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Bc. Zuzana Kroulíková

Tolerogenní dendritické buňky jako nová buněčná terapie v diabetu I. typu

Tolerogenic dendritic cells as a novel cell-based therapy in type 1 diabetes

Diplomová práce

Školitel: MUDr. David Funda, Ph.D.

Praha, 2019

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 12. 8. 2019

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Bc. Zuzana Kroulíková

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Abstract

Utilization of tolerogenic dendritic cells (tolDCs) as a cell-based therapy represents a promising strategy in treatment of autoimmune diseases including type 1 diabetes (T1D). Numerous protocols have been established to generate tolDCs *ex vivo* and their therapeutic effect has been demonstrated in animal models of autoimmune diseases. In this thesis we compared three different variants of such protocols which are based on the combined treatment of bone marrow-derived DCs with vitamin D and dexamethasone applied at different time points of their maturation towards tolDCs. We assessed the efficiency of these protocols in regards of their effect on the expression of co-stimulatory molecules CD40, CD80, CD86, and MHC II and the chemokine receptor CCR7 on the surface of tolDCs. Then, we evaluated the migration pattern of antigen unloaded tolDCs *in vivo* as well as their effect on the induction of immune responses and cell proliferation of lymph node cells. This was achieved by labelling of tolDCs with membrane dye PKH26 and by following their migration path by flow cytometry after intraperitoneal (i.p) or subcutaneous (s.c.) injection into either left or right side of the body. On day 1, 3, 5, 7, and 9, the presence of $PKH26^+$ tolDCs was examined in spleen, pancreatic, mesenteric, inguinal and axillary lymph nodes of NOD mice. Total cell recoveries from these anatomical sites were used as a measure of their migratory capacity. Flow cytometric analysis readily detected live $PKH26^+CD11c^+CD3^-$ tolDCs in spleens and pancreatic lymph nodes after i. p. administration, whereas s. c. injection led to their accumulation preferentially in inguinal and axillary lymph nodes on the respective application side. In addition, we monitored the impact of the above indicated application routes on the prevention of diabetes by tolDCs in the NOD-SCID model by adoptive co-transfer of tolDCs with NOD-derived splenocytes. Our data provide strong evidence that the type of culture protocol along with application route affect tolerogenic properties of tolDCs. Specifically, we established that the capability of tolDCs to migrate to pancreatic lymph nodes and to prevent diabetes in the NOD-SCID adoptive co-transfer model is the most effective when vitamin D and dexamethasone treated tolDCs are administrated via i.p. route. This original data provides a novel experimental platform for further optimizing this protocol, which in a long run, can be potentially used for therapeutic application in future human trials.

Key words

Abstrakt

Tolerogenní dendritické buňky (tolDCs) představují slibný nástroj v rámci buněčné terapie při léčbě autoimunitních onemocnění včetně diabetu I. typu (T1D). Mnoho protokolů je založeno na *ex vivo* generaci tolDCs a jejich terapeutický efekt byl demonstrován na zvířecích modelech autoimunitních onemocnění. V této diplomové práci jsme nejprve porovnali tři různé varianty tolDCs protokolů založených na vitaminu D a dexamethasonu s ohledem na jejich vliv na expresi kostimulačních molekul CD40, CD80, CD86 a MHC II a na expresi chemokinového receptoru CCR7. Dále jsme zhodnotili *in vivo* migraci PKH26 značených antigen-neloadovaných tolDCs a jejich efekt na indukci imunitní odpovědi/buněčné proliferace. TolDCs byly označeny PKH26 a migrace značených buněk byla sledována průtokovou cytometrií po intraperitoneální, subkutánní aplikaci na levé a také na pravé straně těla v 1., 3., 5., 7. a 9. den ve slezině, pankreatických, mesenterických, inguálních a axilárních lymfatických uzlinách NOD myší. Celkové množství buněk bylo použito jako další posuzující parametr. Živé PKH26$^+$CD11c$^+$CD3$^-$ buňky byly jasně detekovány ve slezině a v pankreatických lymfatických uzlinách po intraperitoneální administraci, zatímco subkutánní injekce vedla k akumulaci tolDCs především v inguálních a axilárních lymfatických uzlinách na korespondující straně aplikace. Kromě toho jsme monitorovali také vliv aplikačních cest tolerogenních dendritických buněk na prevenci diabetu v NOD-SCID modelu adoptivního ko-transferu diabetu. Naše data potvrzují, že nejen kultivační protokoly, nýbrž i aplikační cesty ovlivňují tolerogenní vlastnosti tolDCs – např. schopnost tolDCs migrovat do kritických lymfatických orgánů (konkrétně pankreatických lymfatických uzlin) a předcházet diabetu u NOD-SCID modelu adoptivního ko-transferu. Domníváme se, že zvířecí modely diabetu 1. typu mají velký potenciál při optimalizování tolDCs protokolů před jejich translací do lidských klinických studií.

