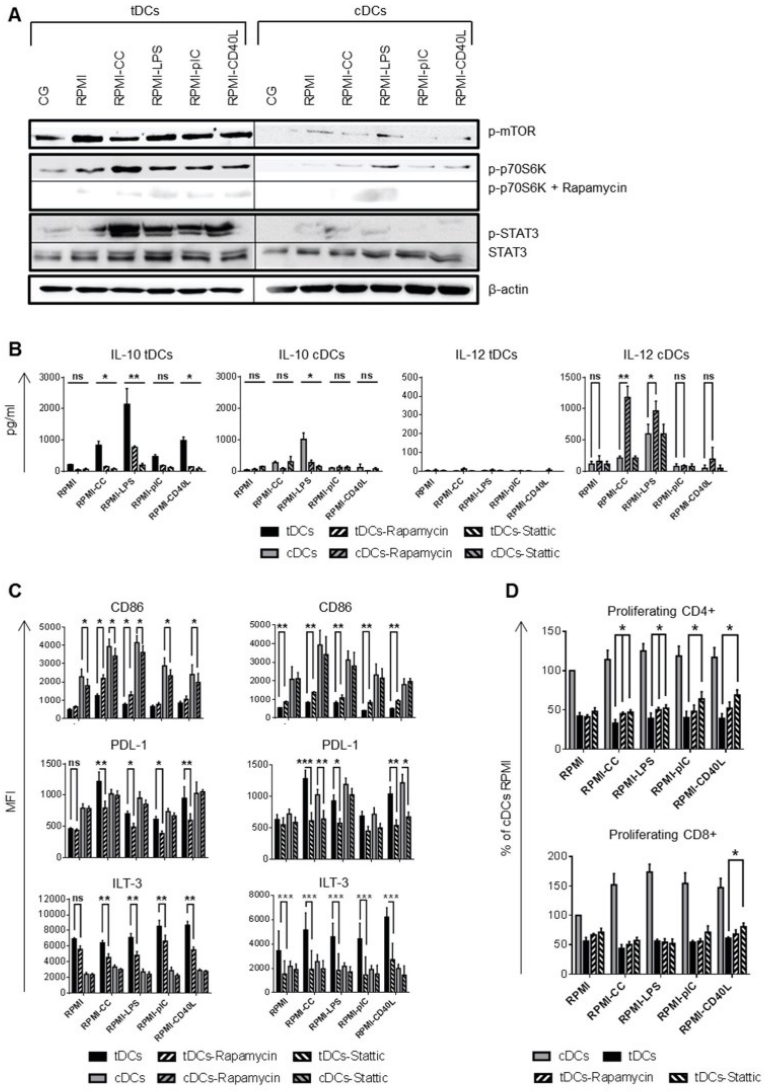

Figure 5: mTOR and STAT3 regulates tolerogenic properties of tDCs after restimulation. DCs were differentiated in Cell Gro in presence (tDCs) or absence of Dex and VitD2 (cDCs) and activated with MPLA (CG). Then, tDCs and cDCs were washed, recultured in complete RPMI without tolerising factors and treated with cytokine cocktail (CC) described in Figure 1B or LPS (1 μg/ml) or polyI:C (25 μg/ml) or CD40L (1000 ng/ml) or they were left unstimulated (RPMI). When indicated, cells were pretreated with mTOR inhibitor rapamycin or STAT3 inhibitor Stattic for 30 min before restimulation. **A.** After 60 min of restimulation western blot analysis for phosphorylated mTOR, p70S6K and STAT3 were performed using specific mAbs. β-actin was used as the equal loading control. One of three experiments performed is shown. **B.** IL-10 and IL-12 production by DCs after 24 h of restimulation was measured by ELISA. **C.** Expression of CD86, PDL-1 and ILT-3 after restimulation with CC, LPS, polyI:C and CD40L in the presence of mTOR inhibitor rapamycin or STAT3 inhibitor Stattic for 24 h was evaluated by FACS analysis. **D.** Before restimulation, tDCs were pretreated with mTOR inhibitor rapamycin or STAT3 inhibitor Stattic and stimulated for 24 h. tDCs were then cocultered with allogeneic T cells. Proliferation was measured on day 6. Data represent mean ± SEM from at least 4 independent experiments. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ (paired *t*-test).

lactate production, tDCs after restimulation revealed higher activity of cellular lactate dehydrogenase, an oxidoreductase enzyme that catalyses the interconversion of pyruvate and lactate, in all the conditions tested compared to cDCs. These data suggest increased glycolysis in Dex/VitD2 tDCs in contrast to cDCs. To test whether mTOR regulates enhanced glycolytic metabolism in tDCs after restimulation, we performed blocking experiments using chemical mTOR inhibitor rapamycin. Rapamycin markedly down-regulated lactate generation in Dex/VitD tDCs (Figure 6B). Thus, restimulation of Dex/VitD tDCs is accompanied by enhanced glycolysis via mTOR activation pathway. Next, we tested whether enhanced glycolysis regulate tolerogenic properties of tDCs after restimulation by adding 10 mM 2-deoxyglucose (2-DG) which acts as an inhibitor of glycolysis and prevents generation of lactate to the DC cultures. Addition of 2-DG to the DCs cultures significantly prevented lactate generation in Dex/VitD tDCs (Figure 6B). Moreover, under these conditions, tDCs failed to up-regulate ILT-3 and PDL-1 molecules (Figure 6C) and markedly decreased IL-10 production (Figure 6D). Expression of CD86 as well as IL-12p70 production remained unaffected upon 2-DG treatment in tDCs in contrast to cDCs. On the other hand, inhibition of glycolysis in tDCs increased partially the ability to induce allogeneic CD4+ as well as CD8+ T cell proliferation (Figure 6E). Taken together, these data show that enhanced glycolysis alters expression of inhibitory molecules, IL-10 production and allostimulatory potential of Dex/VitD tDCs after mimicking *in vivo* subsequent pro-inflammatory activation.

DISCUSSION

Our analysis showed that Dex/VitD tDCs maintain tolerogenic phenotype and function in the inflammatory environment in the absence of tolerogenic factors. We showed for the first time that stability of Dex/VitD tDCs in the inflammatory environment is orchestrated by down-regulated NF-κB, modest activation of p38 MAPK and strong activation of ERK1/2, mTOR and STAT3 molecules that regulate expression of CD86, ILT-3 and PDL-1, production of IL-10 and IL-12p70 and allostimulatory potential of Dex/VitD tDCs.

Recent studies showed a stable tolerogenic phenotype of Dex and/or VitD-treated DCs in terms of maturation markers expression and stable high IL-10 production upon repeated maturation with LPS or pro-inflammatory cytokines [20, 28, 29]. Our data showing stable low to intermediate CD86, CD83 and CD40 expression in contrast to high expression of ILT-3, TIM-3, TLR2 and PDL-1 after restimulation of tDCs corroborate and significantly extend recent findings about the stability of tDCs and indicate the preservation of anti-inflammatory phenotype of tDCs [3, 20]. ILT-3 signaling was shown to result in inhibition of NF-κB and p38 MAPK pathways in DC [30]. TLR-2, ILT-3 and PDL-1 signaling was reported to participate in Tregs induction [20, 31]. In our study, restimulation of tDCs, especially with CC and LPS, led to up-regulation of PDL-1 and ILT-3 expression and stable capacity to induce IL-10 producing CD4+ T cells possessing suppressive capacity. Therefore, our data predict that stable expression of TLR2, ILT-3 and up-regulation of PDL-1 after restimulation of tDCs might play a role in tolerance induction.

We showed that tolerogenic DCs restimulated by inflammatory signals maintained stable cytokine profile with high IL-10 production, up-regulated TGF-β, reduced TNF-α and virtually absent IL-12. The high production of IL-10 together with low production of pro-inflammatory cytokines TNF-α and IL-12 could favor Dex/VitD2 tDCs for immunotherapy.

Consistent with the observed tolerogenic phenotype, Dex/VitD2 tDCs restimulated by inflammatory signals not only showed a reduced ability to induce T cell proliferation, but also were capable of inducing T cells with low IFN-γ and high IL-10 production, by both CD4+ and CD8+ compartments when compared to T cell responses induced by cDCs. The reduction of IFN-γ positive T cells after restimulation with concomitant stable IL-10 positive T cells might be caused by switching T cell response rather toward Th2 due to higher IL-10 production from restimulated tDCs and cDCs [32].

As tDCs reported in this study produced TGF-β, which can induce Tregs as well as Th17 cells [33], we analyzed their Th17 polarizing activity by testing the production of IL-17 from T cells co-cultured with tDCs. We found that Dex/VitD2 tDCs significantly reduced IL-17A production from T cells, even after restimulation with pro-inflammatory stimuli, in contrast to cDCs. As Th17 as well as IFN-γ contributes for pathogenesis of autoimmune diseases [34], the reduction of T cells that secrete IL-17A and IFN-γ might halt or reverse harmful autoimmune processes in subjects with autoimmune disease. Importantly, the low production of IFN-γ and IL-17A with concomitant increased secretion of IL-10 was observed in CD4+ Tregs generated after repetitive stimulation with Dex/VitD2 tDCs and remained similar even upon restimulation with mature DCs. Therefore, cytokine alterations of T cells after priming with Dex/VitD2 DCs cannot be easily explained as the direct result of an insufficient stimulation.

Next, we focused on activation pathways triggered in Dex/VitD2 tDCs upon mimicking subsequent pro-inflammatory activation. We found stable down-regulation of NF-κB pathway in Dex/VitD2 tDCs, further documented by abrogated phosphorylation of IκB-α. In contrast to cDCs, Dex/VitD2 tDCs exhibited low nuclear translocation of NF-κB subunits p65/RelA, RelB and c-Rel that have been shown to up-regulate pro-inflammatory cytokine production [25]. Our data are consistent with the observation that extent of nuclear expression of RelB as a

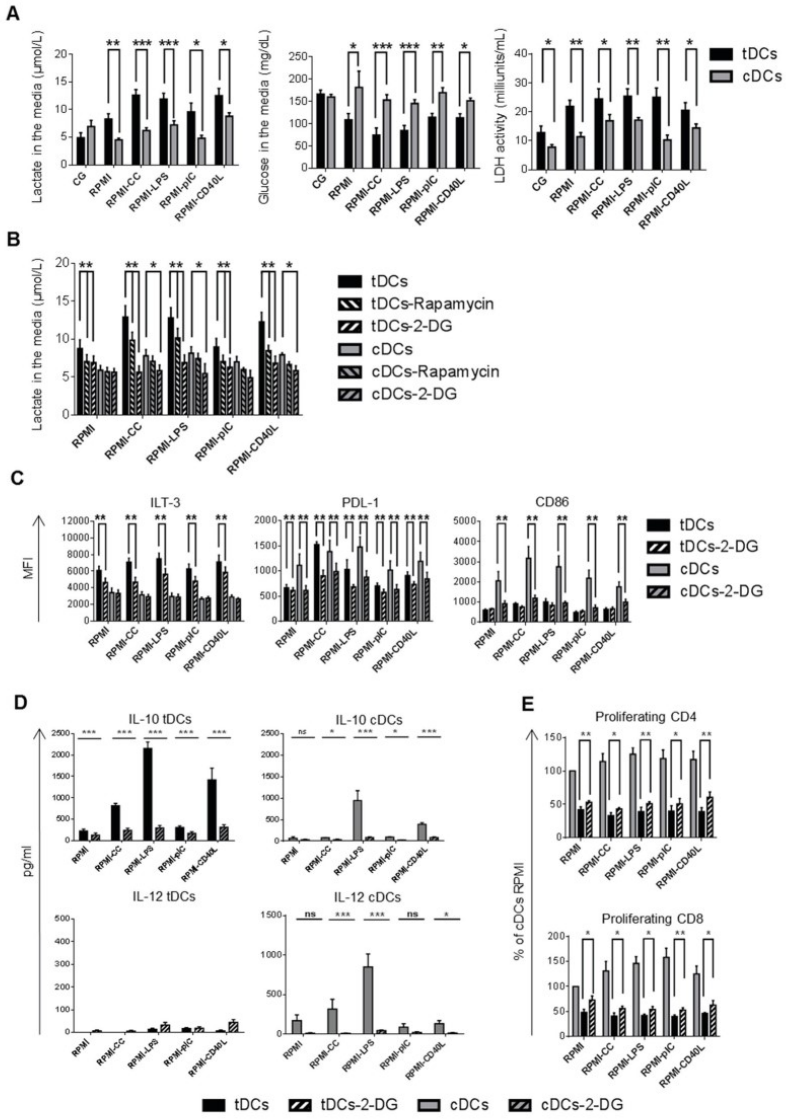

Figure 6: Enhanced glycolysis regulates tolerogenic phenotype and function of Dex/VitD tDCs. DCs were differentiated in Cell Gro in presence (tDCs) or absence of Dex and VitD2 (cDCs) and activated with MPLA (CG). Then, tDCs and cDCs were washed, recultured in complete RPMI without tolerising factors and treated with following stimuli: cytokine cocktail (CC) described in Figure 1B or LPS (1 µg/ml) or polyI:C (25 µg/ml) or CD40L (1000 ng/ml) or they were left unstimulated (RPMI). When indicated, cells were pretreated with rapamycin or 10 mM 2-deoxyglucose (2-DG) for 30 min before restimulation. **A.** 24h later, supernatants were analyzed for the concentration of glucose and lactate as indicator of glycolytic activity. The activity of lactate dehydrogenase (LDH) was analyzed in cell lysates. **B.** 24 h later, suppression of glycolysis by treatment of DCs with rapamycin or 10 mM 2-deoxyglucose 30 min before restimulation was analyzed by evaluating the concentration of lactate in DC supernatants. **C.** ILT-3, PDL-1 and CD86 expression on DCs after 24 h of restimulation in the presence of glycolysis inhibitor 2-deoxyglucose was evaluated by FACS analysis. **D.** IL-10 and IL-12 production by DCs after 24h of restimulation was measured by ELISA. e Before restimulation, tDCs were pretreated with 2-DG and stimulated for 24 h. tDCs were then cocultered with allogeneic T cells. Proliferation was measured on day 6. Data represent mean ± SEM from at least 4 independent experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ (paired *t*-test).

p50/RelB heterodimer in DCs correlates with the degree of maturation [23]. As c-Rel plays a role in IL-12 production [24], down-regulated levels of c-Rel in our tDCs reflect their abrogated ability to produce IL-12 even after secondary stimulation when the tolerogenic agents are absent. High levels of p50 in nucleus of tDCs can reflect the fact that p50 homodimers repress proinflammatory cytokine production but serve as transcriptional activators of IL-10 [25]. The link between high levels of p50 and high production of IL-10 in tDCs is supported by strong reduction of IL-10 production after treatment with NF-κB inhibitor Bay 11-7082 reported previously to block phosphorylation of p50 [35].

Our data support the use of distinct MAPK activation pathways in tDCs *vs* cDCs after restimulation with inflammatory stimuli. In tDCs, activation of p38 MAPK after restimulation is lower compared to cDCs. However, the experiments with p38 MAPK inhibitor show that p38 MAPK plays an important role in IL-10 production and expression of tolerogenic molecules ILT-3 and PDL-1 in tDCs. In contrast, p38 MAPK is markedly activated in cDCs after restimulation and controls mainly IL-12 production with no significant effect on the expression of tolerogenic molecules. We next show the significant ERK1/2 phosphorylation after restimulation with all stimuli tested in tDCs but only after LPS restimulation in cDCs. This might correlate with marked up-regulation of IL-10 production in these stimulatory conditions. Blocking experiments with ERK1/2 inhibitor PD98059 confirmed the role of ERK1/2 in IL-10 production after inflammatory trigger in tDCs and support the role of ERK1/2 activation in IL-10 secretion [36]. Moreover, blocking of ERK1/2 activation partially restored CD86 up-regulation, prevented PDL-1 up-regulation and partially restored allostimulatory potential of tDCs. These data suggest the distinct role of p38 MAPK and ERK in tolerogenic *vs* pro-inflammatory maturation. Corroborating our results, p38 MAPK and ERK were shown to regulate PDL-1 expression in different DCs types [37].

Dex/VitD2 tDCs also express high levels of IDO that remains stable after restimulation. As expression of IDO in tDCs and the ensuing production of tryptophan metabolites has been shown to induce direct suppression of effector T cell activity and concurrent expansion of Tregs [14, 15], stable IDO expression might support tolerogenic properties of Dex/VitD2 tDCs.

Finally, we newly documented that mTOR and STAT3 inhibition led to up-regulated CD86 expression, down-regulated ILT-3 and PDL-1 expression, down-regulated IL-10 production and increased ability to stimulate T cell proliferation in Dex/VitD2 tDCs after restimulation. This phenotype was not observed in control DCs in which surface expression of CD86 was down-regulated but PDL-1 and ILT-3 expression remained similar upon mTOR inhibition. Our data demonstrate the novel and important anti-inflammatory role of mTOR and STAT3 in Dex/VitD2 tDCs and brings additional knowledge about the versatile role of mTOR in DC activation. Recently, the PI3K/mTOR pathway has been documented as a negative regulator of TLR signaling in human monocytes and myeloid DCs. Rapamycin-treated myeloid immune cells display a strong Th1 and Th17 polarization [11] and are capable of blocking the anti-inflammatory effects of dexamethasone [38]. It might suggest that dexamethasone used for generation of our Dex/VitD2 tDCs requires active mTOR for maintaining its anti-inflammatory effects. On the other hand, mTOR was documented to be indispensable for monocyte-derived DC survival and differentiation [11, 12, 39]. Data from our work suggest that in Dex/VitD tDCs, mTOR pathway dictate the maintenance of tolerogenic DC phenotype.

Surprisingly, we showed that enhanced glycolysis modulated via mTOR signaling pathway regulate tolerogenic phenotype and function of Dex/VitD tDCs in the inflammatory environment by modulating CD86, ILT-3 and PDL-1 expression, IL-10/IL-12 ratio and ability to stimulate T cell proliferation. Our data are in a contrast to previous studies showing enhanced glycolysis and PI3/Akt/mTOR signaling pathway being indispensable for pro-inflammatory maturation and function of DCs and T cells [26, 27]. However, in line with our data, Ferreira et al. documented very recently that tolerogenic DCs generated by VitD3 use the activation of glucose metabolism controlled by the PI3/Akt/mTOR signaling pathway to promote tolerogenic phenotype and function [40].

Taken together, we report that our clinical grade Dex/VitD2 tDCs preserve their phenotypic and functional properties upon stimulation with a variety of biologically relevant inflammatory stimuli in the absence of tolerogenic factors. To our knowledge, this study describes for the first time the regulation of key activation pathways after restimulation of tDCs in the absence of tolerogenic agents. Our data show that tDCs employ distinct activation pathways such as p38 MAPK, ERK1/2, IDO, mTOR and STAT3 to maintain their tolerogenic phenotype and immunoregulatory function upon mimicking subsequent pro-inflammatory activation in contrast to cDCs characterized by strong activation of p38 MAPK and NF-κB. Distinct pattern of signaling pathways triggered by inflammatory stimuli can also serve as a feasible and robust identity test that would distinguish inflammatory and tolerogenic DCs in culture. This study on clinical grade tDCs provides a rationale for their testing in the clinical settings, such as in autoimmune diseases or transplantation.

MATERIALS AND METHODS

Reagents and Abs

Flow cytometry: commercial antibodies anti-CD86-FITC (clone 2231 FUN-1), CD274 (PD-L1)-FITC (clone MIH1), CD273 (PD-L2)-PE (clone MIH-18), HLA-DR-PE-Cy7 (clone L243), IFN-γ-FITC (clone 4SB3) were purchased from BD Biosciences; CD83-PerCP-Cy5.5 (clone HB15a) was purchased from Beckman Coulter; CD80-FITC (clone MAB104), CD40-PerCP-eFluor710 (clone 5C3), CD1a-PE-Cy7 (clone HI149) and CD4-PE-Cy7 (clone RPA-T4) were purchased from eBioscience; TLR2-FITC (clone T2.5), TIM-3-PE (clone F38-2E2), IL-10-PE (clone JES3-9D7), KI-67-PE (clone Ki-67) were purchased from BioLegend; CD14-PE-DL594 (clone MEM-15), CD11c-APC (clone BU15), CD3-AF700 (clone MEM-57), CD8-PE-Dy590 (clone MEM-31) were purchased from Exbio; CD85k (ILT-3)-PE (clone 293623), CD85d (IL-T4)-FITC (clone 287219) were purchased from R&D Systems. For western blot, anti-p-p38 MAPK, anti-p-ERK1/2, anti-p-JNK/SAPK, anti-p-IκB-α, anti-IDO, anti-p-mTOR, anti-p-STAT3, anti-p-p70S6K, anti-p38 MAPK, anti-ERK1/2, anti-JNK/SAPK and anti-STAT3 Ab were purchased from Cell Signaling Technology; anti-actin was from BioLegend.

DC differentiation, stimulation and inhibition

Immature DCs were obtained from buffy coats of healthy donors as previously described [18]. Briefly, human peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient and monocytes were separated by allowing 2 h of cell adhesion in 75-cm^2 culture flasks (Nunc). DCs were generated by culturing monocytes for 6 days in GMP-grade Cell Gro DC medium (CellGenix) containing penicillin and streptomycin (100 U/ml and 100 µg/ml, respectively, Gibco) in the presence of GM-CSF (500 IU/ml, Gentaur) and IL-4 (20 ng/ml, CellGenix). Medium and cytokines were replenished on day 3. On day 6, DCs were harvested and seeded in 96-well plates (Nunc) at 1x10^{6}cells/ml. On day 7, immature DCs were activated with vacci grade MPLA (2 µg/ml, Cayla-InvivoGen) for 24 hrs. To induce tDCs, DCs were treated with Dex on day 3 (1 µM, Medochemie) and Dex and VitD2-paricalcitol (1,5 ng/ml, Zemplar, Abbott Laboratories) on day 6. Control DCs (cDCs) were cultured without tolerising factors. For restimulation assays, tDCs and cDCs were washed and recultured in complete RPMI medium (Gibco) containing 5% human AB serum (Invitrogen) in the absence of tolerising factors for 24 h, with or without LPS (1 µg/ml, Sigma-Aldrich), polyI:C (25 µg/ml, Cayla-InvivoGen), megaCD40L™ (1000 ng/ml, Enzo Life Sciences) or mixture of pro-inflammatory cytokines containing IL-1β, TNF-α, IL-6 (all 10 ng/ml) and IFN-γ (100 ng/ml) (all from R&D systems). Signaling inhibitors were added 1 h before the start of experiments under the specified stimulation conditions. SB203580 (p38 MAPK inhibitor at 10 µM), SP600125 (JNK/SAPK inhibitor at 20 µM), PD98059 (ERK1/2 inhibitor at 20 µM), Bay 11-7082 (NF-κB inhibitor at 10 µM), Stattic (STAT3 inhibitor at 5 µM) and rapamycin (mTOR inhibitor at 100 nM) were obtained from Calbiochem and dissolved in dimethyl sulfoxide. Supernatants and cells were collected for further analysis.