Klíčová slova

Diabetes 1. typu, tolerogenní dendritické buňky, buněčná terapie, neobézní diabetická myš (NOD), neobézní diabetická myš s těžkou kombinovanou imunodeficiencí (NOD-SCID), aplikační cesty, *in vivo* migrace, optimalizace protokolu

List of abbreviations

Ag	antigen
AGE	advanced glycation product
ALN	axillary lymph node
APC	antigen presenting cell
BCR	B cell receptor
BMDC	bone marrow dendritic cell
CCR7	C-C chemokine receptor 7
cDC	control dendritic cells
DC	dendritic cells
DE	dual expresser
DEX	dexamethasone
FBS	fetal bovine serum
GAD65	glutamic acid decarboxylase (molecular weigh 65 kDa)
GADA	glutamic acid decarboxylase antibodies
GM-CSF	granulocyte-macrophage colony-stimulating factor
i. d.	intradermal
i. p.	intraperitoneal
i. v.	intravenous
iDC	immature dendritic cell
IL-4	interleukin 4
ILN	inguinal lymph node
LPS	lipopolysacharide
mDC	mature dendritic cell
MHC	major histocompatibility complex
MLN	mesenteric lymph node
MPLA	monophosphoryl lipid A
NOD	non-obese diabetic mouse
NOD-SCID	non-obese diabetic-severe combined immunodeficiency mouse
nVDR	nuclear vitamin D receptor
PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4
PLN	pancreatic lymph node
RAGE	receptor for advanced glycation end products

s. c.	subcutaneous
SPL	splenocyte
T1D	type 1 diabetes
TCR	T cell receptor
tolDC	tolerogenic dendritic cell
Treg	T regulatory cell
vitD	vitamin D