Flow cytometry

Cells (2x10^5/well) were stained with fluorochrome-conjugated mAbs for 30 min at 4°C in PBS, washed and analysed on LSR Fortessa cell analyzer (BD Biosciences). Appropriate isotype controls were included. Data were analyzed using FlowJo software (Tree Star). DCs were gated according to the forward scatter, side scatter and CD11c+ parameters for analysis. Dead cells were excluded from the analysis based on DAPI (4',6-diamidin-2-fenylindol) staining. For intracellular cytokine staining, T cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml, Sigma-Aldrich) plus ionomycin (1 µg/ml, Sigma-Aldrich) for 4-16 h in the presence of Brefeldin A (5 µg/ml, BioLegend) before analysing. After stimulation, cells were washed, incubated in Fixation/Permeabilization Buffer (eBioscience) for 30 min at 4°C, then washed in Permeabilization Buffer (eBioscience) and stained with appropriate monoclonal antibody (mAb) for 30 min at 4°C.

DC cytokine production

Cell supernatants were harvested after 24 h of DC stimulation and frozen at -80°C until analysis. IL-10, IL-12p70, TNF-α and TGF-β concentrations were determined using Luminex assay (MILLIPLEX™ Human Cytokine/Chemokine Kit, Merck Millipore) and ELISA assay (DuoSet ELISA Kit, R&D systems) according to the manufacturer's instructions. Cell supernatants were acidified before measuring TGF-β levels according to the manufacturer's instructions.

DCs and T cells cultures, allostimulatory assay

T cells were obtained from PBMC non-adherent fraction. tDCs or cDCs (2x10^4) were cultured with allogeneic T cells (2x10^5) in complete RPMI medium (Gibco) containing 5% human AB serum (Invitrogen). IL-2 (20 U/ml, PeproTech) was added on day 2, 5 and 7. For primary mixed lymphocyte reaction (MLR) assays, allogeneic T cells (2x10^5) labelled with 5

μM carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) were incubated with tDCs or cDCs ($2x10^4$). T cell proliferation was determined by the sequential dilution of CFSE fluorescence of T cells, as detected by flow cytometry on day 6. For detection of IFN-γ, IL-10 and IL-17A production by T cells, $2x10^4$ tDCs or cDCs were cultured with $2x10^5$ allogeneic T cells. Cytokine production was determined by intracellular staining by flow cytometry on day 6 (IFN-γ) and day 9 (IL-10). IL-17A production from cell culture supernatants was analyzed by ELISA on day 6.

Expansion of regulatory T cells and suppression assay

Naïve CD4+ T cells (donor A) were purified by negative selection with The EasySep™ Human Naïve CD4+ T Cell Enrichment Kit (StemCell Technologies). Naïve CD4+ T cells were plated with allogeneic human leukocyte antigen (HLA)-mismatched Dex/VitD2 tDCs (donor B) in a 10:1 ratio for 6 d in complete RPMI (5% human AB serum) in a 24-well plate. IL-2 (20 U/ml, PeproTech) was added on day 2 and 5. Next, T cells were washed and rested for 2 d with complete RPMI (5% human AB serum) and IL-2 and subsequently restimulated with Dex/VitD2 tDCs under the same condition for 5 d. After 5 days, T cells were recovered and rested for 2 days before use in the suppression assay. T cells primed for two rounds with Dex/VitD2 tDCs are referred to as Tregs. CD4+ Tregs were tested for suppressive capacity in following MLR assay. CD4+ Tregs (donor A) were labeled with Vybrant DiD cell labeling solution (5 μM, Millipore), washed and plated in a round-bottom 96-well plate coated with 1:20 000 anti-CD3 mAb (clone MEM-57) with responder T cells (donor A) and MPLA-matured cDCs (not treated with Dex and VitD2) (donor B). cDCs were from the same donor as the Dex/VitD2 tDCs used to induce Tregs. Cells were plated in a Treg/Tresp/DCs ratio of 10:10:1 or 5:10:1. As additional controls, Tresp and Tregs were cultured alone or with cDCs. After 6 d, cells were recovered and proliferation of responder cells was analyzed by measuring Ki-67 by flow cytometry. Cell culture supernatants were recovered for IL-10, IFN-γ and IL-17A analysis.

Western blot analysis

Cell lysates ($2x10^6$ DCs) were prepared from cells cultured in Cell Gro or recultured in complete RPMI alone or with cytokine cocktail, LPS, polyI:C or CD40L for 1 h as previously described [18]. When indicated, rapamycin (100 nM) was added 1 h before stimulation. Cell lysates were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes before being immunoblotted with indicated specific mAbs. The membranes were revealed by horseradish peroxidase-conjugated secondary Ab (Cell Signaling Technology) using the West Femto Maximum Sensitivity Substrate (Pierce). After stripping, the membranes were reprobed with an appropriate mAb as loading control.

Preparation of nuclear extracts and colorimetric NF-κB assay

Nuclear extracts were prepared from DCs ($2x10^6$) cultured in Cell Gro or recultured in complete RPMI alone or with cytokine cocktail, LPS, polyI:C or CD40L for 90 min using a nuclear extract kit (Active Motif). NF-κB DNA binding activity of p50, p65/RelA, c-Rel and RelB was measured as previously described [18].

Metabolic quantification: lactate, glucose and lactate dehydrogenase measurements

Concentrations of lactate and glucose in DC culture supernatants were measured with Glycolysis Cell-based assay kit (Cayman Chemicals) and Glucose colorimetric assay kit (BioVision), respectively. When indicated, glycolysis was suppressed by treatment with 10 mM 2-deoxyglucose (Sigma) 1 h prior exposition of DCs cultured in Cell Gro into RPMI, LPS, CC, polyI:C or CD40L. LDH activity of the DCs extracts was measured with Lactate dehydrogenase activity assay kit (Sigma).

Statistical analysis

Results were obtained from at least three independent experiments and are given as mean ± SEM. Two-tailed paired t-test was applied for data analysis using GraphPad PRISM 6. A value of $p \leq 0.05$ was considered statistically significant.

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CONFLICTS OF INTEREST

The authors declare no financial or commercial conflict of interest.

Editorial note

This paper has been accepted based in part on peer-review conducted by another journal and the authors' response and revisions as well as expedited peer-review in Oncotarget.

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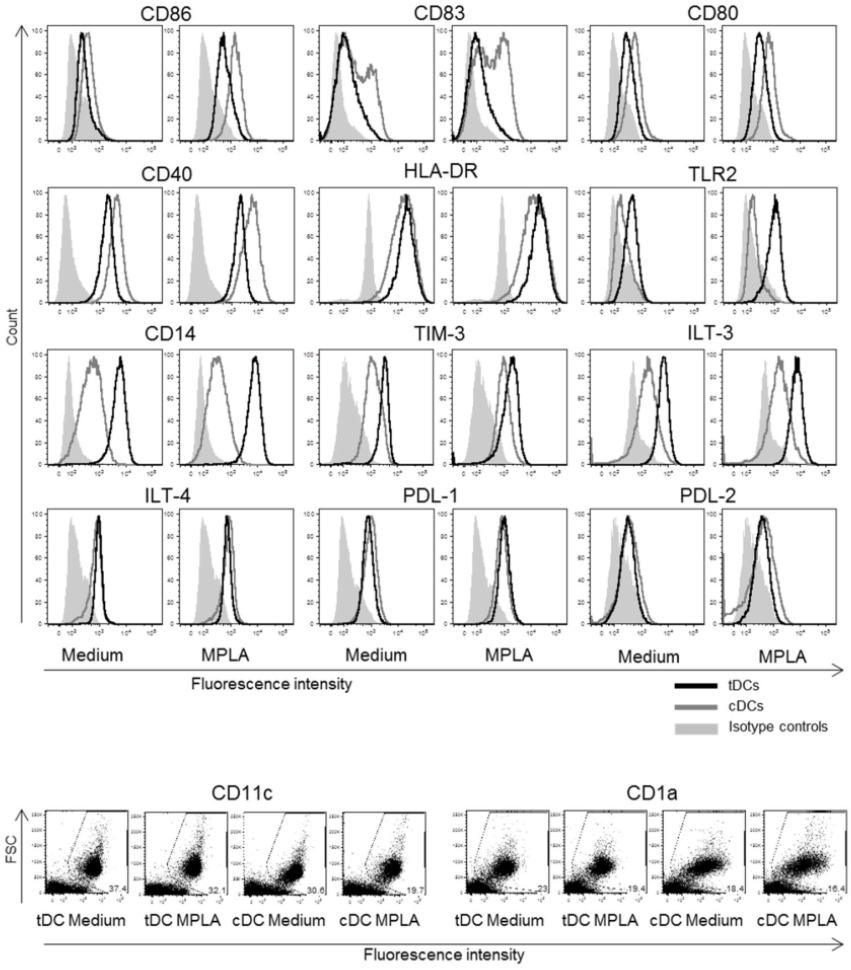
NF-κB, p38 MAPK, ERK1/2, mTOR, STAT3 and increased glycolysis regulate stability of paricalcitol/dexamethasone-generated tolerogenic dendritic cells in the inflammatory environment

Supplementary Material



SUPPLEMENTAL FIGURE 1. Dex/VitD2 tDCs display tolerogenic features. DCs were differentiated in Cell Gro in presence (tDCs) or absence of Dex and VitD2 (cDCs) and then activated with MPLA. (**A**) Surface marker expression on tDCs (black lines) and cDCs (grey lines) before maturation (MEDIUM) and after final maturation with MPLA (MPLA) was evaluated by FACS. Light gray filled histograms represent isotype control mAb staining. (**B**) Representative dot plots of CD11c and CD1a positive cells are shown. Results are shown from representative experiments out of minimal 10 donors.

5.2 PART II: Tolerogenic dendritic cells from poorly compensated type 1 diabetes patients have decreased ability to induce stable antigen-specific T cell hyporesponsiveness and generation of suppressive regulatory T cells

Tolerogenic DCs with their substantial ability to modulate immune responses represent a promising therapeutic tool for the treatment of autoimmune disorders. Given that individuals suffering from an autoimmune disorder have the deregulated immune system with the ongoing autoreactive process, we wanted to test the possibility to generate tolerogenic DCs according to our manufacturing protocol from the blood of T1D patients with the same quality as from healthy individuals. In addition, we wanted to evaluate whether the clinical parameters of T1D patients have an impact on the phenotype and function of tolerogenic DCs, since the study by Segovia-Gamboa showed that metabolic parameters, such as glycemia and a cholesterol level, influenced the quality of IL-10 and TGF-β-conditioned tolerogenic DCs [202].

We demonstrated that dexamethasone/vitamin D_2-treated tolerogenic DCs (referred as Dex/VitD_2 tolerogenic DCs) generated from T1D patients acquired the regulatory phenotype with significantly lower expression of a broad spectrum of maturation-associated markers and higher expression of tolerogenic markers compared with control DCs prepared without tolerizing agents (referred as control DCs). In line with anti-inflammatory surface marker expression, Dex/VitD_2 tolerogenic DCs secreted significantly lower amounts of proinflammatory cytokines and higher amounts of the suppressive cytokine IL-10. However, when we analyzed surface marker expression in the context of HbA_{1c} levels (reflecting long-term glycemic control) of T1D patients, we found that Dex/VitD_2 tolerogenic DCs from patients with low HbA_{1c} levels (HbA_{1c} ≤7.5%), which means with satisfactory glycemic control, expressed significantly higher amounts of PD-L1 and IL-T3 markers than patients with poor glycemic control (HbA_{1c} >7.5%).

Furthermore, we analyzed the stimulatory and suppressive capacity of Dex/VitD_2 tolerogenic DCs generated from well-controlled versus poor controlled patients. We found that GAD65-loaded Dex/VitD_2 tolerogenic DCs from well-controlled patients induced antigen-specific hyporesponsiveness of autologous T cells, which was accompanied by reduction of Th1 and Th17

responses. Moreover, GAD65-loaded Dex/VitD_2 tolerogenic DCs from well-controlled patients were more potent to suppress secretion of proinflammatory cytokines induced by control DCs when compared to Dex/VitD_2 tolerogenic DCs generated from poorly controlled patients. In addition, we found that glycemic control significantly influenced the possibility to induce stable GAD65-specific T cell hyporesponsiveness in T1D patients. Finally, we showed that Dex/VitD_2 tolerogenic DCs of both group of patients promoted the differentiation of naïve $CD4^+$ T cells into $FoxP3^+$ Tregs. However, Tregs from well-controlled patients have better suppressive abilities than Tregs from poorly controlled patients.

In general, given that recent clinical studies have shown that determination of a suitable group of patients could have an absolutely crucial impact on the treatment efficacy, the presented data provide important information about T1D patients who could benefit from treatment with the therapy based on tolerogenic DCs in a potential clinical trial.

My contribution: experimental design, generation of tolerogenic and control DCs, analysis of DC yield, viability and phenotype (flow cytometry and following analysis using FlowJo software; ELISA; Luminex), DC and T cell cultures, analysis of proliferation and cytokine production by T cells, analysis of Treg differentiation from naïve T cells (flow cytometry and following analysis using FlowJo software; ELISA; Luminex), data analysis and interpretation, manuscript and figure preparation.

The Journal of Immunology

Tolerogenic Dendritic Cells from Poorly Compensated Type 1 Diabetes Patients Have Decreased Ability To Induce Stable Antigen-Specific T Cell Hyporesponsiveness and Generation of Suppressive Regulatory T Cells

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Tolerogenic Dendritic Cells from Poorly Compensated Type 1 Diabetes Patients Have Decreased Ability To Induce Stable Antigen-Specific T Cell Hyporesponsiveness and Generation of Suppressive Regulatory T Cells

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Tolerogenic dendritic cells (tolDCs) may offer an interesting intervention strategy to re-establish Ag-specific tolerance in autoimmune diseases, including type 1 diabetes (T1D). T1D results from selective destruction of insulin-producing β cells leading to hyperglycemia that, in turn, specifically affects a patient's immune system. In this study, we prepared monocyte-derived tolDCs modulated by dexamethasone and vitamin D_2 from 31 T1D patients with optimal glycemic control and 60 T1D patients with suboptimal glycemic control and assessed their tolerogenic properties in correlation with metabolic state of patients. tolDCs differentiated from both groups of patients acquired a regulatory phenotype and an anti-inflammatory profile. Interestingly, tolDCs from well-controlled patients expressed higher levels of inhibitory molecules IL-T3 and PD-L1. Additionally, glutamic acid decarboxylase (GAD)65–loaded tolDCs from well-controlled patients decreased significantly primary Th1/Th17 responses, induced stable GAD65-specific T cell hyporesponsiveness, and suppressed markedly control DC-induced GAD65-specific T cell activation compared with poorly controlled patients. The ability of tolDCs from poorly controlled patients to induce durable GAD65-specific T cell hyporesponsiveness was reversed once the control of glycemia improved. In both groups of patients, tolDCs were able to induce regulatory T cells from autologous naive $CD4^+$ T cells. However, regulatory T cells from well-controlled patients had better suppressive abilities. The functionality of tolDCs was confirmed in the adoptive transfer model of NOD-SCID mice where tolDCs delayed diabetes onset. These results suggest that metabolic control of T1D affects the functional characteristics of tolDCs and subsequent effector T cell responses. Metabolic control may be relevant for refining inclusion criteria of clinical trials in the settings of T1D. ***The Journal of Immunology*****, 2017, 198: 000–000.**

Type 1 diabetes (T1D) is a life-long disorder resulting from deficient tolerance to islet β cell Ags, allowing the autoimmune destruction of insulin-producing β cells by $CD4^+$ and $CD8^+$ T cells (1–5). Insulin deficiency together with suboptimal insulin replacement results in complex metabolic derangement with abnormal metabolome. Metabolic derangement may be an important progression factor or may play a role in influencing immunity and pathogenesis of the disease completing the vicious cycle of long-term disease progression (6, 7). Hyperglycemia, a characteristic property of diabetes, contributes to the breakdown of peripheral tolerance (8), affects the function of APCs (9), facilitates the differentiation of proinflammatory Th1/Th17 cells, and suppresses regulatory T cells (Tregs) (10–12).

Long-term insulin treatment of T1D is indispensable for patient survival, but it cannot fully mimic the physiological insulin secretion. The goal for the prevention and/or reversal of T1D is to find an effective approach to restore durable tolerance to halt the immune-mediated loss of β cells. At present, no such therapy exists. So far, many broad-based immunosuppressive and Ag-specific immunoregulatory therapies proved to be effective in an

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Abbreviations used in this article: ctrDC, control dendritic cell; DC, dendritic cell; G1, group 1; G2, group 2; GAD, glutamic acid decarboxylase; Hb, hemoglobin; MPLA, monophosphoryl lipid A; PPD, tuberculin purified protein derivative; T1D, type 1 diabetes; $T_{ctr+GAD}$, T cell from T1D patient cultivated in primary coculture with autologous GAD65–loaded ctrDC; $T_{ctr+PPD}$, T cell from healthy patient cultivated with autologous PPD–loaded ctrDC in primary culture; tolDC, tolerogenic dendritic cell; Treg, regulatory T cell; Tresp, responder T cell; TT, tetanus toxin; $T_{tol+GAD}$, T cell from T1D patient cultivated in primary coculture with autologous GAD65–loaded tolDC; $T_{tol+PPD}$, T cell from healthy patient cultivated with autologous PPD–loaded tolDC in primary culture.

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experimental animal model, but they failed to show a measurable outcome in clinical settings. Some of them seemed to have partial efficacy in selected patient subgroups (13–18).

Tolerogenic dendritic cells (tolDCs) represent a new promising therapeutic approach in prevention of T1D, either alone or in combinatorial therapies (19). TolDCs promote immune tolerance by the induction of hyporesponsiveness or selective apoptosis of autoreactive T cells and induction of different subtypes of Tregs and B regulatory cells (20–23). Induced Tregs can, in turn, transfer the immunoregulatory properties to other T cells and proinflammatory DCs, resulting in maintaining the long-term Ag-specific tolerance (24–26).

We have recently reported a good manufacturing practices–compliant protocol for the generation of dexamethasone/vitamin D_2–modulated human tolDCs that maintain stable regulatory phenotype and ability to induce functional Tregs in the inflammatory environment (27). In the present study, we generated tolDCs from T1D patients and extensively characterized their functional properties with the focus on their capacity to induce functional Tregs. Given the immunomodulatory effect of long-term hyperglycemia, we analyzed tolDCs characteristics separately in patients with satisfactory versus suboptimal glycemic control. We show that the degree of T1D control significantly affects the functional properties of tolDCs and subsequent effector T cell responses.

Materials and Methods

Subjects and animals

Blood samples were obtained from 91 patients diagnosed with T1D and from 11 healthy donors. At the time of the blood sampling, none of the patients had severe diabetic ketoacidosis, nor any active infection, neoplasia, or other comorbidities, except comorbidities related to T1D such as well-controlled thyroiditis or celiac disease (15.5 or 18% of recruited subjects, respectively). Six patients were recruited around the time of diagnosis. Patients were divided into two groups based on the level of glycated hemoglobin (Hb) A_{1c} that reflects a long-term glycemia (Table I). HbA_{1c} of ≤7.5% (58 mmol/mol) was considered as an optimal disease control according to International Society for Pediatric and Adolescent Diabetes guideline (28). Group 1 (n = 31) included patients with optimal T1D control (HbA_{1c} of ≤7.5% [58 mmol/mol]), and group 2 (G2; n = 60) included patients with suboptimal T1D control (HbA_{1c} of >7.5% [58 mmol/mol]). Six patients recruited around the time of diagnosis were included into G2 according to their HbA_{1c}. Healthy donors included six females and five males aged 10–20 y (Table I). All study participants gave informed consent, and the study was approved by the local Ethical Review Committee.

NOD/BomTac and NOD-SCID female mice were obtained from Taconic Europe (Ry, Denmark) and kept under barrier-protected conditions according to the Federation of European Laboratory Animal Science Associations guidelines.

DC generation

Monocytes were purified from peripheral mononuclear cells by density centrifugation (Ficoll-Paque; GE Healthcare), followed by adhesion in 75-cm^2 culture flasks (Nunc) and cultured in CellGro medium (CellGenix) containing 100 μg/ml penicillin-streptomycin (Life Technologies), IL-4 (248 IU/ml; CellGenix), and GM-CSF (500 IU/ml; Gentaur) for 6 d. Medium was refreshed on day 3. TolDCs were generated by adding dexamethasone on day 3 (1 μM; Medochemie) and dexamethasone (1 μM) with vitamin D_2 (Zemplar, 1.5 ng/ml; Abbott Laboratories) on day 6. Control DCs (ctrDCs) were generated without tolerogenic factors. At day 7, tolDCs and ctrDCs were either loaded with glutamic acid decarboxylase (GAD)65 (5 μg/ml; Diamyd Medical) or with tuberculin purified protein derivative (PPD; 1 μg/ml; Statens Serum Institut) or with tetanus toxin (TT; 2 μg/ml; Merck Millipore) or left unpulsed. Finally, DCs were activated with VacciGrade monophosphoryl lipid A (MPLA; 2 μg/ml; Cayla-InvivoGen) for 24 h.

Mouse bone marrow–derived DCs were generated from bone marrow cells of 8-wk-old NOD females in complete RPMI 1640 medium (Lonza) in the presence of GM-CSF (20 ng/ml) and IL-4 (4.5 ng/ml; PeproTech) for 6 d. Fresh medium was added on day 3. TolDCs were induced by adding dexamethasone and vitamin D_2 on day 6 whereas ctrDCs were generated without tolerogenic factors. At day 7, tolDCs and ctrDCs were finally activated with VacciGrade MPLA (2 μg/ml) for 24 h.