Obsah

1. Introduction

Tolerogenic dendritic cells (tolDCs) represent a promising tool for prevention or treatment of autoimmune diseases. In type 1 diabetes (T1D), many animal but also clinical studies investigate the effect of tolDCs in T1D, most of the time as a preventive intervention. Despite the fact, that this field of research is rapidly expanding, many aspects are still not clarified. For example, optimized culture protocols, unspecific effect versus autoantigen-loading, best application routes, single or repeated application or cell dose, *in vivo* survival and dying patterns etc.
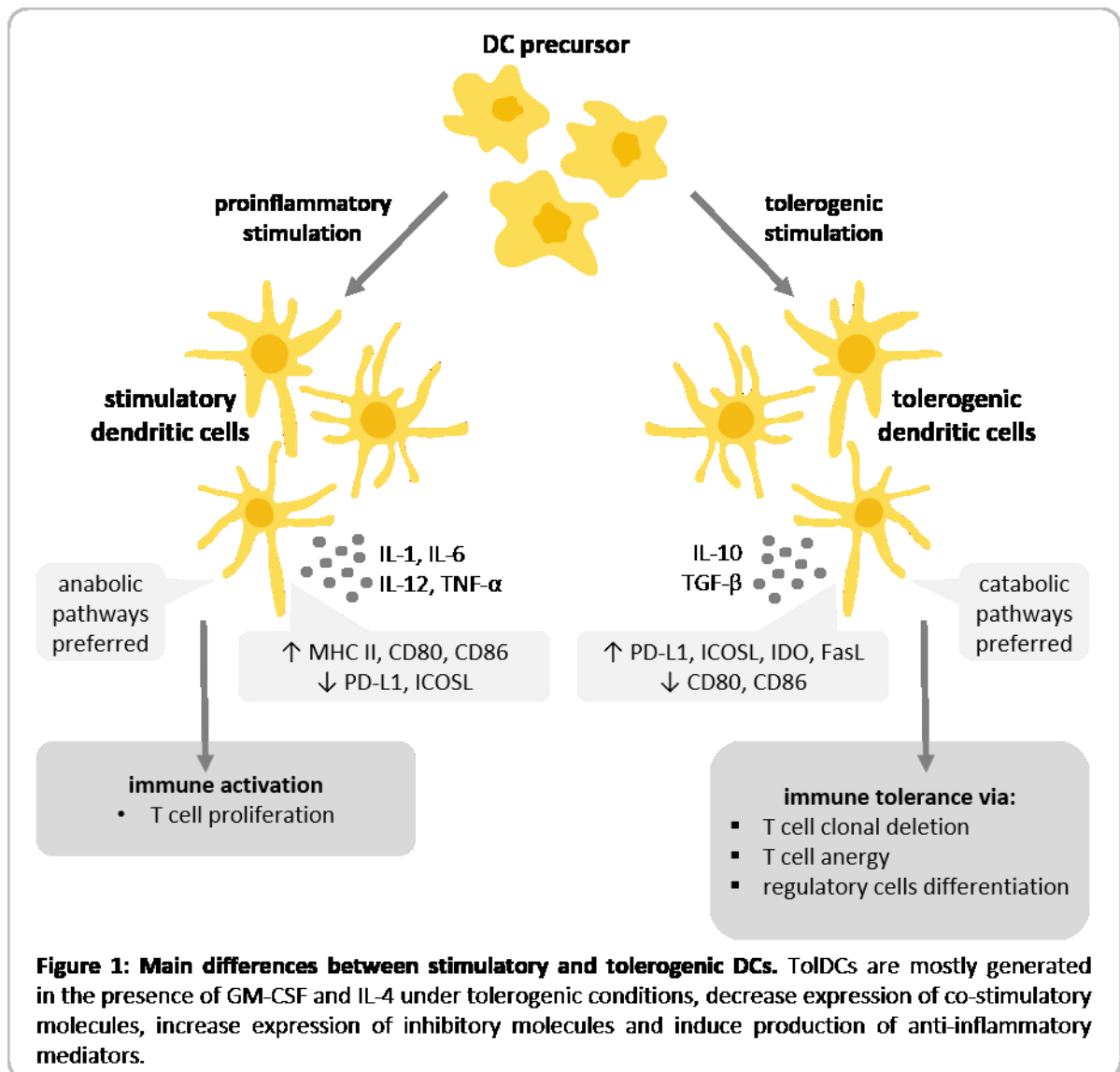
Several clinical trials with tolDCs in T1D and in other autoimmune pathologies are completed or ongoing. These therapies are in general confirmed as safe and well tolerated but often only in phase I, thus without known therapeutic outcomes. Suboptimal protocols of tolDCs may lower overall enthusiasm to invest more resources into this otherwise very promising intervention strategy. For this reason, full clarification of tolDCs properties and well tested protocols in experimental models are required. This thesis describes some aspects of an optimization strategy for prevention of T1D by using the NOD and NOD-SCID mouse models as one more step towards subsequent clinical applications.

1.1 Tolerogenic dendritic cells

Dendritic cells (DCs) play a pivotal role in the regulation of the immune system. Specifically, they are highly potent in a specific induction of immune response via the stimulation of lymphocytes and they are also necessary in tolerance induction and maintaining of immune-homeostasis. They are the most efficient antigen-presenting cells (APC) and their activity leads to a precise polarization and differentiation of effector or regulatory cell subpopulations (Steinman and Cohn 1973, Steinman and Witmer 1978).

Poorly immunogenic tolerogenic dendritic cells (tolDCs) provide induction of self-tolerance or non-danger stimuli. The functional difference between immunogenic and tolerogenic DCs phenotype and capacity tightly depends on maturation state and maturation environment. Contrary to stimulatory DCs, typical tolDCs features in general are decreased level of co-stimulatory molecules (e. g. CD80, CD86), increased amount of co-inhibitory molecules (e. g. PD-L1, ICOSL) and the capacity to induce secretion of immunosuppressive cytokines (e. g. IL-10, TGF-β) (Ganguly, Haak et al. 2013, Osorio, Fuentes et al. 2015).

Other variation between stimulatory and tolerogenic DCs is the distinct metabolic profile. TolDCs are characterized by a shift toward the catabolic pathways, oxidative phosphorylation, mitochondrial oxidative activity and fatty acid oxidation compared to activated DCs (Krawczyk, Holowka et al. 2010, Everts, Amiel et al. 2012, Danova, Klapetkova et al. 2015; cit. as per Grohová, Dáňová et al. 2019). The main differences between stimulatory and tolerogenic DCs are showed in **Figure 1**.