Flow cytometry

Cells were stained with following fluorochrome-conjugated Abs: anti–CD86-FITC (2231 FUN-1), CD274 (PD-L1)–FITC (MIH1), and HLA-DR–PE-Cy7 (L243) purchased from BD Biosciences; CD83-PerCP-Cy5.5 (HB15a) purchased from Beckman Coulter; CD4-PE-Cy7 (RPA-T4) and FOXP3–Alexa Fluor 488 (PCH101) purchased from eBioscience; TLR2-FITC (T2.1), CD25-PerCP-Cy5.5 (BC96), Ki-67–PE (Ki-67), IFN-γ–Pacific Blue (4S.B3), IL-17A–Alexa Fluor 647 (BL168), and CD127–Alexa Fluor 647 (A019D5) purchased from BioLegend; CD14-PE-DL594 (MEM-15), CD11c-allophycocyanin (BU15), CD3–Alexa Fluor 700 (MEM-57), and CD8-PE–Dyomics 590 (MEM-31) purchased from Exbio; and CD85k (IL-T3)–PE (293623) purchased from R&D Systems. Mouse DCs were characterized by using following mAbs purchased from eBioscience: anti–CD11c-APC (clone N418), anti–CD40-PerCP-eFluor 710 (clone 1C10), anti–CD80-FITC (clone 16-10A1), anti–CD86-PE (clone GL1), and anti–MHC class II (I-A/I-E) (clone MS/114.15.2). Data were acquired by LSRFortessa and LSR II flow cytometers (BD Biosciences) and analyzed using FlowJo software (Tree Star). $CD11c^+$ $DAPI^-$ DCs and $CD3^+$ T cells were included into the analysis. Appropriate isotype controls were included. For intracellular detection of IFN-γ and IL-17A on day 6, T cells were restimulated with PMA (50 ng/ml; Sigma-Aldrich) plus ionomycin (1 μg/ml; Sigma-Aldrich) for 4 h in the presence of brefeldin A (5 μg/ml; BioLegend). Intracellular Ki-67, IFN-γ, IL-17A, or FOXP3 detection was assessed using a fixation/permeabilization buffer kit (eBioscience) with appropriate mAb.

Cytokine analysis

Levels of secreted IL-10, IL-12p70, IL-6, IL-9, TNF-α, IL-23, IFN-γ, and IL-17A were determined in cell-free culture supernatants using Luminex assay (Milliplex human cytokine/chemokine kit; Merck Millipore) according to the manufacturer's instructions. Data were acquired using Luminex MAGPIX.

DC/T cell cultures

DC/T cells cultures were carried out in complete RPMI 1640 containing 5% human AB serum. In patients with T1D, unlabeled T cells (2×10^5) were stimulated with unpulsed or GAD65-loaded autologous tolDCs or ctrDCs (2×10^4) for 6 d. In healthy donors, unlabeled T cells (2×10^5) were stimulated with unpulsed or PPD-loaded autologous tolDCs or ctrDCs (2×10^4) for 6 d. For tolDC suppression assay, additional GAD65-loaded tolDCs in T1D patients or PPD-loaded tolDCs in healthy donors were added to the cultures containing T cells and Ag-loaded ctrDCs (tolDC/ctrDC ratio was 0.25:1, 0.5:1, and 1:1). For tolerance assays, T cells from primary cultures were harvested on day 6, stained with 3 μM CFSE, and restimulated with GAD65- or PPD-loaded ctrDCs in T1D patients, respective with PPD- or TT-loaded ctrDCs in healthy donors, at a 10:1 T cell/DC ratio for an additional 6 d. T cell proliferation and cytokine production were determined by flow cytometry, Luminex assay, and ELISA (R&D Systems).

Treg induction and functional test

Naive $CD4^+$ T cells (2×10^5) purified by negative selection kit (EasySep human naive $CD4^+$ T cell enrichment kit; StemCell Technologies) from autologous T cells were stimulated with unpulsed or GAD65-loaded tolDCs or ctrDCs (2×10^4) for 9 d (primary cultures). IL-2 (20 U/ml) was added on days 2, 5, and 7. Induction of Tregs was evaluated by flow cytometry. IL-10 production was evaluated by ELISA (R&D Systems). For expansion of Tregs from primary cultures, GAD65-loaded tolDC-induced T cells from primary cultures were washed and restimulated with GAD65-loaded tolDCs under the same condition for 5 d. Then, T cells were recovered and rested for 2 d in the presence of IL-2 (20 U/ml). Tregs primed for two rounds with tolDCs were then labeled with Vybrant DiD cell-labeling solution (5 μM; Molecular Probes), washed, and cultured with autologous responder T cells (Tresps) and GAD65-loaded ctrDCs. The Tresp/GAD65-loaded ctrDC ratio was 10:1, and the Treg/Tresp ratio was 0.25:1, 0.5:1, and 1:1. After 6 d, Tresp proliferation was analyzed by flow cytometry.

Adoptive transfer and diabetes monitoring

Diabetogenic splenocytes were isolated from 13-wk-old prediabetic NOD female donors (n = 10) and cotransferred by i.p. route (5×10^6) in a

volume of 300 μl of PBS with 2×10^6 mouse tolDCs or no DCs (control) into the 8-wk-old NOD-SCID female recipients. Diabetes incidence was ascertained twice weekly in tail vein blood. Confirmation of diabetes was noted by two consecutive positive glycemia (>12 mmol/l) readings.

Statistical analysis

Results obtained from at least three independent experiments are given as mean ± SEM. Owing to limited amounts of blood sample, not all patients are involved in all experiments. A two-tail paired or unpaired *t* test was applied for data analysis using GraphPad Prism 6. Probability levels for correlation were determined using the Pearson correlation test. A p value ≤ 0.05 was considered statistically significant. The cumulative diabetes incidence in animal experiments was assessed using the Kaplan–Meier estimation and contingency tables. A log-rank test and χ^2 test were used for comparisons between groups.

Results

TolDCs from well-controlled patients display higher levels of IL-T3 and PD-L1

We first evaluated whether long-term hyperglycemia (as documented by HbA_{1c}) affects the phenotype of monocyte-derived DCs in 91 T1D patients. TolDCs were generated using dexamethasone/vitamin D_2 and activated with MPLA, a nontoxic alternative of LPS, to obtain alternatively activated tolDCs with improved tolerogenic properties as reported previously (29) (Table I).

In both groups of patients (group 1 [G1], HbA_{1c} of ≤7.5%; G2, HbA_{1c} of >7.5%) and in healthy donors, immature as well as MPLA-activated tolDCs had lower surface expression of CD86, CD83, CD40, and HLA-DR but higher expression of CD14, TLR2, and inhibitory molecule IL-T3 than did ctrDCs (Fig. 1A, Supplemental Fig. 1). Expression of inhibitory molecule PD-L1 was lower on tolDCs compared with ctrDCs, but MPLA-activated tolDCs displayed a higher PD-L1/CD86 ratio (Fig. 1B) that was shown to correlate with reduced T cell stimulatory capacity (30). Interestingly, expression of IL-T3 and PD-L1 was significantly higher on tolDCs from G1 patients compared with tolDCs from G2 patients, and the PD-L1 level gradually decreased with an increasing HbA_{1c} level (Fig. 1C). These data suggest that hyperglycemia in patients with T1D downregulates IL-T3 and PD-L1 expression on monocyte-derived tolDCs.

In both groups of patients and in healthy donors, MPLA-activated tolDCs produced higher levels of IL-10 and significantly lower quantities of IL-6, TNF-α, IL-23, and IL-12p70 compared with ctrDCs. There were no significant differences among G1 and G2 patients and healthy donors (Supplemental Fig. 2).

TolDCs from well-controlled patients induce autologous T cell hyporesponsiveness accompanied by reduction of primary Th1 and Th17 responses

Then, we investigated the capacity of GAD65-loaded tolDCs from G1 and G2 or PPD-loaded tolDCs from healthy donors to modulate autologous T cell responses by analyzing T cell proliferation and Th1/Th17 induction on day 6 of coculture.

Compared to Ag-loaded ctrDCs, $CD4^+$ T cells (Fig. 2A) as well as $CD8^+$ T cells (Supplemental Fig. 3A) cocultured with Ag-loaded tolDCs exhibited reduced proliferation and IFN-γ production, observed equally in both G1 and G2 irrespective of patients' metabolic state, as well as in healthy donors (Fig. 2A). In contrast, numbers of IL-17A–producing $CD4^+$ T cells were significantly reduced only in G1 patients and healthy donors but not in G2 patients (Fig. 2A). Supernatants from autologous primary cultures of T cells with GAD65-loaded tolDCs from G1 patients contained significantly lower concentrations of Th1-related cytokines IFN-γ and TNF-α and Th-17 related cytokine IL-17A, IL-9, and IL-23 and upregulated levels of IL-10 compared with cultures with ctrDCs (Fig. 2B). However, in G2 patients, we detected significantly lower levels of IFN-γ, TNF-α, and IL-9, but not IL-17A and IL-23. Therefore, in G1 patients, tolDCs exhibited a better ability to induce hyporesponsiveness of autologous GAD65-reactive T cells accompanied by a reduction of Th1 and Th17 cytokines compared with G2 patients.

Interestingly, in these primary cultures we observed not only differences in T cell hyporesponsiveness induced by tolDCs between well- and poorly controlled patients but we also found differences in the baseline characteristics of T cell responses induced by ctrDCs.

In well-controlled G1 patients, $CD4^+$ T cells showed a significantly stronger Ag-specific proliferation and IFN-γ and IL-17A production (Fig. 2A) after GAD65-loaded ctrDC stimulation. In contrast, $CD4^+$ T cells from poorly controlled G2 patients had markedly increased homeostatic (Ag-independent) proliferation as well as IFN-γ and IL-17A production induced by unpulsed ctrDCs, whereas they had lower Ag-specific $CD4^+$ T cell responses after stimulation with GAD65-loaded ctrDCs (Fig. 2A). Similar results were obtained for $CD8^+$ T cells (Supplemental Fig. 3A). In accordance with this finding, we observed the superior production of IFN-γ, IL-17A, and IL-9 in supernatants from T cell and GAD65-loaded ctrDC cultures of G1 but not G2 patients (Fig. 2B). Interestingly, the basal IFN-γ levels produced by unstimulated T cell from G2 were increased in comparison with those from G1 (Fig. 2B).

Next, we asked whether the distinct characteristics of T cell responses induced by ctrDCs in well- versus poorly controlly patients could be associated with different clinical parameters of T1D patients, including HbA_{1c} level, triglyceride level, patient age, age of disease onset, and duration of the disease. Correlation analysis showed that $CD4^+$ T cell Ag-specific proliferation as well as IFN-γ and IL-17A production induced by GAD65-loaded ctrDCs gradually decreased with increasing HbA_{1c}, whereas T cell responses to unpulsed ctrDCs increased (Fig. 2C). Similar results were observed for $CD8^+$ T cells (Supplemental Fig. 3B). There was no correlation between additional clinical parameters and T cell responses (data not shown). Overall, these data show that hyperglycemia leads to increased homeostatic T cell proliferation that, in turn, negatively modulates the GAD65 specificity of T cell responses.

Table I. Characterization of the patients with T1D and healthy donors evaluated in the study

	G1	G2	HD
Number (female/male)	31 (1:1.4)	60 (1.4:1)	11 (1.2:1)
Age: mean ± SD; range (y)	15.1 ± 2.5; 8–18	15.9 ± 2; 10–19	16.6 ± 3.1; 10–20
T1D duration: mean ± SD; range (y)	4.1 ± 4.6; 0–13	6.3 ± 5; 0–17	
HbA_{1c}: mean ± SD; range (%)	6.9 ± 0.5; 5.6–7.5	10.2 ± 1.9; 7.9–16.3	
HbA_{1c}: mean ± SD; range (mmol/mol)	52.4 ± 5.6; 38–58	87.8 ± 20.2; 63–155	

HD, healthy donors.

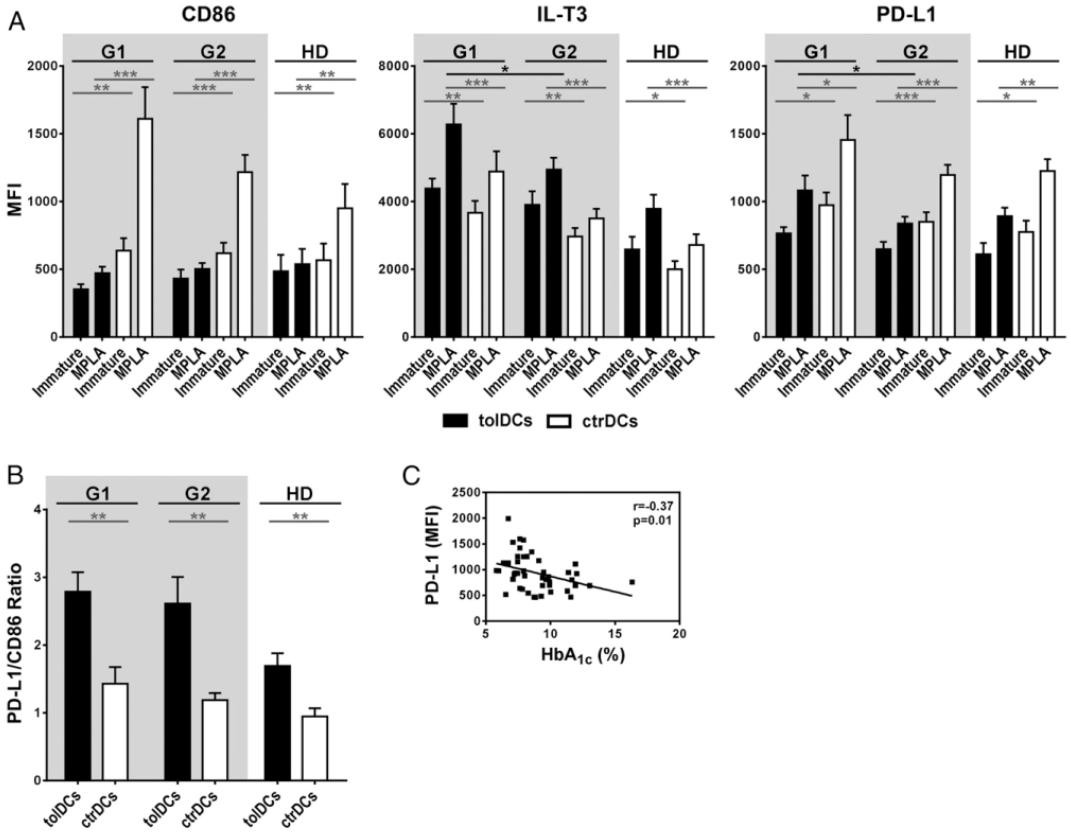

FIGURE 1. Different expression of regulatory molecules IL-T3 and PD-L1 on tolDCs from patients with optimal (G1; HbA_{1c} of ≤7.5%, n = 23) and suboptimal (G2; HbA_{1c} of >7.5%, n = 50) glycemic control and healthy donors (HD; n = 11). (**A**) Surface expression of CD86, IL-T3, and PD-L1 on immature or MPLA-activated tolDCs (filled bars) or ctrDCs (open bars) as analyzed by flow cytometry. Data represent mean fluorescence intensity (MFI) ± SEM from at least 10 experiments. (**B**) Ratios of PD-L1 (MFI)/CD86 (MFI) expressed on MPLA-activated tolDCs and ctrDCs. (**C**) Correlation of PD-L1 expression with HbA_{1c}. Each point represents the MFI value from an individual patient. The r values indicate the correlation index according to Pearson analysis. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

The ability of tolDCs to induce stable Ag-specific T cell hyporesponsiveness depends on the glycemic control of patients with T1D

To this point, we found that tolDCs, especially from well-controlled patients, induced hyporesponsiveness of autologous GAD65-reactive T cells accompanied by a reduction of Th1 and Th17 cytokines. Next, we tested the stability and Ag specificity of T cell hyporesponsiveness induced by tolDCs from T1D patients and healthy donors in a two-step culture system. To address this issue, T cells from T1D patients were cultivated in primary coculture with either autologous GAD65-loaded tolDCs ($T_{tol+GAD}$) or GAD65-loaded ctrDCs ($T_{ctr+GAD}$) for 6 d. Then, to test the stability of T cell hyporesponsiveness, T cells were recovered from primary cultures, stained with CFSE, and subsequently restimulated with autologous GAD65-loaded ctrDCs in secondary cultures. To test whether the induction of hyporesponsiveness by tolDCs was Ag specific, T cells recovered from primary cultures were subsequently restimulated with unrelated Ag, PPD. In healthy donors, T cells were cultivated with either autologous PPD-loaded tolDCs ($T_{tol+PPD}$) or PPD-loaded ctrDCs ($T_{ctr+PPD}$) in primary cultures and subsequently restimulated with PPD-loaded ctrDCs (to test stability) or TT-loaded ctrDC (to test Ag specificity) in secondary cultures (the experimental scheme is shown in Fig. 3A).

As shown in Fig. 3, upon restimulation with GAD65-loaded ctrDCs, $T_{tol+GAD}$ from well-controlled G1 patients but not from poorly controlled G2 patients showed impaired proliferative potential (Fig. 3B) and lower IFN-γ and IL-17A production (Fig. 3C) when compared with $T_{ctr+GAD}$. Importantly, $T_{tol+GAD}$ from G1 proliferated readily to an unrelated Ag as seen for PPD-loaded ctrDC restimulation. Furthermore, tolerance to GAD65 (calculated as the percentage of proliferating $T_{ctr+GAD}$ minus the percentage of proliferating $T_{tol+GAD}$ upon restimulation with GAD65-loaded ctrDCs) inversely correlated with HbA_{1c} levels or the time since diagnosis for both $CD4^+$ T cells (Fig. 3D) and $CD8^+$ T cells (Supplemental Fig. 3C). We found no association between tolerance to GAD65 and other clinical parameters of patients (data not shown). The induction of stable and Ag-specific T cell hyporesponsiveness was observed also in healthy donors (Fig. 3B, 3C). Overall, these data suggest that hyperglycemia has a strong impact on the stability and GAD65 specificity of tolDC-primed T cell hyporesponsiveness in T1D patients.

Next, we asked whether the dysfunction of tolDCs to induce stable tolerance in autoreactive T cells in poorly controlled patients could be reversed following correction of glucose alteration. To answer this question, tolDCs were generated from two diabetic

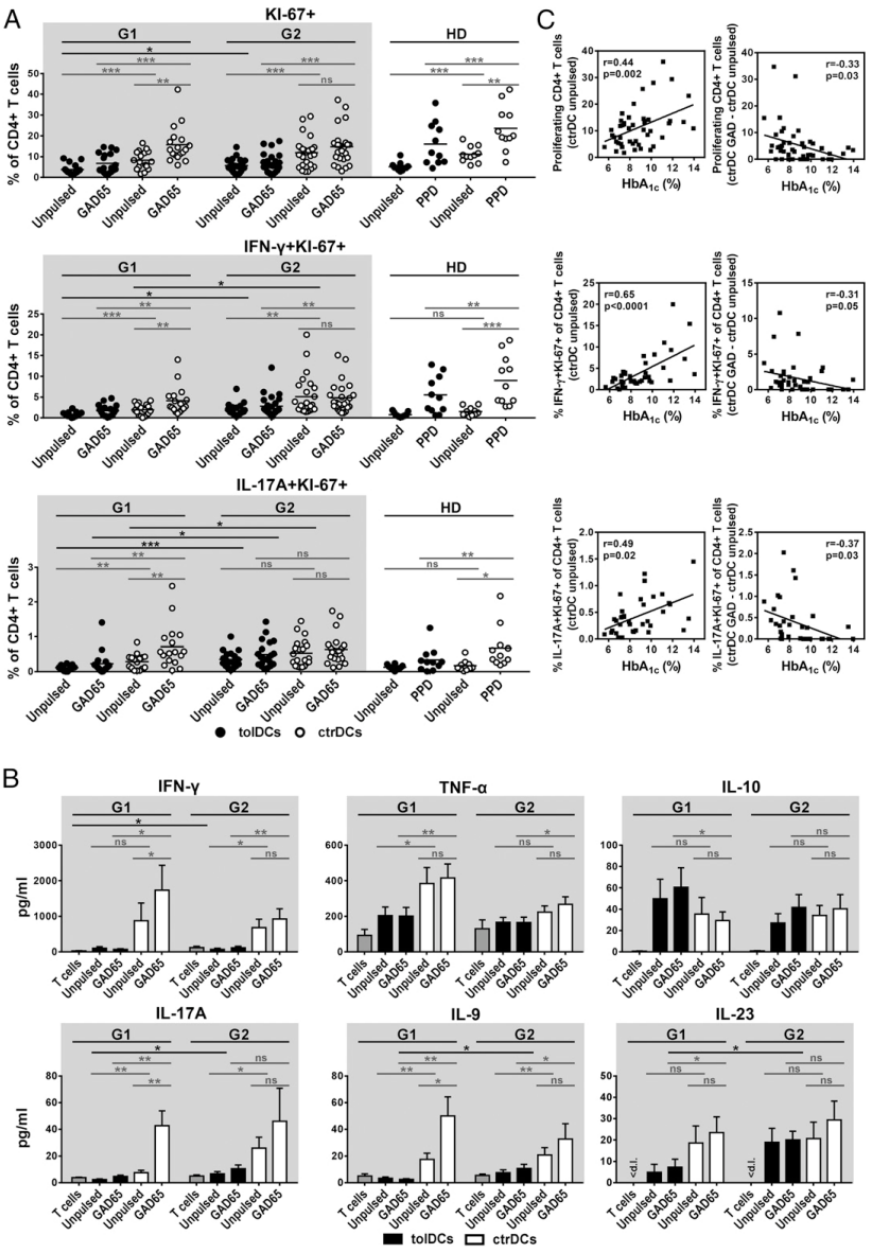

FIGURE 2. Analysis of T cell responses induced by tolDCs or ctrDCs of patients with optimal (G1; HbA_{1c} of ≤7.5%, n = 18) and suboptimal (G2; HbA_{1c} of >7.5%, n = 29) glycemic control and healthy donors (HD; n = 11). (**A**) Proliferation, percentage of IFN-γ$^+$Ki-67$^+$ T cells, and percentage of IL-17A$^+$Ki-67$^+$ T cells from CD4$^+$ T cells induced by unpulsed or Ag (GAD65 or PPD)–loaded tolDCs (●) or ctrDCs (○) as analyzed by intracellular flow cytometry on day 6 of coculture. Each point represents the value from an individual donor from 10 experiments. (**B**) Cytokine concentrations in 6-d culture supernatants of unstimulated T cells (gray bars) and T cells stimulated with unpulsed or GAD65-loaded tolDCs (black bars) or ctrDCs (open bars) from G1 (n = 20) and G2 (n = 32) patients as quantified by Luminex assay. Data represent means ± SEM from at least 10 experiments. (**C**) Correlation analyses of CD4$^+$ T cell responses and HbA_{1c} of T1D patients. The correlation between the percentage of proliferating T cells, the percentage of IFN-γ$^+$KI-67$^+$ T cells, or the percentage of IL-17A$^+$Ki-67$^+$ T cells from CD4$^+$ T induced by unpulsed ctrDCs and HbA_{1c} (left panel) is shown. The correlation of GAD65-specific proliferation, induction of IFN-γ$^+$Ki-67$^+$ T cells, or IL-17A$^+$Ki-67$^+$ T cells from CD4$^+$ T cells (given as the percentage T cells responding to stimulation with GAD65-loaded ctrDCs minus the percentage of T cells responding to stimulation with unpulsed ctrDCs) with HbA_{1c} (right panel) is shown. Ki-67, IFN-γ, and IL-17A were detected by intracellular flow cytometry at day 6. Each point represents the value from an individual patient from 10 experiments. The r values indicate the correlation index according to Pearson analysis. *$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.001$. ns, not significant; <d.l., below detection limit.