Figure 1: Main differences between stimulatory and tolerogenic DCs. TolDCs are mostly generated in the presence of GM-CSF and IL-4 under tolerogenic conditions, decrease expression of co-stimulatory molecules, increase expression of inhibitory molecules and induce production of anti-inflammatory mediators.
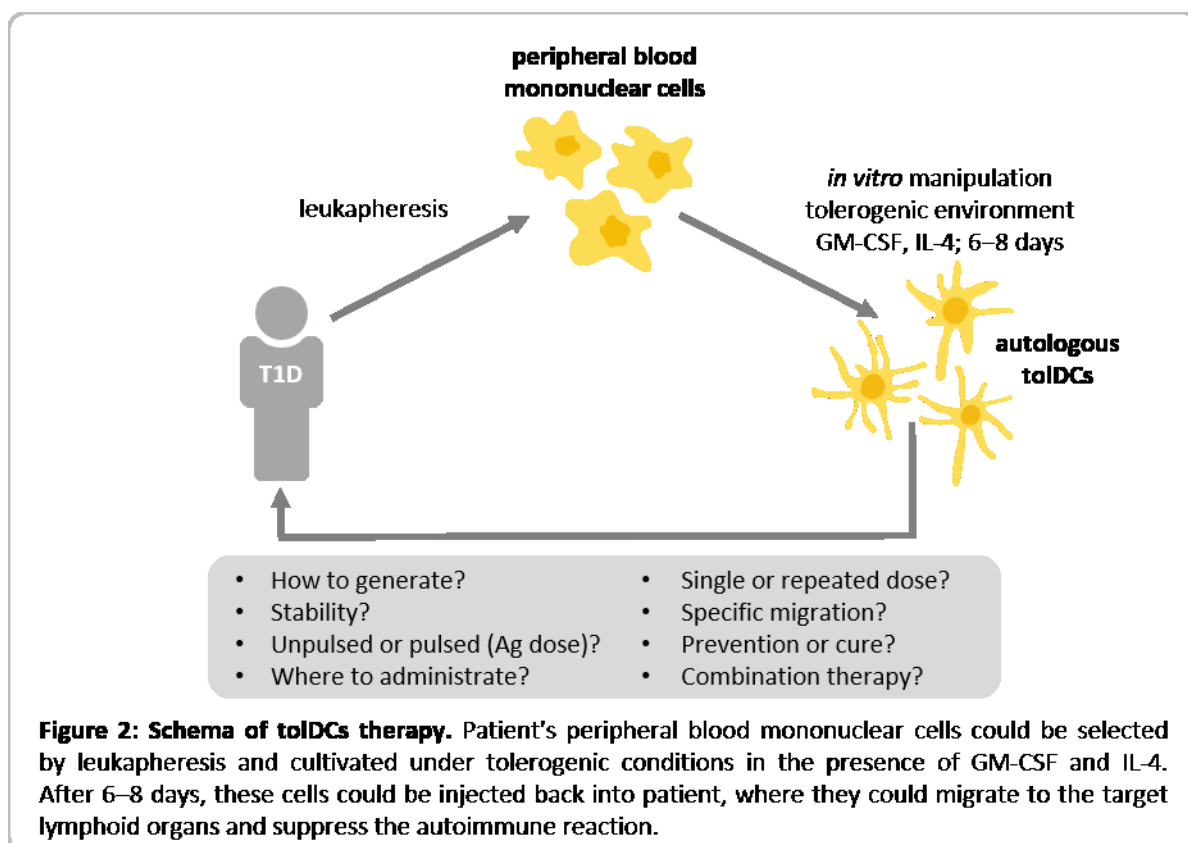
Because of the lower expression of co-stimulatory molecules and on the contrary the increased level of inhibitory molecules, tolDCs are able to promote lymphocyte tolerance via T cell clonal deletion (via Fas/FasL interaction pathways), T cell anergy induction (as a result of Ag recognition without sufficient co-stimulation), and regulatory T cells differentiation (Steinman and Nussenzweig 2002; cit. as per Osorio, Fuentes et al. 2015). Co-inhibitory signaling via PD-L1 expressed on tolDCs promote the expansion of Foxp3$^+$ T regulatory cells (Wang, Pino-Lagos et al. 2008), tolDCs also contribute to induction of regulatory B cells (Di Caro, Phillips et al. 2013) and Tr1 cells (Levings, Gregori et al. 2005, Battaglia, Stabilini et al. 2006). Other mediators with immunosuppressive properties produced by tolDCs represent e.g. indoleamine 2,3-dioxygenase (IDO) or hemoxygenase-1.