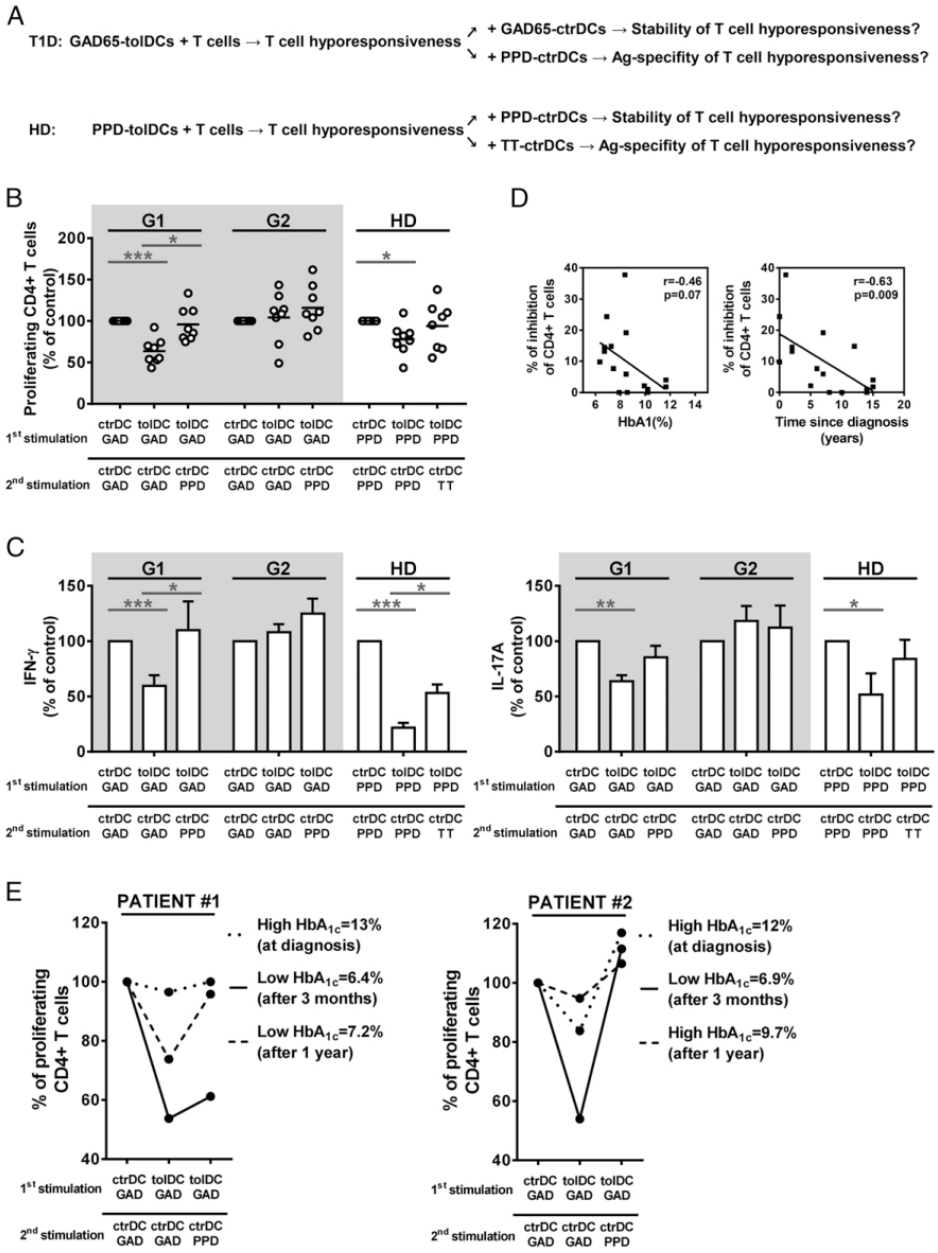

FIGURE 3. Induction of stable GAD65-specific T cell hyporesponsiveness. (**A**) Experimental scheme: T cells from patients with optimal (G1; HbA_{1c} of ≤7.5%, $n = 8$) and suboptimal (G2; HbA_{1c} of >7.5%, $n = 8$) glycemic control previously stimulated with GAD65-loaded ctrDCs ($T_{ctr+GAD}$) or tolDCs ($T_{tol+GAD}$) were stained with CFSE and rechallenged with GAD65- or PPD-loaded ctrDCs. T cells from healthy donors (HD; $n = 8$) previously stimulated with PPD-loaded ctrDCs ($T_{ctr+PPD}$) or tolDCs were stained with CFSE and rechallenged with PPD- or TT-loaded ctrDCs. (**B**) T cell proliferation detected as CFSE dilution at day 6. Each point represents the value from an individual donor's sample from five experiments. $T_{ctr+GAD}$ upon restimulation with GAD65-loaded ctrDCs were set as 100% for T1D patients and $T_{ctr+PPD}$ upon restimulation with PPD-loaded ctrDC were set as 100% for HD. (**C**) Secretion of IFN-γ and IL-17A detected in 6-d supernatants by Luminex assay. Data represent means ± SEM of secretion calculated as percentage of controls ($T_{ctr+GAD}$ upon restimulation with GAD65-loaded ctrDCs were set as 100% for T1D patients and $T_{ctr+PPD}$ upon restimulation with PPD-loaded ctrDCs were set as 100% for HD) from five experiments. (**D**) The correlation analysis of the HbA_{1c} level or time since diagnosis with the inhibition of proliferation (calculated as the percentage of proliferating $T_{ctr+GAD}$ minus the percentage of proliferating $T_{tol+GAD}$ upon restimulation with GAD65-loaded ctrDCs). Each point represents the value from one patient's sample. (**E**) TolDCs were generated and T cells isolated from two diabetic patients at three points: at T1D onset, when HbA_{1c} was markedly increased (patient 1, HbA_{1c} of 13%; patient 2, HbA_{1c} of 12%); after 3 mo, when patients were well controlled (patient 1, HbA_{1c} of 6.4%; patient 2, HbA_{1c} of 6.9%); and after 1 y, when patients differed in HbA_{1c} (patient 1, HbA_{1c} of 7.2%; patient 2, *(Figure legend continues)*

patients at different time points: at the time of diagnosis when HbA_{1c} was markedly increased (patient 1, HbA_{1c} of 13%; patient 2, HbA_{1c} of 12%), 3 mo later when HbA_{1c} decreased and patients were well controlled (patient 1, HbA_{1c} of 6.4%; patient 2, HbA_{1c} of 6.9%), and 1 y since diagnosis when patients differed in HbA_{1c} (patient 1, HbA_{1c} of 7.2%; patient 2, HbA_{1c} of 9.7%). The ability of GAD65-loaded tolDCs to induced stable Ag-specific T cell tolerance was tested. As documented in Fig. 3E, the major improvement in the induction of stable GAD65-specific T cell hyporesponsiveness was achieved in poorly controlled patients once they became well controlled. However, whereas tolDCs still induced T cell hyporesponsiveness in well-controlled patient 1 at 1 y after diagnosis, the tolerogenic potential of tolDCs in patient 2 with increased HbA_{1c} was lost. These case reports further indicate that the ability of tolDCs to induce GAD65-specific T cell hyporesponsiveness depends on the glycemic control.

TolDCs from patients with satisfactory T1D control suppress ctrDC-induced proinflammatory cytokine production

The ability of tolDCs to inhibit ctrDC-induced Ag-specific T cell responses would be important for successful tolDC therapy. To test the suppressive ability of tolDCs, GAD65-loaded tolDCs were titrated into a culture comprised of autologous GAD65-loaded ctrDCs and T cells and the cytokine production was analyzed (the experimental scheme is shown in Fig. 4A). Fig. 4B shows that GAD65-loaded tolDCs from G1 patients significantly reduced GAD65-loaded ctrDC-induced IFN-γ and IL-9 secretion (in all ratios tested) and TNF-α and IL-17A secretion (at 1:0.5 and 1:1 tolDC/ctrDC ratios). In G2 patients, only reduction of GAD65-loaded ctrDC-induced IFN-γ (at 1:1 tolDC/ctrDC ratio) was observed. The suppressive effect of tolDCs on ctrDC-induced IL-17A and IL-9 production (cytokine levels detected in ctrDC/T cell cultures minus cytokine levels detected in cultures of ctrDC/tolDC/T cell at a 1:1:10 ratio) correlated negatively with HbA_{1c} level (both IL-17A and IL-9: $r = -0.39$, $p = 0.05$, Fig. 4C) and for IL-17A with the time since diagnosis (IL-17A: $r = -0.31$, $p = 0.09$, data not shown). We found no association between suppressive effect of tolDCs and other clinical parameters of patients (data not shown). In both groups, we detected the significant upregulation of ctrDC-induced IL-10 production by GAD65-loaded tolDCs (in all ratios tested). Overall, these data suggest that hyperglycemia affects the ability of tolDCs to suppress Th1/Th17 responses induced by ctrDCs.

TolDCs induce differentiation of suppressive Tregs from naive $CD4^+$ T cells

The ability of tolDCs to induce functional Tregs is crucial for the induction of the long-lasting regulation of autoimmune processes ongoing in a patient's body. Thus, we tested tolDC capacity to induce Tregs from autologous naive $CD4^+$ T cells. As shown in Fig. 5B, particularly GAD65-loaded tolDCs were able to induce high numbers of $CD4^+Ki\text{-}67^+CD25^{high}CD127^{low}FOXP3^+$ Tregs when compared with ctrDCs. This tendency was more pronounced in well-controlled G1 patients (Fig. 5C). Moreover, only GAD65-loaded tolDCs from G1 induced superior production of IL-10 in T cells compared with GAD65-loaded ctrDCs (Fig. 5D).

Previously, we documented that IL-10–producing Tregs generated by repetitive priming of $CD4^+$ naive T cells with dexamethasone/vitamin D_2–induced tolDCs from healthy donors display suppressive properties (27). In the present study, we tested whether metabolic control in T1D patients influences the suppressive properties of tolDC-induced Tregs. For this purpose, Tregs were expanded after two rounds of stimulation with GAD65-loaded tolDCs and then titrated into a culture comprising of autologous GAD65-loaded ctrDCs and autologous Tresps (the experimental scheme is shown in Fig. 5A).

We found that Tregs from G1 patients significantly inhibited Tresp proliferation in a dose-dependent manner (Fig. 5E). In G2 patients, the suppressive effect of Tregs on Tresp proliferation was less pronounced. Addition of T cells primed with GAD65-loaded ctrDCs (referred as T_{ctr}) did not affect the Tresp proliferation. IL-10–producing Tregs induced by GAD65-loaded tolDCs are thus functional and are able to suppress proliferation of responder T cells.

Dexamethasone/vitamin D_2–modulated NOD DCs delayed diabetes development in the adoptive transfer model of NOD-SCID mice

To test that our protocol for generating tolDCs led to diabetes prevention in vivo, tolDCs were generated from bone marrow cells of NOD mice by using dexamethasone and vitamin D_2. Previous studies showed that to prevent diabetes onset and induce Ag-specific tolerance in animal model of diabetes, Ag supply might not be required in DC-based therapies for T1D (31–34). It is suggested that unpulsed DCs could pick up diabetogenic Ags in vivo and present them in a tolerogenic fashion (35). Such unpulsed autologous tolDCs were confirmed to be safe and exhibited some benefits in a phase 1 clinical trial in T1D patients without inducing unspecific systemic immunosuppression (19). Therefore, we used unpulsed bone marrow–derived MPLA-activated tolDCs. As shown in Supplemental Fig. 4, MPLA-activated tolDCs had a semimature phenotype with lower expression of CD40, CD80, and CD86 molecules and a similar level of MHC class II (I-A/I-E) expression compared with MPLA-activated ctrDCs. We carried out adoptive transfer experiments, in which tolDCs were cotransferred with diabetogenic splenocytes from prediabetic NOD females into the NOD-SCID recipients. As shown in Fig. 6, significant delay of diabetes onset was observed in NOD-SCID mice given a single injection of tolDCs (2×10^6, $p < 0.05$) compared with control mice (PBS). No significant effect on prolongation of diabetes onset was observed in ctrDC recipients.

Discussion

TolDCs have been intensively studied during the past years for their potential use in the treatment of autoimmune diseases (36). In this study, we analyzed the impact of metabolic state and disease control on functional properties of tolDCs generated from patients with T1D to further investigate the potential group of patients targeted for tolDC immunotherapy.

We show that tolDCs generated from T1D patients acquire tolerogenic phenotype, comparable to tolDCs differentiated from monocytes of healthy donors (27), and that our protocol for generating tolDCs led to diabetes prevention when tested in an adoptive transfer model of diabetes into NOD-SCID recipients. In addition to previous studies on tolDCs from T1D patients (37, 38),

HbA_{1c} of 9.7%). The ability of GAD65-loaded tolDCs to induced stable, Ag-specific T cell tolerance was tested as described in (A). Data show T cell proliferation detected as CFSE dilution at day 6. Each point represents the value of proliferating $CD4^+$ T cells from total $CD4^+$ T cells. $T_{ctr+GAD}$ upon restimulation with GAD65-loaded ctrDC were set as 100%. The r values indicate the correlation index according to Pearson analysis. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

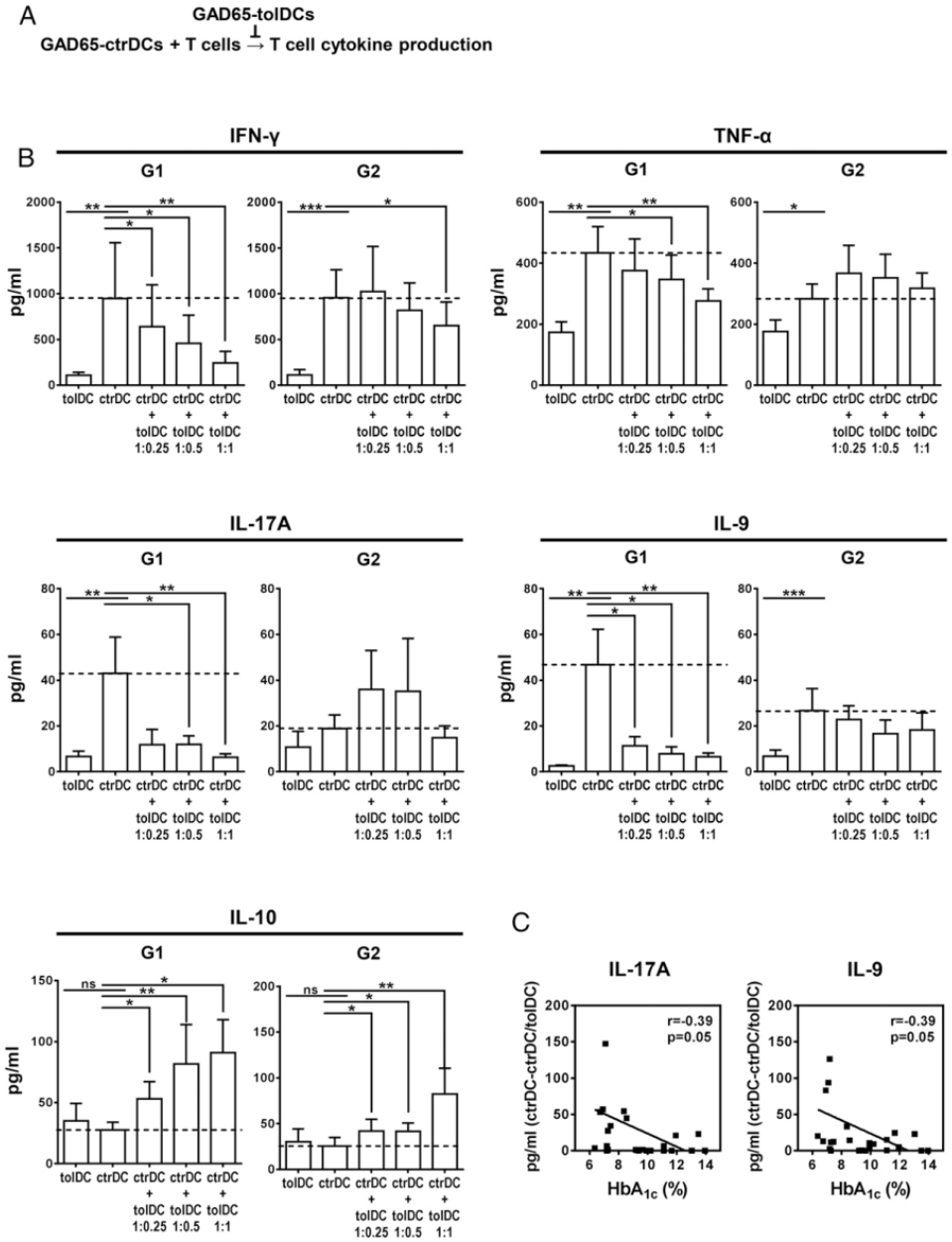

FIGURE 4. Suppression of ctrDC-induced T cell cytokine secretion by tolDCs. (**A**) Experimental scheme. GAD65-loaded tolDCs were titrated into a culture comprising of autologous GAD65-loaded ctrDCs and T cells. (**B**) Cytokine levels in supernatants from 6-d cocultures of T cells and autologous GAD65-loaded ctrDCs with various number of GAD65-loaded tolDCs added to cultures (T cell/ctrDC/tolDC ratios were 10:1:0.25, 10:1:0.5, and 10:1:1) from patients with optimal (G1; HbA_{1c} of ≤7.5%, $n = 10$) and suboptimal (G2; HbA_{1c} of >7.5%, $n = 17$) glycemic control as quantified by Luminex assay. Data represent means ± SEM from five experiments. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$. (**C**) Correlation analysis of the HbA_{1c} level with the suppressive effect of tolDCs on ctrDC-induced IL-17A and IL-9 production (calculated as cytokine levels detected in ctrDC/T cell cultures minus cytokine levels detected in cultures of ctrDC/tolDC/T cells at a 1:1:10 ratio). Each point represents the value from one patient's sample. The r values indicate the correlation index according to Pearson analysis.

we show that tolDCs upregulate IL-T3 and TLR2 and have a higher PD-L1/CD86 ratio. The high expression of IL-T3, PD-L1, and TLR2 might favor high capacity of tolDCs to induce tolerance, as signaling through these molecules participates in Treg induction (21, 39). Interestingly, IL-T3 and PD-L1 expression was markedly lower on tolDCs from patients with poor glycemic

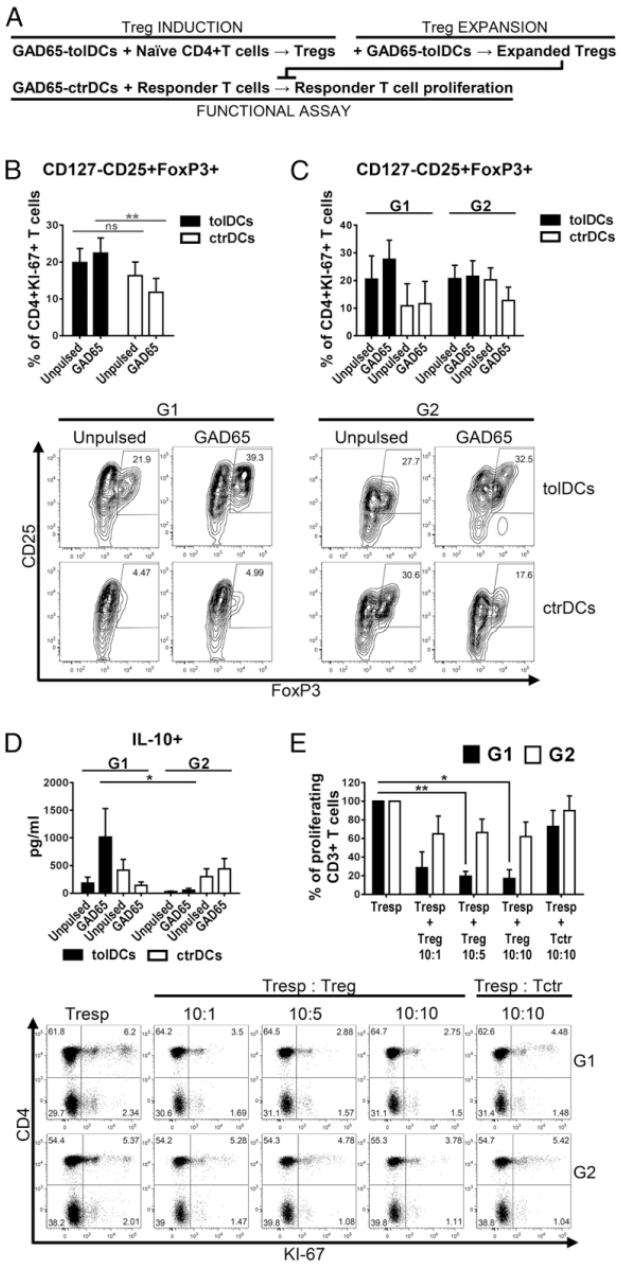

FIGURE 5. Phenotypic and functional analysis of Tregs induced from naive $CD4^+$ T cells of patients with HbA_{1c} of ≤7.5% (G1) or HbA_{1c} of >7.5% (G2). (**A**) Experimental scheme. (**B**) Percentages of $CD25^{high}CD127^{low}FOXP3^+$ from $CD4^+Ki\text{-}67^+$ T cells induced by unpulsed or GAD65-loaded tolDCs (filled bars) or ctrDCs (open bars) from overall patients and (**C**) from G1 and G2 patients separately as detected by flow cytometry at day 9 are shown. Representative dot plots from G1 and G2 patients are shown. (**D**) Secretion of IL-10 detected in 9-d supernatants from T cells stimulated by tolDCs (filled bars) or ctrDCs (open bars) by ELISA. Data represent means ± SEM from five experiments of 12 donors. (**E**) GAD65-loaded tolDCs were incubated with naive $CD4^+$ T cells for two rounds of priming. Induced Tregs were plated with Tresps and GAD65-loaded ctrDCs for 6 d (Treg/Tresp/DC ratios were 1:10:1, 5:10:1, and 10:10:1). Naive $CD4^+$ T cells primed with GAD65-loaded ctrDCs (Tctr) served as control for crowding. Proliferation (Ki-67 expression) of Tresps was analyzed by flow cytometry. The percentage inhibition of Tresp proliferation in G1 (filled bars, $n = 4$) and G2 (open bars, $n = 11$) is shown (mean ± SEM for three independent experiments). Representative dot plots from G1 and G2 patients are shown. $*p \leq 0.05$, $**p \leq 0.01$. ns, not significant.

control compared with patients with optimal disease control. Hyperglycemia was shown to attenuate the expression of the vitamin D receptor and to impair its function (40, 41). This could, in turn, attenuate the effect of vitamin D as a tolerogenic agent that was shown to induce IL-T3 and PD-L1 expression on monocytes and DCs (21, 42). Moreover, given that hyperglycemia activates proinflammatory signaling pathways in patient blood cells (11, 43, 44), it is possible that the well-controlled patients have a better capability to overcome this proinflammatory phenotype in the process of tolDC generation.

Successful tolDC therapy may be achieved by suppression and/or anergy of ongoing autoreactive responses induced by resident immunogenic DCs and induction of long-lasting Ag-specific tolerance. We show that tolDCs from patients with better glycemic control significantly inhibited, in a dose-dependent manner, ctrDC-induced secretion of Th1 and Th17 cytokines in contrast to poorly controlled patients. Our observation that HbA_{1c} levels correlate with the degree of suppression of ctrDC-induced IL-17A and IL-9, but not IFN-γ, secretion might be clarified in part by the observation that high glucose activates predominantly IL-17 and IL-6 signaling

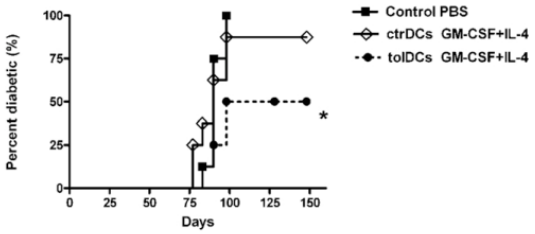

FIGURE 6. TolDCs suppress diabetes in the adoptive transfer model of NOD-SCID mice. DCs were generated from bone marrow cells of 8-wk-old NOD females in the presence of GM-CSF plus IL-4 and further treated with dexamethasone/vitamin D_2 (tolDCs) or without tolerogenic agents (ctrDCs). PBS-resuspended tolDCs (●) or ctrDCs (◇) (in 300 μl, 2×10^6) were injected i.p. (left side of the belly) with 5×10^6 diabetogenic splenocytes isolated from 13-wk-old prediabetic NOD females ($n = 10$) to 8-wk-old NOD-SCID female recipients ($n = 6$ in each group). Control NOD-SCID mice received PBS-resuspended splenocytes only (■). Data show cumulative incidence of diabetes in NOD-SCID recipients of DCs. Representative results of one out of three adoptive transfer experiments are shown. $*p < 0.05$ versus control (PBS), Kaplan–Meier log-rank test.

in lymphocytes from T1D patients without the impact on IFN-γ (11). Th17 T cells have also the ability to produce IL-9, and a higher frequency of IL-9$^+$IL-17$^+$ double-positive cells was found in patients with T1D in comparison with healthy individuals (45, 46). Recently, hyperglycemia was shown to positively correlate with numbers of IL-9–producing Th9 cells and CD4$^+$ IL-9$^+$IL-17$^+$ double-positive cells in T1D patients (46). Moreover, the reduced PD-L1 expression on tolDCs from poorly controlled patients might reduce the tolDC ability to suppress the Th17 response. Indeed, the combination of reduced PD-L1, CD80, and CD86 expression on DCs reportedly boosted Th17 immunity in ovarian cancer patients (47). Because both Th1 and Th17 participate in the development of T1D, the ability of tolDCs from well-controlled patients to suppress efficiently both Th1/Th17 responses might favor the success of tolDC immunotherapy. Upregulation of the IL-17, IFN-γ, and IL-9 pathways and Th1/Th17 plasticity in peripheral blood of children with T1D was observed (4, 11, 46, 48). In NOD mice, double deficiency in IL-17 and IFN-γ signaling, in contrast to an IFN-γ single knockout, suppressed the development of diabetes (49, 50).

Furthermore, we show that tolDCs from well-controlled patients mediate stable GAD65-specific T cell hyporesponsiveness. However, the autoreactive T cells from patients with poor T1D control were difficult to tolerize. Interestingly, they exhibited higher basal non–GAD65-specific proliferation, higher Th1/Th17 cytokine production, and lower specific responses to GAD65 in primary cultures. Our data are in accordance with two previous studies on IL-10/TGF-β–modulated tolDCs from T1D patients (37, 38). In the study by Segovia-Gamboa et al. (37), Ag-specific T cell tolerance was lost in patients with high homeostatic T cell proliferation, and the magnitude of T cell suppression inversely correlated with the levels of HbA_{1c} and cholesterol. These data suggest that autoantigen-specific T cell responses in the primary cultures might help in the prediction of the outcome of tolDC immunotherapy.

The failure of T cell tolerance induction in patients with poor disease control might have several explanations. First, patients with poor disease control may exhibit more preactivated T cells with hyperactive effector/memory phenotype and increased IFN-γ production (51), probably as a consequence of aggressive autoimmune process and chronic hyperglycemia. Hyperglycemia-driven oxidative stress exerts a global effect on T cells as documented by epigenetic modification of naive T cells, T cell hyperresponsiveness (52), polarization of naive T cells into proinflammatory Th1/Th17 cells (10, 11), and diminished regulation of IL-7–mediated T cell survival and homeostatic expansion (53). These autoreactive effector/memory T cells may be difficult to tolerize possibly due to the phenomenon of nonexhausted T cells existing in autoimmunity characterized by persistent effector function, low expression of coinhibitory molecules, and the high potential to proliferate and to produce IFN-γ instead of IL-10 (54). Next, T cell anergy is induced in an Ag-specific manner (55); however, we observed that primary T cell GAD65-specific responses in poorly controlled patients were markedly reduced. Indeed, chronic hyperglycemia markedly decreased T cell GAD65-specific proliferation as well as IFN-γ$^+$ and IL-17A$^+$ T cell induction. Similarly, the suppressive effect of high blood glucose on mitogen-stimulated proliferative responses of T cells in BALB/c mice with streptozotocin-induced T1D was observed (56). Our data suggest that also duration of T1D might affect T cell tolerance induction. In our study, the magnitude of T cell suppression inversely correlated with HbA_{1c} and disease duration. This might be explained in part by composition of G1 versus G2. G1 consists of a higher percentage of patients (68%) with short-term disease duration of <3 y, in contrast to G2 where only 35% had disease duration of <3 y. This observation might help with the timing of immune intervention.

Collectively, these data imply that the failure of T cell tolerance induction in poorly controlled patients is connected with metabolic and immunological disorders observed in T1D patients. Importantly, our data suggest that induction of T cell anergy could be recovered in T1D patients once they become well controlled. However, large numbers of patients are needed to confirm this observation.

Induction of Tregs is one of the mechanisms contributing to tolDC-induced tolerance (21). We found that cocultures of naive T cells and GAD65-loaded tolDCs from patients with low HbA_{1c} levels led to the induction of high levels of CD4$^+$Ki-67$^+$CD25^{high} CD127^{low}FOXP3$^+$ Tregs and high levels of IL-10, which can in turn potentiate the suppressive potential of Tregs. However, we cannot distinguish whether IL-10 is secreted by classical FOXP3$^+$ Tregs or another IL-10–producing adaptive type 1 Treg shown to be induced by repetitive priming of naive T cells with vitamin D_3 or vitamin D_3/dexamethasone–induced tolDCs (21, 27). Of great interest, we document that IL-10–producing Tregs expanded by tolDCs from patients with optimal T1D control have suppressive abilities.

Taken together, we show that the tolDC ability to modulate T cell responses is influenced by the metabolic state of patients with T1D. Hyperglycemia affects the current activation state of patient T cells, the tolerogenic phenotype of monocyte-derived DCs, and subsequently the outcome of T cell responses with a great impact on the IL-17 family. TolDCs of patients with lower HbA_{1c} and shorter disease duration have better capacity to induce stable Ag-specific hyporesposiveness, suppress ctrDC-induced T cell activation, and promote differentiation of functional Tregs. Importantly, improvement of glycemic control restores the ability of tolDCs to tolerize autoreactive T cells. Overall, our findings provide important information for the definition of inclusion criteria in clinical studies exploiting tolDCs in T1D prediction.

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Disclosures

L.P.-J., K.D., and R.Š. are named inventors in a related patent, "Tolerogenic Dendritic Cells, Methods of Producing the Same, and Uses Thereof" (U.S. Provisional Application No. 62/066,994), which describes methods for the

preparation of stable semi-mature tolerogenic DC. The other authors have no financial conflicts of interest.

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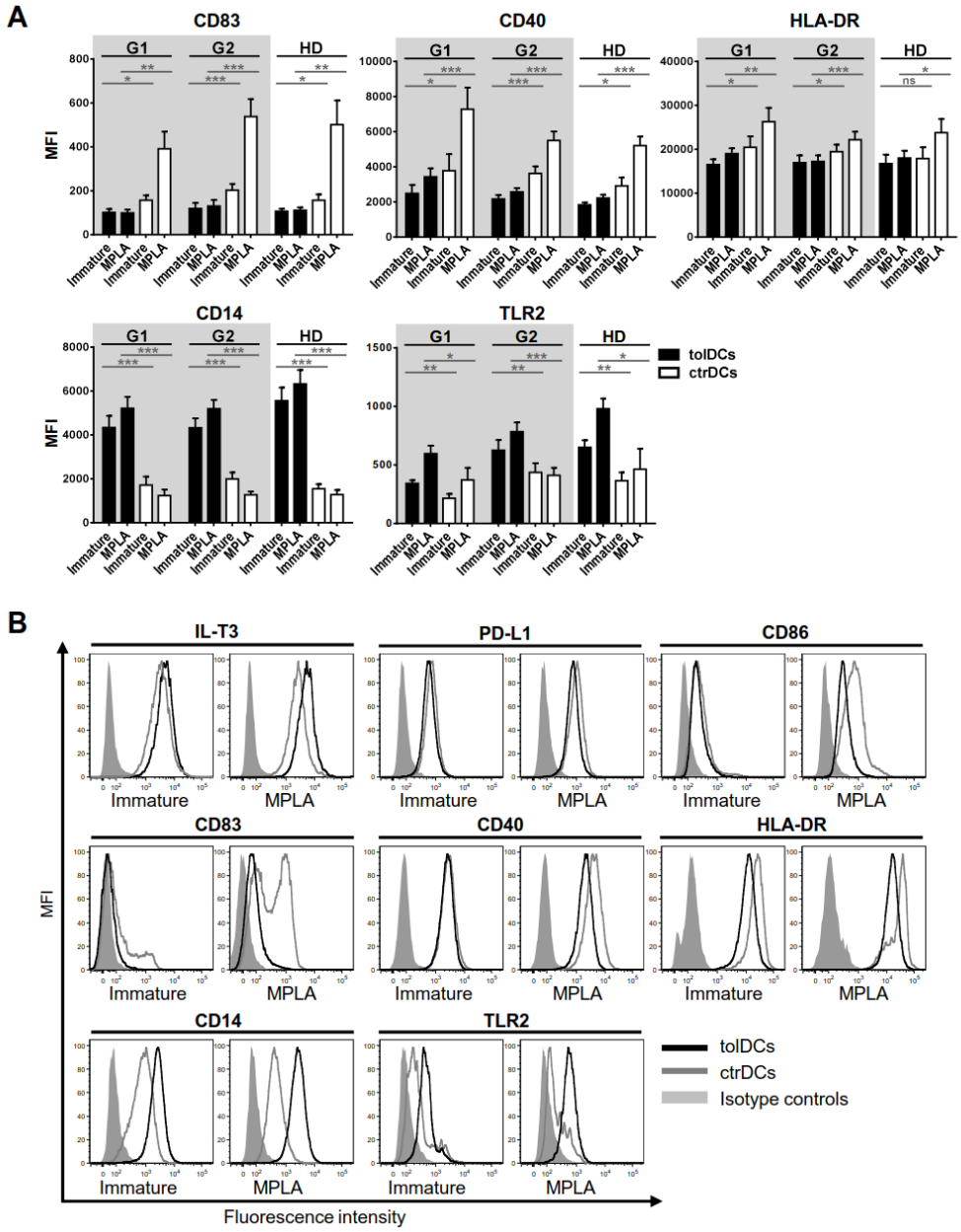
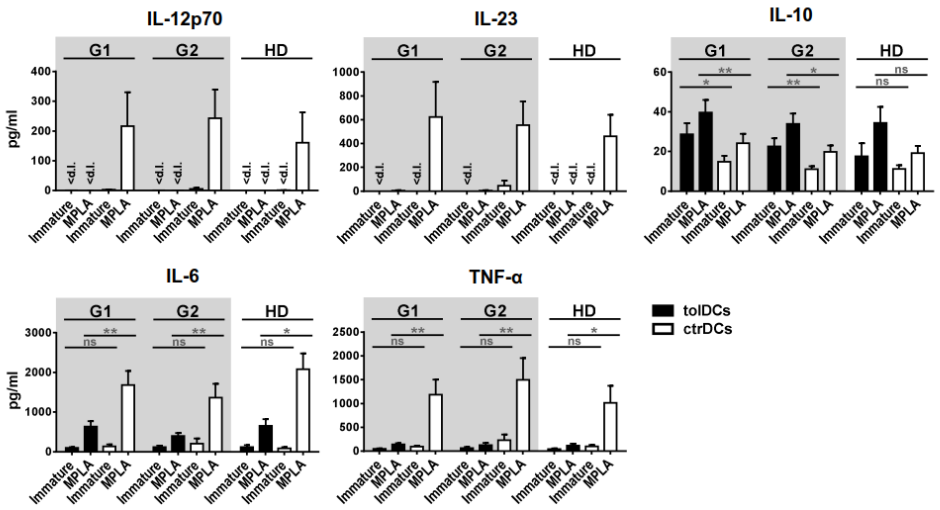
Supplemental Figure 1.



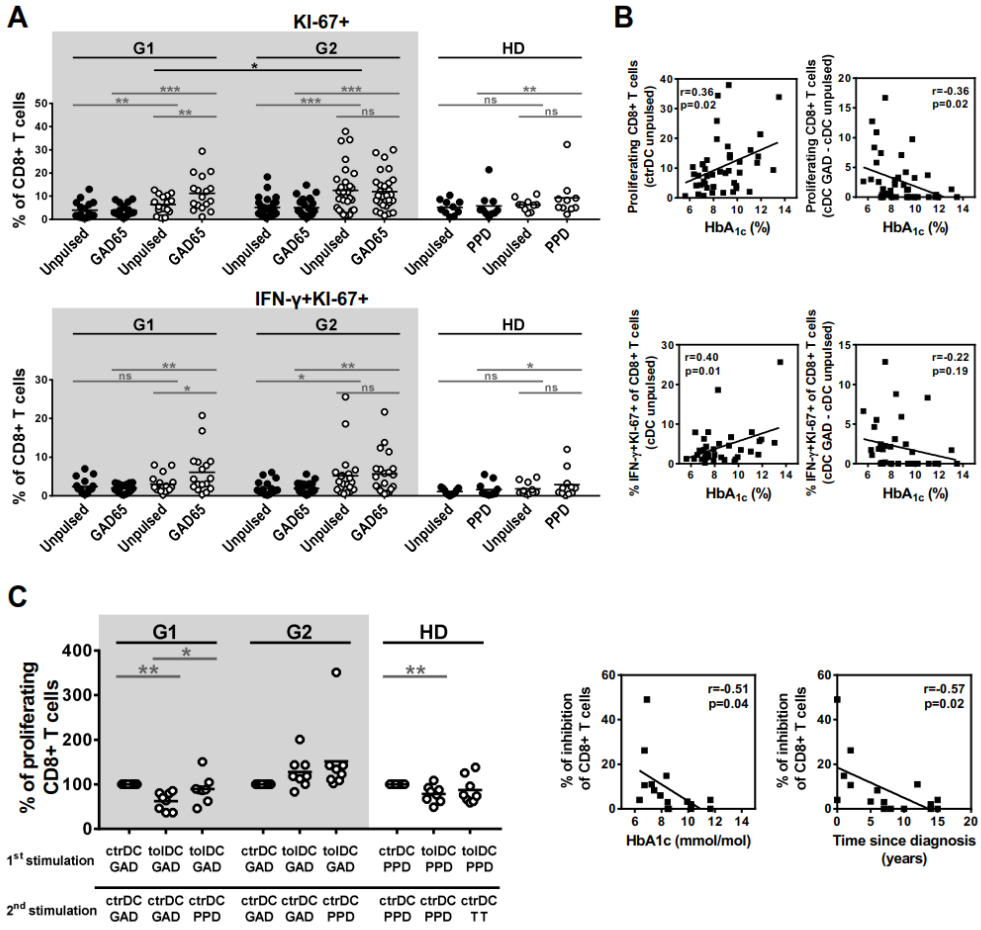
Supplemental Figure 1. The phenotype of DCs from patients with optimal (G1; HbA_{1c}≤7.5%, n=23) and suboptimal (G2; HbA_{1c}>7.5%, n=50) glycemic control and healthy donors (HD; n=11). Surface marker expression on immature or MPLA-activated tolDCs (black) or ctrDCs (white) as analyzed by flow cytometry. (**A**) Data represent means of MFI (mean fluorescence intensity) ± SEM from at least 10 experiments. (**B**) Representative histograms of one T1D patient. Light grey filled histograms represent isotype control mAb staining. *p≤0.05, **p≤0.01, ***p≤0.001; ns, non significant

Supplemental Figure 2.



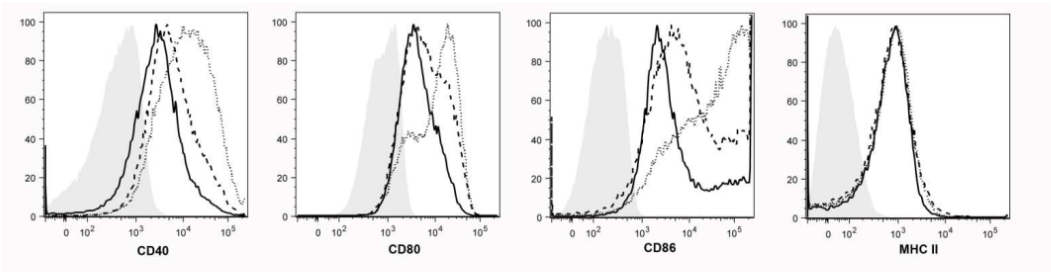
Supplemental Figure 2. The cytokines secreted by DCs from patients with optimal (G1; HbA_{1c}≤7.5%, n=26) and suboptimal (G2; HbA_{1c}>7.5%, n=29) glycemic control and healthy donors (HD; n=11). Cytokine concentrations in culture supernatants of tolDCs (black bars) or ctrDCs (white bars) as determined by Luminex assay 24 h after MPLA activation. Data represent means ± SEM from 3 experiments. *p≤0.05, **p≤0.01, ***p≤0.001; ns, non significant; <d.l., below detection limit

Supplemental Figure 3.



Supplemental Figure 3. Analysis of CD8+ T cell responses induced by tolDCs or ctrDCs of patients with optimal (G1; HbA_{1c}≤7.5%, n=18) and suboptimal (G2; HbA_{1c}>7.5%, n=29) glycemic control and healthy donors (HD; n=11). (**A**) The proliferation and the percentage of IFN-γ+KI-67+ T cells from CD8+ T cells induced by unpulsed or antigen (GAD65 or PPD)-loaded tolDCs (black circles) or ctrDCs (white circles) as analyzed by intracellular flow cytometry on day 6 of co-culture. Each point represents the value from an individual donor from 10 experiments. (**B**) Correlation analyses of T cell responses and HbA_{1c} of T1D patients. The correlation between the percentage of proliferating T cells or the percentage of IFN-γ+KI-67+ T cells from CD8+ T induced by unpulsed ctrDCs and HbA_{1c} (left panel). The correlation of GAD65-specific proliferation or induction of IFN-γ+KI-67+ T cells from CD8+ T cells (given as % T cells responding to stimulation with GAD65-loaded ctrDCs – % T cells responding to stimulation with unpulsed ctrDCs) with HbA_{1c} (right panel). KI-67 and IFN-γ were detected by intracellular flow cytometry at day 6. Each point represents the value from an individual patient from 10 experiments. (**C**) T cells from patients with optimal (G1; HbA_{1c}≤7.5%, n=8) and suboptimal (G2; HbA_{1c}>7.5%, n=8) glycemic control previously stimulated with GAD65-loaded ctrDCs ($T_{ctr+GAD}$) or tolDCs ($T_{tol+GAD}$) were stained with CFSE and rechallenged with GAD65- or PPD-loaded ctrDCs. T cells from healthy donors (HD; n=8) previously stimulated with PPD-loaded ctrDCs ($T_{ctr+PPD}$) or tolDCs were stained with CFSE and rechallenged with PPD- or TT-loaded ctrDCs. T cell proliferation was detected as CFSE dilution at day 6. Each point represents the value from an individual donor's sample. $T_{ctr+GAD}$ upon restimulation with GAD65-loaded ctrDC were set as 100% for T1D patients and $T_{ctr+PPD}$ upon restimulation with PPD-loaded ctrDC were set as 100% for HD. The correlation analysis of the HbA_{1c} level or time since diagnosis with the inhibition of proliferation (calculated as % of proliferating $T_{ctr+GAD}$ - % of proliferating $T_{tol+GAD}$ upon restimulation with GAD65-loaded ctrDCs). Each point represents the value from one patient's sample. r = correlation index according to Pearson's analysis. *p≤0.05, **p≤0.01, ***p≤0.001; ns, not significant

Supplemental Figure 4.



Supplemental Figure 4. Maturation phenotype of NOD tolDCs. Expression of CD40, CD80, CD86 and MHC II (I-A/I-E) surface markers on tolDCs (dashed), ctrDCs (dotted), and immature iDCs (solid), prepared without 20 h maturation step by MPLA, cultivated in the presence of GM-CSF+IL-4. Isotype controls are shown as grey overlays. Example of at least 3 experiments.

5.3 PART III: Testing and optimization of a tolerogenic DC manufacturing protocol for potential application of the tolerogenic DC-based therapeutic product in a clinical trial

After the establishment of the manufacturing protocol and comprehensive *in vitro* testing of properties of tolerogenic DCs generated from healthy donors as well as T1D patients, we prepared two publications summarizing the results. Moreover, we also prepared two patent applications which describe methods for the preparation of stable semimature tolerogenic DCs and T1D patient selection for tolerogenic DC-based treatment. In the next step, we tested the possibility to produce tolerogenic DCs according to the established protocol on a scale and with sufficient quality for potential clinical testing.

In the process of transition from preclinical to clinical studies, changes in scale, culture materials, handling and processing procedures can significantly affect the quality and efficacy of the final product. Main aspects related to the transition of the tolerogenic DC manufacturing process for clinical studies are as follows: a source of peripheral blood mononuclear cells (PBMCs), a method for monocyte enrichment and implementation of cryopreservation.

The first aspect is a source of PBMCs for the generation of monocyte-derived DCs. Compared with preclinical experiments where the whole blood or buffy coats are typically used, PBMCs are usually obtained from leukapheresis products in clinical trials. Leukapheresis products, in comparison to the whole blood, contains an enriched leukocyte fraction. Therefore, it allows obtaining high numbers of monocytes for the generation of sufficient numbers of tolerogenic DCs [203]. The second aspect is the selection of a monocyte separation method. Adherence to plastic, immunomagnetic separation and elutriation are three basic methods applied for monocyte isolation. Given that the isolation of monocytes based on their ability to adhere to plastic does not allow the complete removal of all the remaining immune cell populations from monocytes, the final product of monocyte-derived tolerogenic DCs contains other immune cells in addition to $CD11c^+$ tolerogenic DCs, mainly T cells. Therefore, the utilization of this method leads to a DC-based therapeutic product with high numbers of contaminating T cells and the low proportion of DCs. In contrast, an advantage of this method is its inexpensiveness. Compared with plastic adherence, the utilization of immunomagnetic separation and elutriation provides a highly pure final DC-based

therapeutic product. Immunomagnetic separation is based on positive selection of monocytes using anti-CD14 antibody. These antibodies can be directly linked with magnetic beads (MACS technology, Miltenyi Biotec) or they can be linked via a specific construct (EasySep technology, STEMCELL Technologies) [204]. Elutriation is a method for separation of particles based on their size and density. The advantage of elutriation compared with immunomagnetic separation is no application of xenogeneic antibodies for monocyte targeting [205,206]. Additionally, monocytes isolated via immunomagnetic separation and elutriation can be subsequently differentiated into DCs in hydrophobic culture bags. The main advantage of a closed system of culture bags over culture flasks is a decreased risk of contamination. Moreover, it facilitates the scale-up and automation. Regarding the yield, phenotype and function, most studies reported no marked differences between DCs generated in culture bags and culture flasks [207]. The last aspect is implementation of tolerogenic DC cryopreservation until application in case that the treatment consists of multiple doses.

We tested the production of clinical-scale tolerogenic DCs based on our manufacturing protocol described in Dáňová *et al*., 2015 and 2017 [208,209]. Compared with our previous *in vitro* experiments, we implemented the necessary, abovementioned changes associated with modification of a tolerogenic DC manufacturing procedure to clinical purpose. We used leukapheresis products as a source of PBMCs and we incorporated cryopreservation of the final product. Regarding the monocyte isolation method, we initially used adherence to plastic for monocyte enrichment, which we used in our manufacturing protocol reported in Dáňová *et al*., 2015 and 2017. However, given the higher number of processed monocytes, we had to use different culture flasks and culture plates for DC differentiation and maturation. Based on a study showing that tolerogenic DCs pulsed with an antigen can exacerbate instead of suppress T1D onset in the mouse model of T1D [190], we optimized the generation of antigen-unloaded tolerogenic DCs. Finally, we tested a lower concentration of MPLA (1 μg/ml versus previously tested 2 μg/ml) for final tolerogenic DC maturation to assess the possibility to reduce the amount of MPLA. The low dose of MPLA was also used for the generation of dexamethasone/vitamin D_3-treated tolerogenic DCs in a clinical trial with patients suffering from RA [199]. We used leukapheresis products from healthy donors for initial testing and optimization of the manufacturing process. Nevertheless, it is possible that parameters of T1D patients' leukapheresis products will differ from that of healthy donors, which can influence the quality and quantity of the final tolerogenic DC-based product.

Evaluation of the quality and quantity of the generated tolerogenic DCs was based on our previous tests described in Dáňová *et al*., 2015 and 2017 and consisted of analysis of tolerogenic DC yield, phenotype, phenotypic stability, function and other specific parameters of the final product. The DC yield was calculated as a number of tolerogenic DCs obtained from monocytes used for DC generation. Complementary to yield, the total number of obtained tolerogenic DCs is important for determination of a total number of treatment doses and a number of tolerogenic DCs per dose in a clinical study. Analysis of the DC phenotype and phenotypic stability was based on the examination of maturation-associated or tolerogenic marker expression and secretion of IL-10 and IL-12. DC function was measured as a stimulatory capacity in the allogeneic lymphocyte reaction. The examined parameters of the final product were purity (a percentage of $CD11c^+$ DCs in the final product), viability and the so-called combined parameter, which showed a percentage of viable $CD11c^+$ tolerogenic DCs in the final product. Assays for analysis of DC parameters and phenotype were also tested and optimized in order to establish routine tests for tolerogenic DC quality control in a clinical trial. Complementary, functional assays were tested and optimized as a basis for the establishment of the so-called potency test for potential next phases of tolerogenic DC clinical testing. This test should reflect suppressive function of tolerogenic DCs. The requirements for quality control tests and potency assays are simplicity, as they are routinely used during the clinical study, and the accurate reflection of the tolerogenic DC phenotype and function.

Tolerogenic DCs manufactured according to our protocol (referred as Dex/VitD_2 tolerogenic DCs) with all of the implemented changes, were able to acquire a tolerogenic phenotype with low expression of maturation-associated markers CD80, CD83 and CD86 and increased expression of tolerogenic markers IL-T3, TLR2 and CD14 compared with DCs prepared without tolerizing factors dexamethasone and vitamin D_2 (referred as control DCs). Moreover, in comparison to control DCs, Dex/VitD_2 tolerogenic DCs secreted high amounts of IL-10 and no IL-12 after restimulation with LPS. Finally, we verified that Dex/VitD_2 tolerogenic DCs generated from leukapheresis products and with incorporated cryopreservation step retained the lower T cell stimulatory capacity compared with control DCs as demonstrated by low numbers of induced proliferating and IFN-γ-producing T cells. We also found that the decrease in MPLA dose did not significantly alter the regulatory phenotype of Dex/VitD_2 tolerogenic DCs and that DCs activated with 1 μg/ml MPLA displayed the same functional properties as DCs activated with 2 μg/ml MPLA (Figure 4).

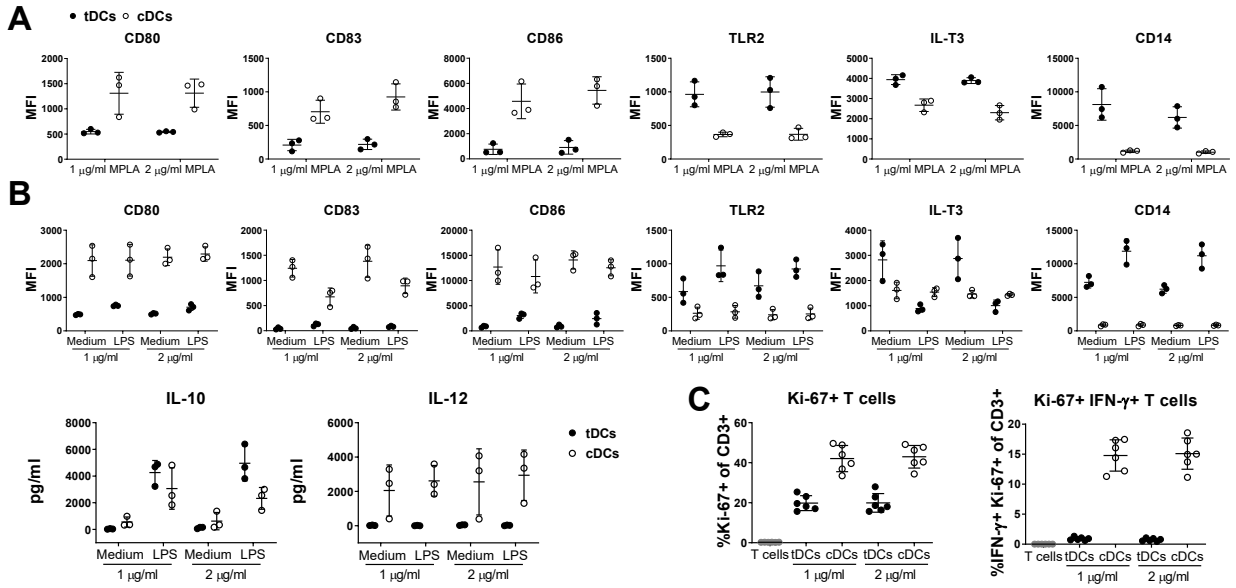


Figure 4. Dex/VitD$_2$ tolerogenic DCs generated from leukapheresis products of healthy individuals displayed the regulatory phenotype and function. DCs were generated in CellGenix GMP DC media supplemented with IL-4 and GM-CSF in the presence (Dex/VitD$_2$ tolerogenic DCs, tDCs) or absence (control DCs, cDCs) of dexamethasone and vitamin D$_2$. DCs were activated with 1 or 2 μg/ml MPLA, frozen and stored in liquid nitrogen. Then, DCs were thawed and (**A**) analyzed for surface marker expression or (**B**) cultured in RPMI with 5% human AB serum (Medium) without tolerizing factors or restimulated with LPS for 24 hours prior to analysis. Surface molecule expression was analyzed by flow cytometry. Secretion of IL-10 and IL-12p70 was analyzed using ELISA. (**C**) Thawed DCs were cultivated with allogeneic purified CFSE-stained CD3$^+$ T cells from 2 different donors. The percentage of Ki-67$^+$ (proliferating) and Ki-67$^+$IFN-γ$^+$ T cells from CD3$^+$ T cells induced in response to stimulation with tDCs or cDCs was detected by intracellular staining and analyzed by flow cytometry on day 4. Individual values of marker expression (expressed as mean fluorescence intensity, MFI, or %) or values of secreted cytokines (pg/ml) from 3 donors are shown. Horizontal lines represent mean values ± SD. Dex/VitD$_2$ tolerogenic and control DCs were generated by Sotio a.s. Technical Operations – Process Development team[1].

However, parameters as the DC purity and viability as well as the overall number of the generated Dex/VitD$_2$ tolerogenic DCs were lower than we expected. We did not find significant differences in these parameters between Dex/VitD$_2$ tolerogenic DCs matured with 1 and 2 μg/ml MPLA (Table 3). However, we observed nonsignificant trend towards the higher purity, viability and combined parameter of Dex/VitD$_2$ tolerogenic DCs matured with 1 μg/ml MPLA compared with DCs matured with 2 μg/ml MPLA.

[1] I would like to thank Mgr. Kateřina Pokorná Ph.D., Mgr. Veronika Novotná, Ing. Kateřina Ferschmannová and Adam Těhník from Sotio a.s. Technical Operations – Process Development department for excellent collaboration.

Table 3. DC purity, viability, combined parameter and DC yield of cryopreserved Dex/VitD$_2$ tolerogenic DCs generated from leukapheresis products of healthy individuals.

Dex/VitD$_2$ tolerogenic DCs		
	1 µg/ml MPLA	2 µg/ml MPLA
Purity (% CD11c$^+$ DCs)	41.66 ± 8.01	40.14 ± 3.70
Viability (% DAPI$^-$ DCs)	67.04 ± 10.41	60.88 ± 17.18
Combined Parameter	28.48 ± 9.20	24.79 ± 8.58
DC yield	20.35 ± 6.55	20.43 ± 7.28

Dex/VitD$_2$ tolerogenic DCs were generated in CellGenix GMP DC media supplemented with IL-4 and GM-CSF in the presence of dexamethasone and vitamin D$_2$ and then were activated with MPLA. The DC purity, viability and combined parameter after freezing and thawing were assessed by flow cytometry and DC count was assessed using Bürker chamber. The DC yield was calculated as a number of obtained Dex/VitD$_2$ tolerogenic DCs from monocytes used for DC generation. Mean ± SD values obtained from 3 donors are shown. Experiment and analysis was performed by Sotio a.s. Technical Operations – Process Development team.

In order to increase the yield and purity of Dex/VitD$_2$ tolerogenic DCs, we decided to further optimize the manufacturing protocol. Therefore, in the second phase of this part, we tested the CliniMACS Prodigy® automated system for CD14$^+$ monocyte separation, which allows to avoid density centrifugation over Ficoll-Paque media and a subsequent adherence step. Therefore, this method should provide a better proportion of DCs in the final product. CD14$^+$ separated monocytes were subsequently cultivated either in culture flasks or in culture bags with the aim of finding the best procedure for Dex/VitD$_2$ tolerogenic DC generation. Based on the study by Bell *et al*. [199] and our results with Dex/VitD$_2$ tolerogenic DCs generated by adherence, where we did not observe significant differences, we applied 1 µg/ml instead of 2 µg/ml MPLA for the final Dex/VitD$_2$ tolerogenic DC activation.

First, we analyzed the yield, purity and viability of generated Dex/VitD$_2$ tolerogenic DCs. We found that in comparison to plastic adherence, utilization of CD14$^+$ monocyte separation by the CliniMACS Prodigy® monocyte enrichment system significantly improved the percentages of CD11c$^+$ DCs in the final product. Additionally, the purity of Dex/VitD$_2$ tolerogenic DCs generated by CD14$^+$ monocyte separation was not affected by the subsequent cultivation method, as the percentages of CD11c$^+$ DCs differentiated in culture flasks or bags were comparable and remained unchanged during the production process. We did not find significant differences in the viability of Dex/VitD$_2$ tolerogenic DCs prepared by plastic adherence or by CD14$^+$ monocyte separation;

however, we observed a trend towards the lower viability of DCs generated via $CD14^+$ monocyte separation with subsequent differentiation in culture bags. The highest combined parameter displayed tolerogenic DCs generated by $CD14^+$ monocyte separation and cultivation in culture flasks (Figure 5). In contrast, application of the new separation method did not lead to Dex/$VitD_2$ tolerogenic DC yield improvement (data not shown).

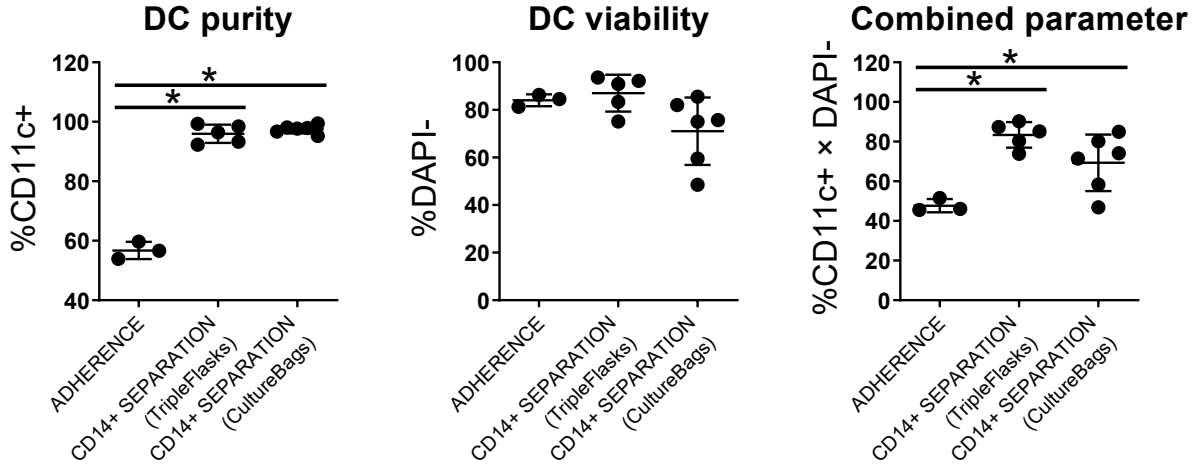


Figure 5. $CD14^+$ monocyte separation using the CliniMACS Prodigy® cell separator significantly increased the purity and combined parameter of Dex/$VitD_2$ tolerogenic DCs generated from leukapheresis products of healthy individuals. Monocytes were obtained via adherence or via $CD14^+$ separation using the CliniMACS Prodigy® cell separator and differentiated into DCs in triple flasks or culture bags. Dex/$VitD_2$ tolerogenic DCs were generated in CellGenix GMP DC media supplemented with IL-4 and GM-CSF in the presence of dexamethasone and vitamin D_2 and then were activated with MPLA. The DC purity (a percentage of $CD11c^+$ DCs) and viability (a percentage of $DAPI^-$ DCs) after freezing and thawing were analyzed by flow cytometry. Individual values from minimum of 3 donors are shown. Horizontal lines represent mean values ± SD. *$p \leq 0.05$ (unpaired Mann-Whitney test). $CD14^+$ monocyte separation and Dex/$VitD_2$ tolerogenic DC generation were performed by Sotio a.s. Technical Operations – Process Development team.

Further, we analyzed the phenotype of generated Dex/$VitD_2$ tolerogenic DCs. We found that application of $CD14^+$ separation procedure for monocyte isolation led to a slight decrease of activation molecule expression (with an exception of CD86 expression in case of Dex/$VitD_2$ tolerogenic DCs generated by $CD14^+$ monocyte separation with subsequent cultivation in culture bags) while did not reduce expression of tolerogenic markers (Figure 6), which could even improve the regulatory potential of Dex/$VitD_2$ tolerogenic DCs.

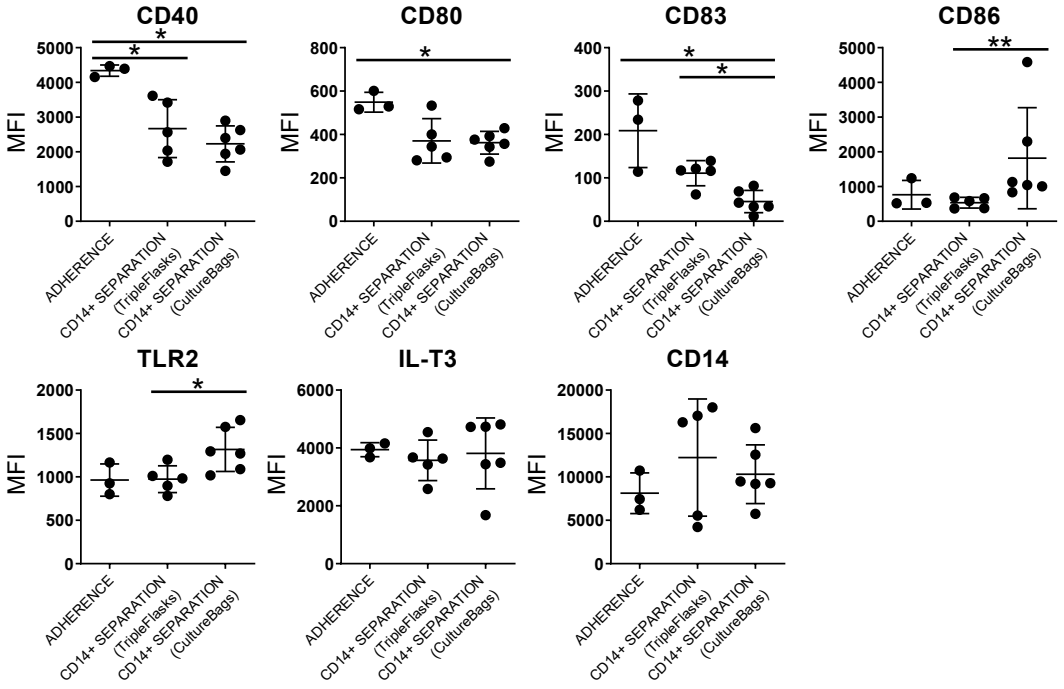


Figure 6. CD14$^+$ monocyte separation using the CliniMACS Prodigy® cell separator did not alter the regulatory phenotype of Dex/VitD$_2$ tolerogenic DCs generated from leukapheresis products of healthy individuals. Monocytes were obtained via adherence or via CD14$^+$ separation using the CliniMACS Prodigy® cell separator and differentiated into DCs in triple flasks or culture bags. Dex/VitD$_2$ tolerogenic DCs were generated in CellGenix GMP DC media supplemented with IL-4 and GM-CSF in the presence of dexamethasone and vitamin D$_2$ and then were activated with MPLA. Expression of surface markers after freezing and thawing was analyzed by flow cytometry. Individual values of marker expression (expressed as mean fluorescence intensity, MFI) from minimum of 3 donors are shown. Horizontal lines represent mean values ± SD. *p ≤ 0.05, **p ≤ 0.01 (unpaired Mann-Whitney test). CD14$^+$ monocyte separation and Dex/VitD$_2$ tolerogenic DC generation were performed by Sotio a.s. Technical Operations – Process Development team.

In the next step, we analyzed the viability and phenotypic stability of Dex/VitD$_2$ tolerogenic DCs upon cultivation in media with human serum and upon restimulation with LPS, which mimics the potential application of Dex/VitD$_2$ tolerogenic DCs to patients and strong activation stimulus. We found that Dex/VitD$_2$ tolerogenic DCs generated from CD14$^+$ monocyte separated by the CliniMACS Prodigy® cell separator differentiated in culture flasks had significantly better viability upon cultivation in media with human serum without tolerogenic factors compared with Dex/VitD$_2$ tolerogenic DCs prepared by monocyte adherence. In addition, the nonsignificant trend to the higher viability of both types of Dex/VitD$_2$ tolerogenic DCs generated from CD14$^+$ separated monocytes was obvious upon LPS restimulation. The higher initial purity and viability was then reflected in higher combined parameter of both types Dex/VitD$_2$ tolerogenic DCs generated from

$CD14^+$ separated monocytes compared with DCs generated from monocytes obtained by adherence upon DC cultivation in media with human serum as well as upon LPS restimulation (Figure 7).

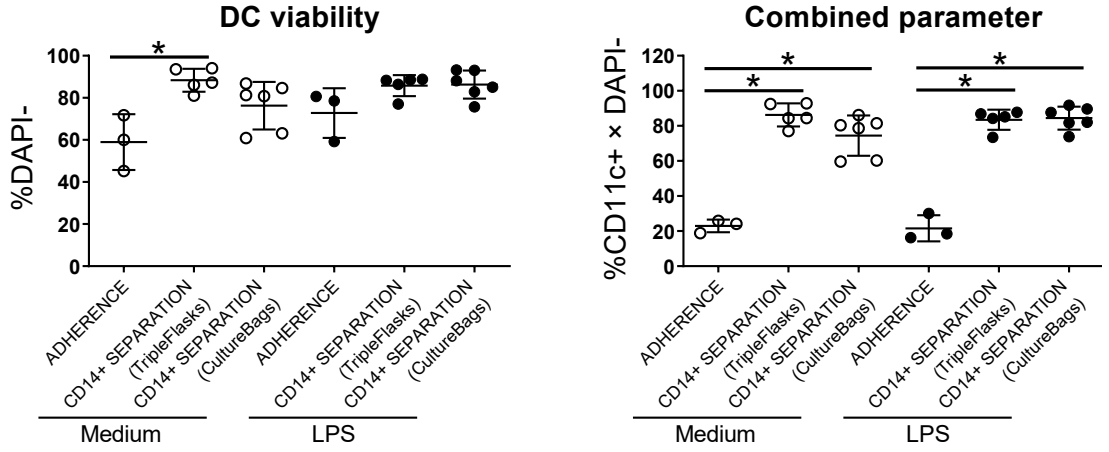


Figure 7. Dex/VitD$_2$ tolerogenic DCs generated by CD14$^+$ monocyte separation using the CliniMACS Prodigy® cell separator displayed the better viability and combined parameter upon restimulation with LPS. Monocytes were obtained via adherence or via CD14$^+$ separation using the CliniMACS Prodigy® cell separator and differentiated into DCs in triple flasks or culture bags. Dex/VitD$_2$ tolerogenic DCs were generated in CellGenix GMP DC media supplemented with IL-4 and GM-CSF in the presence of dexamethasone and vitamin D$_2$ and then were activated with MPLA. MPLA-matured Dex/VitD$_2$ tolerogenic DCs were frozen and stored in liquid nitrogen. Then, DCs were thawed and cultured in RPMI with 5% human AB serum (Medium) without tolerizing factors or restimulated with LPS for 24 hours prior to analysis. The percentage of CD11c$^+$ DCs and viability (a percentage of DAPI$^-$ DCs) were analyzed by flow cytometry. Individual values from minimum of 3 donors are shown. Horizontal lines represent mean values ± SD. *$p \leq 0.05$ (unpaired Mann-Whitney test). CD14$^+$ monocyte separation and Dex/VitD$_2$ tolerogenic DC generation were performed by Sotio a.s. Technical Operations – Process Development team.

Regarding the phenotype, Dex/VitD$_2$ tolerogenic DCs prepared by CD14$^+$ monocyte separation retained low expression of maturation-associated markers, which did not largely upregulate after LPS treatment. One exception was CD86 molecule, which expression was slightly increased compared with Dex/VitD$_2$ tolerogenic DCs prepared by monocyte adherence. Interestingly, the expression of tolerogenic markers TLR2, IL-T3 and CD14 was rather higher on Dex/VitD$_2$ tolerogenic DCs prepared from CD14$^+$ separated monocytes compared with DCs prepared by monocyte adherence. We did not observe major differences in activation and tolerogenic marker expression between Dex/VitD$_2$ tolerogenic DCs generated by CD14$^+$ monocyte separation and subsequent cultivation in triple flasks or culture bags (Figure 8). When we analyzed production of IL-10 and IL-12 in cell culture supernatants upon LPS restimulation, we found that Dex/VitD$_2$ tolerogenic DCs generated from CD14$^+$ separated monocytes with subsequent cultivation in triple

flasks secreted comparable amounts of IL-10 as Dex/VitD$_2$ tolerogenic DCs generated from monocytes obtained by adherence. In contrast, Dex/VitD$_2$ tolerogenic DCs generated from CD14$^+$ separated monocytes with subsequent cultivation in culture bags secreted significantly lower levels of IL-10. Irrespective of the subsequent cultivation method, both types of Dex/VitD$_2$ tolerogenic DCs generated using CD14$^+$ monocyte separation produced absolutely no IL-12 (Figure 8).

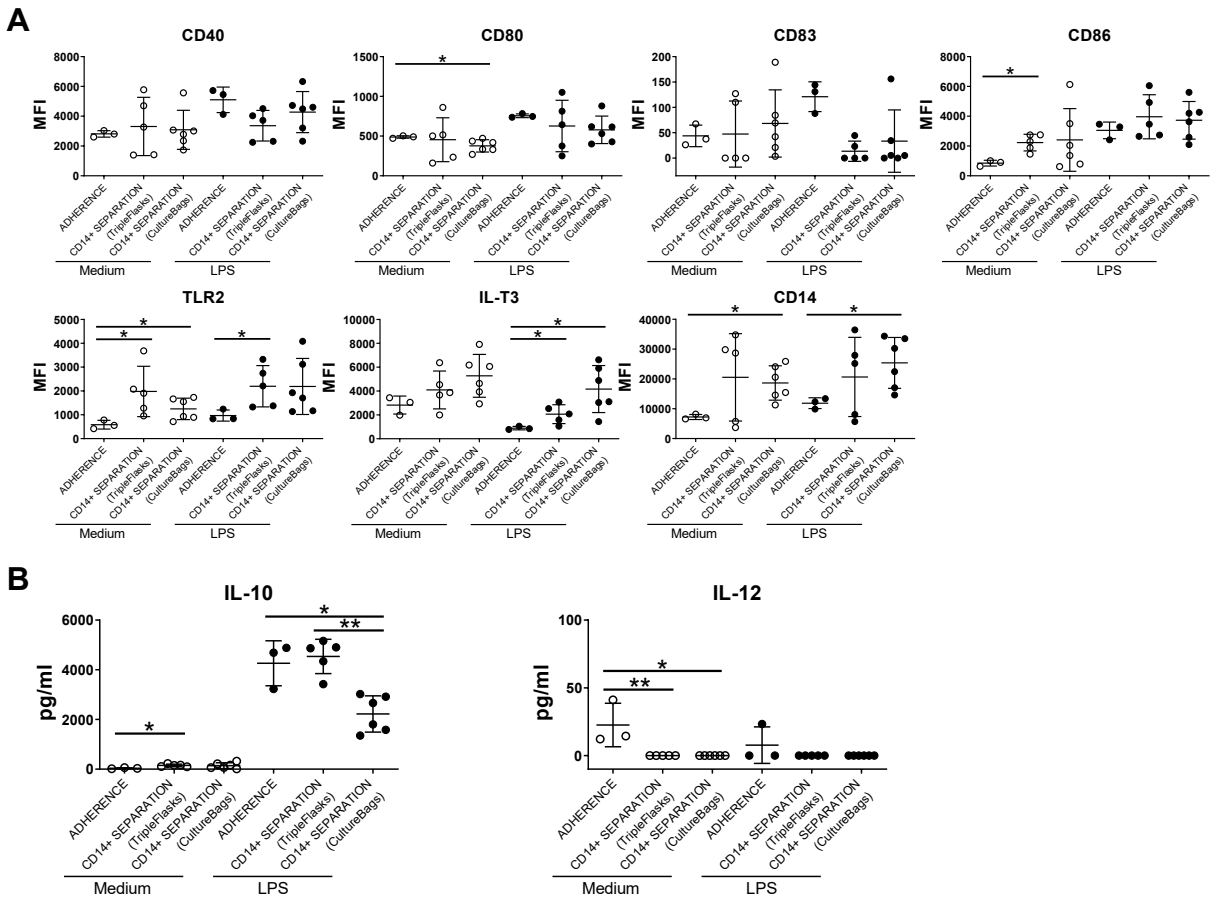


Figure 8. CD14$^+$ monocyte separation using the CliniMACS Prodigy® cell separator did not alter the stability of the regulatory phenotype of Dex/VitD$_2$ tolerogenic DCs. Monocytes were obtained via adherence or via CD14$^+$ separation using the CliniMACS Prodigy® cell separator and differentiated into DCs in triple flasks or culture bags. Dex/VitD$_2$ tolerogenic DCs were generated in CellGenix GMP DC media supplemented with IL-4 and GM-CSF in the presence of dexamethasone and vitamin D$_2$ and then were activated with MPLA. MPLA-matured Dex/VitD$_2$ tolerogenic DCs were frozen and stored in liquid nitrogen. Then, DCs were thawed and cultured in RPMI with 5% human AB serum (Medium) without tolerizing factors or restimulated with LPS for 24 hours prior to analysis. (**A**) Surface molecule expression was analyzed by flow cytometry. (**B**) Secretion of IL-10 and IL-12p70 was analyzed using ELISA. Individual values from minimum of 3 donors are shown. Horizontal lines represent mean values ± SD. *p ≤0.05, **p ≤ 0.01 (unpaired Mann-Whitney test). CD14$^+$ monocyte separation and Dex/VitD$_2$ tolerogenic DC generation were performed by Sotio a.s. Technical Operations – Process Development team.

Next, we tested the function of Dex/VitD$_2$ tolerogenic DCs. We used a mixed lymphocyte reaction to test the stimulatory capacity of antigen-unloaded Dex/VitD$_2$ tolerogenic DCs. We stained purified CD3$^+$ T cells with CFSE, which allowed us to distinguish T cells originally coming from DC samples in case of Dex/VitD$_2$ tolerogenic DCs generated by monocyte adherence and allogeneic T cells used for the examination of tolerogenic DC stimulatory capacity (Figure 9). Subsequently, we stimulated CFSE-stained CD3$^+$ T cells with either tolerogenic or control DCs, and after 4 days, the cultures were restimulated with PMA and ionomycin in order to evaluate INF-γ production in proliferating T cells. We found that Dex/VitD$_2$ tolerogenic DCs generated from monocytes prepared using the CliniMACS Prodigy® cell separator, irrespective of the subsequent cultivation method, induced significantly lower T cell proliferation (Ki-67$^+$ T cells) and INF-γ production compared with control DCs prepared without tolerizing factors. Moreover, the numbers of proliferating Ki-67$^+$ T cells induced by both types of Dex/VitD$_2$ tolerogenic DCs generated from CD14$^+$ separated monocytes were significantly lower compared with the numbers induced by Dex/VitD$_2$ tolerogenic DCs generated by monocyte adherence. The levels of IFN-γ-producing Ki-67$^+$ T cells induced by Dex/VitD$_2$ tolerogenic DCs generated from monocytes obtained by adherence and by CD14$^+$ separation were comparable (Figure 9). These data point to the low stimulatory capacity of Dex/VitD$_2$ tolerogenic DCs prepared from isolated CD14$^+$ monocytes.

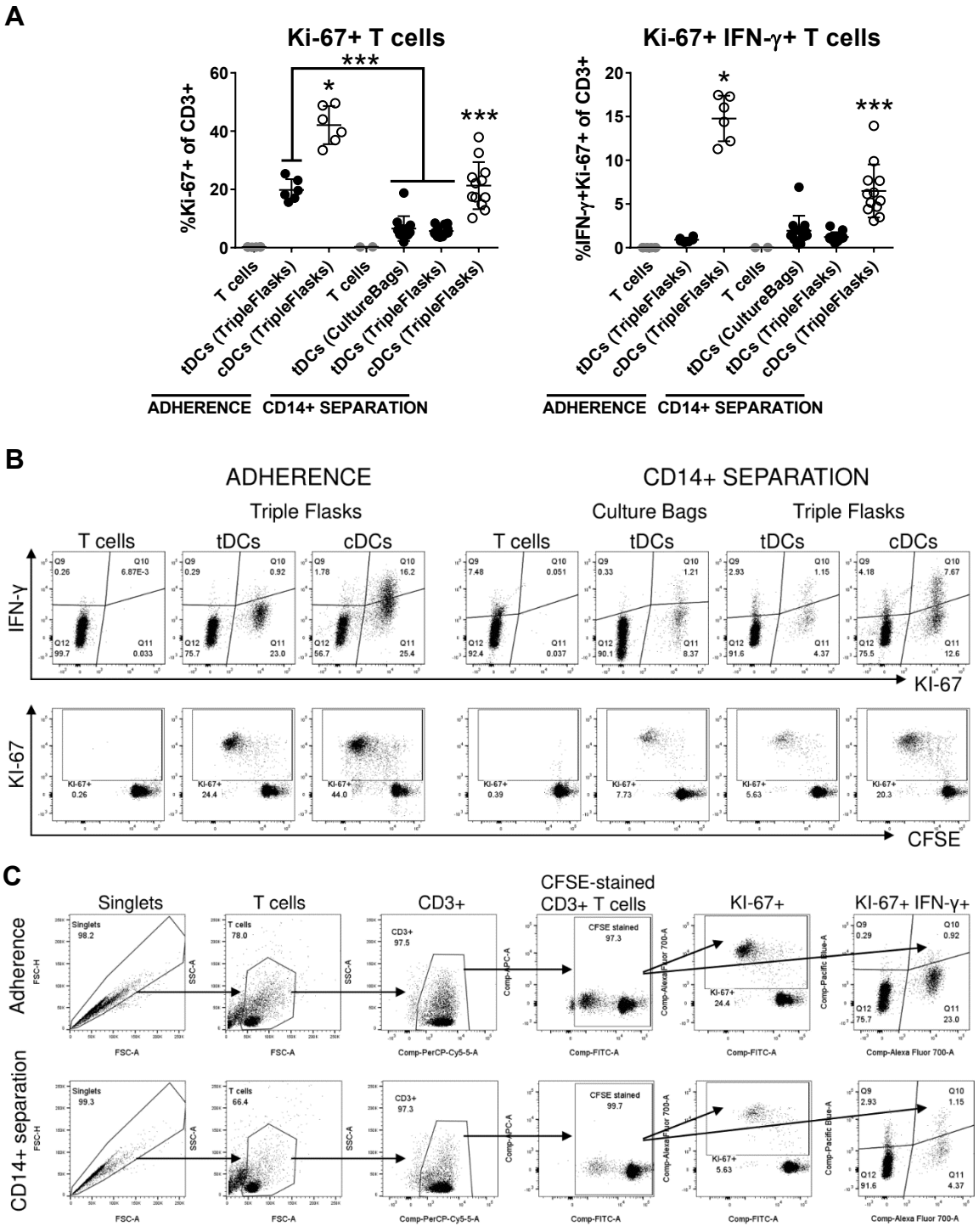


Figure 9. Dex/VitD_2 tolerogenic DCs generated by $CD14^+$ monocyte separation using the CliniMACS Prodigy® cell separator displayed the low stimulatory capacity. Monocytes were obtained via adherence or via $CD14^+$ separation using the CliniMACS Prodigy® cell separator and differentiated into DCs in triple flasks or culture bags. DCs were generated in CellGenix GMP DC media supplemented with IL-4 and GM-CSF in the presence (Dex/VitD_2 tolerogenic DCs, tDCs) or in the absence (control DCs, cDCs) of dexamethasone and vitamin D_2 and then were activated with MPLA. MPLA-matured DCs were frozen and

stored in liquid nitrogen, then were thawed, washed and cultivated with allogeneic purified CFSE-stained $CD3^+$ T cells from 2 different donors. The percentage of Ki-67^+ (proliferating) and Ki-67+IFN-γ^+ T cells from $CD3^+$ T cells induced in response to stimulation with tDCs or cDCs was detected by intracellular staining and analyzed by flow cytometry on day 4. (**A**) Individual values from minimum of 3 donors are shown. Horizontal lines represent mean values ± SD. *$p \leq 0.05$, ***$p \leq 0.001$ (Adherence vs. $CD14^+$ separation: unpaired Mann-Whitney test; tDCs vs. cDCs: paired Wilcoxon t-test). (**B**) Representative dot plots and (**C**) the gating strategy are shown. $CD14^+$ monocyte separation and DC generation were performed by Sotio a.s. Technical Operations – Process Development team.

Finally, we analyzed the ability of Dex/$VitD_2$ tolerogenic DCs generated by CliniMACS Prodigy® to suppress proliferation of T cells induced by unspecific stimulation using anti-CD3/CD28 antibody-coated beads. We developed this test for routine evaluation of suppressive abilities of Dex/$VitD_2$ tolerogenic DCs. Moreover, in comparison to previous assay testing the capacity of Dex/$VitD_2$ tolerogenic DCs to induce T cell proliferation and IFN-γ production, we do not need to generate control DCs, which serve as a control inducing strong T cell activation. In addition, avoiding generation of control DCs would allow us to use all separated monocytes for tolerogenic DC generation, so we could increase the total amount of generated Dex/$VitD_2$ tolerogenic DCs. This assay is not suitable for analysis of suppressive abilities of Dex/$VitD_2$ tolerogenic DCs prepared by monocyte adherence, as T cells that remain in the final product after the non-specific monocyte enrichment by adherence technique compete for anti-CD3/CD28 antibody-coated beads.

Therefore, we stimulated purified allogeneic CFSE-stained $CD3^+$ T cells with anti-CD3/CD28 antibody-coated beads (1:1 ratio) and we added the increasing numbers of Dex/$VitD_2$ tolerogenic DCs to the cultures (0.2, 0.4 and 0.8:1 DC/T cell ratio). The suppressive effect of Dex/$VitD_2$ tolerogenic DCs on T cell proliferation was investigated after 3 days. We found that both types of Dex/$VitD_2$ tolerogenic DCs prepared from separated $CD14^+$ monocytes, cultivated in triple flasks as well as in culture bags, were able to significantly inhibit T cell proliferation induced by anti-CD3/CD28 antibody-coated beads in the highest ratio (Figure 10).

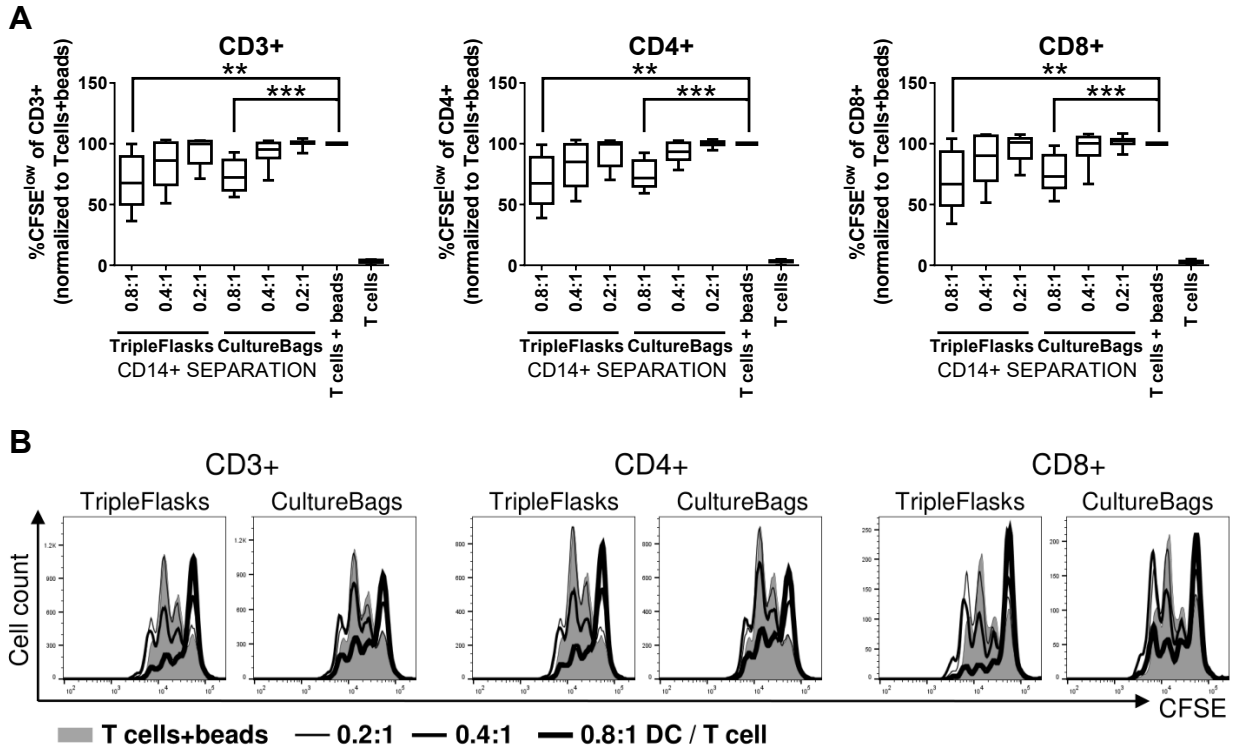


Figure 10. Dex/VitD$_2$ tolerogenic DCs generated by CD14$^+$ monocyte separation using the CliniMACS Prodigy® cell separator inhibited T cell proliferation induced by anti-CD3/CD28 antibody-coated beads. Monocytes were obtained via adherence or via CD14$^+$ separation using the CliniMACS Prodigy® cell separator and differentiated into DCs in triple flasks or culture bags. Dex/VitD$_2$ tolerogenic DCs were generated in CellGenix GMP DC media supplemented with IL-4 and GM-CSF in the presence of dexamethasone and vitamin D$_2$ and then were activated with MPLA. MPLA-matured DCs were frozen and stored in liquid nitrogen, then were thawed, washed and cultivated with allogeneic purified CFSE-stained CD3$^+$ T cells from 2 different donors at a ratio of 0.8, 0.4 or 0.2:1 (DC/T cell) and anti-CD3/CD28 antibody-coated beads (at a ratio of 1:1 T cell/bead). T cells stimulated only with beads and unstimulated T cells served as a control. T cell proliferation was analyzed by flow cytometry on day 3. (**A**) Data from 5 (the group Triple Flasks) or 6 donors (the group Culture Bags) are expressed as boxplots. **p ≤ 0.01, ***p ≤ 0.001 (paired Wilcoxon t-test). (**B**) Representative histograms are shown. CD14$^+$ monocyte separation and Dex/VitD$_2$ tolerogenic DC generation were performed by Sotio a.s. Technical Operations – Process Development team.

Together with a growing number of clinical studies exploiting DC-based immunotherapy for the cancer treatment, there is also a growing number of studies showing the impact of the monocyte isolation method on the phenotype and function of generated DCs and thereby on their final efficacy in clinical trials. A significant improvement in DC yield, purity and viability was shown in DCs generated from CD14$^+$ purified monocytes (MACS technology, Miltenyi Biotec) compared with DCs prepared from monocytes obtained by adherence [210]. Regarding the phenotype and function, only a minor effect of the monocyte isolation method on surface marker expression was

described. However, DCs generated from $CD14^+$ purified monocytes were shown to secrete lower levels of IL-10, IL-12 and TNF-α after LPS treatment [211]. Interestingly, DCs generated by monocyte adherence were shown to be more potent activators of Th1 immune response in comparison to DCs generated from $CD14^+$ purified monocytes that induced higher IL-4 and lower IFN-γ production in T cells and thus favoring the Th2 type immune response [211,212]. These data suggest that the MACS technology could be beneficial for the preparation of tolerogenic DCs; nevertheless, data evaluating the impact of the monocyte isolation method on the phenotype and function of tolerogenic DCs are very limited.

We observed that utilization of the CliniMACS Prodigy® system for isolation of $CD14^+$ monocytes significantly improved numbers of $CD11c^+$ DCs in the final tolerogenic DC-based therapeutic product compared with tolerogenic DCs that were generated from monocytes obtained via adherence. The CD14-based separation method did not affect the viability and capacity of Dex/$VitD_2$ tolerogenic DCs to acquire a stable, regulatory phenotype with low surface expression of maturation-associated molecules, high expression of tolerogenic markers and high secretion of IL-10. Moreover, we verified the low stimulatory capacity of Dex/$VitD_2$ tolerogenic DCs generated from separated $CD14^+$ monocytes in the allogeneic lymphocyte reaction. Indeed, Dex/$VitD_2$ tolerogenic DCs generated from $CD14^+$ separated monocytes induced significantly lower numbers of proliferating and comparable proportion of IFN-γ positive T cells in comparison to Dex/$VitD_2$ tolerogenic DCs generated from monocytes obtained by adherence.

Additionally, we did not observe major differences between Dex/$VitD_2$ tolerogenic DCs generated in culture flasks or bags. The only exception was secretion of IL-10 after LPS restimulation where we found significantly higher production in case of Dex/$VitD_2$ tolerogenic DCs differentiated in culture flasks. This observation is in line with the study by Kurlander *et al*. showing the significant impact of DC cultivation in culture bags on cytokine production. In fact, they showed that DCs generated in culture bags produced markedly lower levels of IL-10 and IL-12 compared with DCs prepared in flasks. Although the group did not investigate mechanisms that are responsible for the reduced cytokine production, they suggested the effect of altered or interrupted adherence-dependent intracellular signaling, which may be necessary for sufficient cytokine production [213]. We also observed a trend to slightly better viability after thawing and upon cultivation in media without tolerogenic factors in case of Dex/$VitD_2$ tolerogenic DCs differentiated in culture flasks.

However, we did not see significant differences in suppressive function of Dex/VitD$_2$ tolerogenic DCs generated in culture flasks or bags. Given the slightly better viability, markedly higher production of IL-10 and lower costs, Dex/VitD$_2$ tolerogenic DC differentiation in triple flasks can be considered a slightly better option. Nevertheless, other parameters should be address before a final decision is made, such as a migratory potential and the ability to induce Treg differentiation, which might be also affected [213].

Importantly, we established the assay examining the suppressive capacity of Dex/VitD$_2$ tolerogenic DCs. Indeed, Dex/VitD$_2$ tolerogenic DCs generated from separated CD14$^+$ monocytes were able to suppress T cell proliferation induced by anti-CD3/CD28 antibody-coated beads. However, the ratio of tolerogenic DCs to T cells had to be very high in order to overcome strong T cell activation induced by anti-CD3/CD28 antibody-coated beads and/or allogeneic setup of the assay. Therefore, further optimization of the assay conditions is recommended. Finally, we found that application of 1 μg/ml instead of 2 μg/ml MPLA did not significantly affect the phenotype and function of tolerogenic DCs. All these findings were used for improvement of the manufacturing protocol for the generation of our tolerogenic DC-based therapeutic product.

Taken together, in this part we optimized Dex/VitD$_2$ tolerogenic DC manufacturing process. Moreover, we evaluated that analysis of Dex/VitD$_2$ tolerogenic DC purity, viability and combined parameter, analysis of maturation-associated as well as tolerogenic markers by flow cytometry and production of IL-10 and IL-12 upon LPS stimulation by ELISA can be used as quality control tests. Additionally, suppression of T cell proliferation induced by anti-CD3/CD28 antibody-coated beads will be further optimized for the potency assay establishment.

The next step of the project will be generation of three batches of Dex/VitD$_2$ tolerogenic DCs according to the optimized protocol in GMP conditions in clean rooms with utilization of optimized tests for evaluation of their quality and quantity. Results of preclinical testing and GMP-generated batches will then represent a basis for setting of release criteria and other parameters of Dex/VitD$_2$ tolerogenic DCs for potential clinical study.

6 GENERAL DISCUSSION

The main goal for the treatment of autoimmune diseases is permanent suppression of pathological autoimmune reactions and the re-establishment of immune tolerance. Various approaches of immunotherapy have been tested in T1D. Despite promising results from preclinical research of those therapies in animal models of T1D or from initial clinical testing, no immunotherapy has been approved for T1D treatment to date. The main problem of tested approaches is a gradual loss of the effect once the treatment ends. A possible explanation is that the tested treatments did not fundamentally affect the immune system setting. In this regard, cell-based therapies, such as Treg-based therapy, SC-based therapy or AHST, seem to be promising because they have the potential to reset immune system responses. However, they have not yet provided unequivocally optimistic prospects. *Ex vivo* polyclonally expanded Tregs have been reported to change to a naïve instead of a memory phenotype, which would be desired for long-term tolerance induction. Moreover, they disappeared from the blood circulation over time, and the second Treg dose did not recapitulate the increase in Treg proportion in patients [134,135,214]. After AHST, the subset of memory T cells was the most abundant population of T cells detected during long-term follow-up in patients, indicating that the immunosuppressive regimen prior to SC transplantation might not have sufficiently eliminated potentially autoreactive and pathogenic memory T cells, which then could re-expand. This suggestion is also supported by the observation that the T cell repertoire diversified after AHST, but the variability declined with time, and memory T cells, including autoreactive clones, outnumbered the recent thymic emigrants [125].

Tolerogenic DCs represent another type of cell-based immunotherapy. The first clinical trial examining tolerogenic DCs was conducted in 2011 with patients suffering from autoimmune T1D. Since then, tolerogenic DCs have been tested in numerous clinical trials for the treatment of not only T1D but also MS, RA, CD or transplant rejection. The ability to comprehensively modulate immune responses is the key advantage of tolerogenic DCs. In fact, tolerogenic DCs can employ various mechanisms for immune system regulation to secure the restoration of balance between regulatory and effector immune responses in patients suffering autoimmune diseases. These mechanisms involve effector T cell inhibition or their modulation towards noninflammatory subtypes, deletion of activated T cells and induction of suppressive T cell subsets [144,215]. In our studies, we observed that tolerogenic DCs generated from healthy donors using dexamethasone

and vitamin D_2 (referred as Dex/VitD_2 tolerogenic DCs) were able to induce antigen-specific T cell hyporesponsiveness, inhibit T cell proliferation, IFN-γ and IL-17 production and suppress immunogenic DC-induced T cell activation [208,209]. This DC suppressive potential is crucial for T1D treatment, as Th1 and Th17 cells producing IFN-γ and IL-17, respectively, are responsible for pathological immune responses and beta cell killing. The ability of tolerogenic DCs to induce the development of regulatory T and B cells is important for long-lasting tolerance induction. The capacity of tolerogenic DCs to promote various subsets of suppressive T cells and B cells has been shown in various studies *in vitro*. In this regard, we observed that Dex/VitD_2 tolerogenic DCs were potent inductors of IL-10-producing Tregs, which demonstrated the substantial suppressive capability of inhibiting responder T cell proliferation and IFN-γ as well as IL-17 production. Importantly, the results from clinical trials have also shown increased regulatory cells in the peripheral blood of patients during tolerogenic DC administration [144].

Although tolerogenic DCs can be generated according to various protocols, they share an immature or a semimature phenotype with reduced expression of activation markers and costimulatory molecules, increased expression of inhibitory molecules, increased production of suppressive mediators and substantial suppressive capabilities. After application to patients, tolerogenic DCs can encounter proinflammatory signals that normally induce DC activation. Therefore, the stability of the tolerogenic DC regulatory phenotype and function is the key requirement for the tolerogenic DCs utilized in clinical trials. The results from the first part of this study showed that Dex/VitD_2 tolerogenic DCs displayed a tolerogenic phenotype and function even upon restimulation with LPS, poly(I:C), proinflammatory cytokines or CD40 ligand, which represent relevant DC activation signals and thereby mimic the possible situation *in vivo*. Indeed, we demonstrated that Dex/VitD_2 tolerogenic DCs did not largely upregulate expression of CD40, CD83, CD86 and HLA-DR and secretion of IL-12 and TNF-α after 24 hours of restimulation. In contrast, they retained high expression of TLR2, IL-T3, PD-L1, and CD14 and secretion of TGF-β and IL-10. More importantly, in line with the phenotype, we demonstrated that repeatedly activated Dex/VitD_2 tolerogenic DCs were still poor inducers of T cell proliferation and production of IFN-γ and IL-17. In contrast, they maintained the ability to induce IL-10-producing T cells with strong suppressive potential [208]. Therefore, we significantly broadened previous observations of stable surface marker expression and IL-10 production by dexamethasone plus vitamin D_3-treated tolerogenic DCs restimulated with LPS, proinflammatory cytokines or peptidoglycan [179]. In addition, we

found that p38 MAPK, ERK and NF-κB were important molecules that played a role in the maintenance of IL-10 production, tolerogenic marker (PD-L1 and IL-T3) expression and low T cell stimulatory capacity of Dex/VitD_2-treated tolerogenic DCs. These findings are in line with previously published data implicating those molecules in the regulation of DC activation and induction of the tolerogenic phenotype in DCs [216].

More importantly, we revealed that the mTOR/STAT3 pathway regulated the metabolism of Dex/VitD_2 tolerogenic DCs towards increased glucose consumption, which contributed to maintenance of the regulatory phenotype and function of Dex/VitD_2 tolerogenic DCs in the proinflammatory environment. The critical role of the metabolic state for tolerogenic DCs was reported in two other publications in 2015. Ferreira *et al*. showed that tolerogenic DCs generated using vitamin D_3 show an upregulation of both glycolysis and oxidative phosphorylation. Their subsequent analysis revealed that glucose availability and enhanced glycolysis, in contrast to oxidative phosphorylation, are essential for induction and maintenance of the tolerogenic DC phenotype and function. Moreover, in agreement with our data, glucose metabolism and the tolerogenic status of DCs are regulated via the PI3K/Akt/mTOR axis [152]. Malinarich *et al*. also reported an increased glycolytic capacity and reserve and enhanced mitochondrial content and oxidative phosphorylation activity in tolerogenic DCs generated using vitamin D_3 and dexamethasone. In this case, tolerogenic DC function is dependent on fatty acid oxidation as an energy source for oxidative phosphorylation [217]. Therefore, our and other recent studies have provided new insights into important molecular mechanisms for induction and maintenance of the tolerogenic DC phenotype and function. The metabolic state and its regulation are probably key factors in those processes [218].

Highly stable tolerogenic DCs represent a promising tool that is now being widely tested in clinical trials. The possibility to generate tolerogenic DCs from blood precursors of T1D patients has been previously tested using IL-10 in combination with TGF-β [202,219], and anti-sense oligonucleotides against CD40, CD80, CD86 or vitamin D_3 have recently been used for the preparation of tolerogenic DCs in clinical trials with T1D patients. In the second part of this study, we showed that vitamin D_2 and dexamethasone could also be used for the generation of tolerogenic DCs with sufficient quality and quantity from blood precursors of patients suffering from T1D. We demonstrated that Dex/VitD_2 tolerogenic DCs induced T cell hyporesponsiveness with inhibited T

cell proliferation and production of IFN-γ and IL-17, which was antigen-specific against GAD65. Moreover, our Dex/VitD$_2$ tolerogenic DCs were potent inductors of FoxP3$^+$ Tregs [209].

Interestingly, in both previously published studies, which investigated the *in vitro* suppression of memory T cells by IL-10/TGF-β-treated tolerogenic DCs in T1D patients, T cells were tolerized in only a subgroup of patients. A study reported by Segovia-Gamboa *et al*. showed that the potential to induce stable hyporesponsiveness in T cells by IL-10/TGF-β tolerogenic DCs was linked to the ability of T cells to proliferate in an antigen-specific fashion against insulin or GAD65. In turn, levels of antigen-specific T cell responses negatively correlated with the patient's cholesterol and HbA$_{1c}$ levels. Moreover, the potential to tolerize T cells was lower in newly diagnosed patients, which could also be associated with the patient's metabolic parameters, as newly diagnosed patients had higher HbA$_{1c}$ and cholesterol levels when compared to nonnewly diagnosed patients [202]. The HbA$_{1c}$ level reflects glycemic control in T1D patients because HbA$_{1c}$ is formed during the process of nonenzymatic glycation as a consequence of hemoglobin exposure to plasma glucose [46].

Based on these findings, we evaluated the effect of HbA$_{1c}$ on the phenotype of Dex/VitD$_2$-treated tolerogenic DCs and their ability to tolerize T cells. We found that long-term hyperglycemia impaired the capacity of Dex/VitD$_2$ tolerogenic DCs to induce T cell hyporesponsiveness with reduced T cell proliferation and Th1 and Th17 cytokine production. Notably, the ability to suppress IL-17 production was affected by the patient's HbA$_{1c}$ status. Additionally, hyperglycemia had a strong impact on the stability and antigen-specificity of T cell suppression, and it also influenced the capacity of Dex/VitD$_2$ tolerogenic DCs to suppress immunogenic DC-induced production of proinflammatory cytokines by T cells. Finally, the key role of the HbA$_{1c}$ level for potential tolerogenic DC treatment efficacy was obvious based on the ability of Dex/VitD$_2$ tolerogenic DCs to promote differentiation of regulatory T cells with substantial suppressive potential, which was largely lost in patients with poor glycemic control. In addition to HbA$_{1c}$, we also observed a less prominent effect of disease duration on the possibility to tolerize T cells by Dex/VitD$_2$ tolerogenic DCs in T1D patients, as the level of T cell suppression inversely correlated not only with HbA$_{1c}$ but also with disease duration. Analogously to the study by Segovia-Gamboa *et al*., those two parameters might be related, as 68% of patients in the well-controlled group had suffered from T1D for less than 3 years, whereas only 35% of the patients in the group with poor glycemic had a

short disease duration while the rest had suffered from T1D for more than 3 years. Interestingly, clinical studies with anti-CD3 antibodies have also repeatedly shown efficacy only in a subgroup of T1D patients. The autoimmune process is better suppressed in patients who are relatively young, with a short disease duration and with good metabolic control of T1D (HbA_{1c} level, insulin dose) at the time of study enrollment. Finally, the effect of the patient's HbA_{1c} level on drug efficacy has also been mentioned in the case of treatment with abatacept and AHST [108,126].

Regarding the patient's age and disease duration, it is possible that the pathological process is not fully developed and thereby still sensitive to suppression in younger patients and patients with a short disease duration. Indeed, autoreactive T cells with less antigen specificity are present in the pancreas of T1D patients at the time of diagnosis and increase in number over time [54]. Additionally, besides antigen spreading, bystander activation of different immune cell types may occur, which may strengthen the pathological process. Alternatively, with a longer time since diagnosis and with an increasing age of T1D patients, as the beta cell mass is gradually destroyed, the number of beta cells may decline to below a critical limit for regeneration, or, if still present, they may be unable to functionally recover [54,60].

An elevated blood glucose level, reflected by a high HbA_{1c} parameter, can be directly associated with beta cell destruction or with the deregulation of immune cells towards a proinflammatory phenotype. A broad spectrum of effects of long-term hyperglycemia on immune cells has been described. In the human monocyte cell line THP-1 or peripheral blood monocytes, hyperglycemia or high glucose-induced expression of proinflammatory mediators such as chemokines, IL-1β, TNF-α, IL-6 and others has been described. Moreover, monocytes exposed to high glucose levels *in vitro* show decreased surface expression of the HLA-DR molecule, which can significantly influence their antigen-presenting capabilities. Additionally, hyperglycemia or high glucose affects the phagocytic and chemotactic capacity of monocytes. Similar effects have also been observed in macrophages [220,221]. Analogously, T cells exposed to hyperglycemia or high glucose levels display a proinflammatory cytokine profile with dominant IL-6 and IL-17 family expression [67]. Advanced glycation end products (AGEs; glycated macromolecules) drive the differentiation of naïve $CD4^+$ T cells towards the proinflammatory Th1/Th17 subsets and inhibit the suppressive function of Tregs. Moreover, the frequency of Tregs is inversely correlated with the HbA_{1c} level in T1D patients [222,223]. Interestingly, high glucose or AGEs elevates the level of resting

cytokine production or proliferation of monocytes and T cells and, more importantly, impairs induction of the antigen-specific T cell response [224]. Those observations are in line with our results and those of Segovia-Gamboa *et al*. showing high basal T cell proliferation and, in turn, the inability to induce an antigen-specific response in poorly controlled patients with high HbA_{1c} levels [202,209]. Taken together, those findings strongly indicate that long-lasting hyperglycemia significantly influences immune cells towards a proinflammatory state. Therefore, it is very likely that the immunopathological response in patients with poor disease control is more difficult to suppress irrespective of the applied immunotherapy. Nevertheless, in the case of therapy based on tolerogenic DCs, the effect may be more pronounced, as monocytes as a source for DC differentiation are exposed to a hyperglycemic environment in patients with poor glycemic control. Therefore, it may be impossible to efficiently tolerize them with vitamin D and dexamethasone. Indeed, we observed that tolerogenic DCs generated from patients with good versus poor glycemic control differed in expression of the tolerogenic marker PD-L1 and IL-T3 [209].

In the third part of this study, we evaluated the possibility of generating Dex/$VitD_2$ tolerogenic DCs according to our manufacturing protocol, of the desired quality and quantity for potential clinical application. Despite changes in the production process that are indispensable for clinical-scale manufacturing (leukapheresis products instead of whole blood or buffy coats as a source of PBMCs, different plastic material, cryopreservation step), Dex/$VitD_2$ tolerogenic DCs displayed a good regulatory phenotype and function. However, we further tested different monocyte separation and cultivation methods to improve the DC yield and other important parameters of tolerogenic DC-based products such as purity and viability.

Monocyte separation based on plastic adherence with subsequent cultivation in a culture flask is a well-established, routinely used method for monocyte-derived DC generation. However, this method does not allow the complete removal of all the remaining immune cell populations from monocytes. Therefore, the final product of monocyte-derived tolerogenic DCs has a lower purity and contains other immune cells in addition to $CD11c^+$ tolerogenic DCs, mainly T cells, which can markedly influence the efficacy of tolerogenic DC-based therapy in clinical trials. In comparison to adherence, a significant improvement in DC yield, purity and viability was shown when monocytes were separated with anti-CD14 antibody using the immunomagnetic-based method (MACS technology, Miltenyi Biotec) [210]. An additional advantage of monocyte

immunomagnetic separation is the possibility to subsequently differentiate DCs in hydrophobic culture bags, which means in a closed system with a reduced risk of contamination. Moreover, immunomagnetic separation with subsequent cultivation in bags facilitates the scale-up and automation of the manufacturing process [207].

We observed that, in comparison to adherence, immunomagnetic separation of $CD14^+$ monocytes significantly improved the proportion of viable $CD11c^+$ DCs in the final tolerogenic DC-based therapeutic product. However, the DC yield was not improved. The regulatory phenotype and function of tolerogenic DCs generated from $CD14^+$ separated monocytes were not affected, as DCs recapitulated the low surface expression of maturation-associated molecules, high expression of tolerogenic markers and high secretion of IL-10. In fact, we observed that Dex/$VitD_2$ tolerogenic DCs generated from $CD14^+$ separated monocytes induced significantly lower numbers of proliferating T cells in comparison to DCs generated from monocytes obtained by adherence. Moreover, Dex/$VitD_2$ tolerogenic DCs generated from separated $CD14^+$ monocytes were able to suppress T cell proliferation induced by anti-CD3/CD28 antibody-coated beads. Regarding the comparison of culture flasks and bags, we did not observe major differences in the DC phenotype and function. The only exception was significantly higher IL-10 production upon LPS restimulation in the case of Dex/$VitD_2$ tolerogenic DCs differentiated in culture flasks, potentially because of altered or missing adherence-dependent intracellular signaling in DCs generated in culture bags, which could be indispensable for sufficient cytokine production [213]. Tolerogenic DCs differentiated in culture flasks also displayed a trend towards slightly better viability after defrosting and upon cultivation in medium without tolerogenic factors. Based on these observations, tolerogenic DCs generated from monocytes using the immunomagnetic separation method with a subsequent differentiation step in triple flasks was considered the best option. Nevertheless, the migratory potential and ability to induce Treg differentiation should also be addressed before a final decision is made, as those features represent other important parameters of clinical-grade tolerogenic DCs and might be affected by the distinct monocyte separation method [213]. Finally, we also suggested assays that can be used for control of the quality and suppressive capacity of Dex/$VitD_2$ tolerogenic DCs generated for potential clinical study.

Thus far, tolerogenic DC-based therapy has been demonstrated in several clinical trials, which showed a well-tolerated safety profile and promising biological effects, especially an increase in

regulatory cell frequencies *in vivo* after the treatment. Nevertheless, other studies are necessary to validate the efficacy of this approach. To achieve the best possible results, important questions concerning the number of applied DCs, dosing schedule, and route of delivery, as well as the suitable group of patients and treatment timing, must be addressed. The findings presented in this study argue strongly in favor of the further development of tolerogenic DC-based therapy.

7 CONCLUSIONS

PART I:

1. We established the GMP-compliant protocol for tolerogenic DC generation using vitamin D_2, the immunosuppressive drug dexamethasone and MPLA.

2. We verified the stability of the regulatory phenotype and suppressive function of tolerogenic DCs upon restimulation with various proinflammatory stimuli, which is a key requirement for the application of tolerogenic DCs in clinical trials. Moreover, we revealed an important role of metabolic regulations and specific signaling pathways in the maintenance of the regulatory status of tolerogenic DCs.

3. Based on these data, an international patent application was prepared ("Tolerogenic Dendritic Cells, Methods of Producing Same and Uses Thereof").

PART II:

4. Tolerogenic DCs generated from patients with T1D according to the established protocol acquired regulatory phenotype, induced antigen-specific Th1/Th17 hyporesponsiveness and induced suppressive $FoxP3^+$ Tregs.

5. We found that long-term blood hyperglycemia is a crucial factor influencing functional characteristics of tolerogenic DCs prepared from T1D patients, which represents the important stratification marker for the settings of potential clinical study.

6. Based on these data, an international patent application was prepared ("Tolerogenic Dendritic Cells").

PART III:

7. We optimized the manufacturing process for generation of a tolerogenic DC-based product using the CliniMACS Prodigy® system for $CD14^+$ monocyte isolation, which improved the proportion of $CD11c^+$ tolerogenic DCs in the final product.

8. We developed and optimized new tests that could be used for control of the quality and suppressive capacity of tolerogenic DCs.

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