1.1.1 Tolerogenic dendritic cells as a cell-based therapy

Because of tolDCs properties, these cells represent a new promising strategy for prevention or treatment of autoimmune diseases. In chronic autoimmune responses, tolDCs could specifically target the pathogenic reaction and contribute to restoring the immunological balance. In addition to that, tolDCs display a migratory capacity from site of application to relevant target tissues (Creusot, Yaghoubi et al. 2009). This is the other advantageous and promising attribute for a potential cell-based immunotherapy.

The effect of tolDCs *in vivo* was demonstrated for the first time by Steinman's group. Pulsed immature DCs, generated under GM-CSF and IL-4 conditions, were injected into healthy donors. It was documented that tolDCs administration was safe and well tolerated and, primarily, led to a specific suppression of CD8$^+$ effector T cells (Dhodapkar, Steinman et al. 2001, Dhodapkar and Steinman 2002).

At this moment, clinical trials with tolDCs have been completed or are ongoing in fields of type 1 diabetes, rheumatoid arthritis, Crohn's disease or multiple sclerosis (**Table 1**). For clinical application, tolDCs are usually prepared from peripheral blood mononuclear cells (PBMCs) (from few milliliters of peripheral blood) commonly in the presence of GM-CSF and IL-4 for 6–8 days and are called as monocyte-derived DCs. A simplified scheme of tolDCs cell-based therapy with unanswered questions and needed optimizations is depicted in **Figure 2**.

Figure 2: Schema of tolDCs therapy. Patient's peripheral blood mononuclear cells could be selected by leukapheresis and cultivated under tolerogenic conditions in the presence of GM-CSF and IL-4. After 6–8 days, these cells could be injected back into patient, where they could migrate to the target lymphoid organs and suppress the autoimmune reaction.

The first human trial using tolDCs was accomplished for the treatment of T1D (Giannoukakis, Phillips et al. 2011). A total of ten adult patients between 18 and 60 years of age received unmanipulated or *ex vivo* antisense oligonucleotides-treated autologous dendritic cells. This cell-based therapy was documented as safe and well tolerated and a higher frequency of B220$^+$ CD11c$^-$ B cells was reported.

In rheumatoid arthritis (RA) results of two phase I clinical trials with tolDCs have been published. In the first one, twelve RA patients received a repeated dose of Ag-pulsed tolDCs subcutaneously in the area around inguinal lymph nodes (Joo et al. 2014). Further, eighteen HLA-risk genotype-positive RA patients obtained a single intradermal dose of autologous dendritic cells treated with NF-κB inhibitor exposed to citrullinated peptides (Benham, Nel et al. 2015). Finally, tolDCs have been also investigated in inflammatory arthritis, Ag-pulsed tolDCs were administrated arthroscopically into the inflamed knee joint but no systemic clinical or immunomodulatory effects were found (Bell, Anderson et al. 2017).

At last, one completed phase I study with tolDCs in Crohn's disease was also published. Nine patients were included and received a single or repeated intraperitoneal injection of unloaded tolDCs. Interestingly, this is the only clinical trial with intraperitoneal injection

of tolDCs in humans (Jauregui-Amezaga, Cabezón et al. 2015). Details and published data of some of the representative completed and ongoing clinical trials with tolDCs are given in **Table 1**.

High-risk individuals (based on family history, HLA genotype and presence of autoantibodies) could be ideal candidates for DC-based immunotherapy because tolDCs are more effective in disease prevention than reversal (**Table 4**). This cell-based therapy could be useful also for patients with recent onset of T1D and with still remaining and viable β cells. In this case, tolDCs could alter autoimmunity and provide time for the β cell regeneration and sufficient insulin production.

Moreover, tolerogenic properties of tolDCs could be enhanced by various combination therapies which include e.g. mRNA electroporation, involving of distinct DCs subpopulations, combination of multiple administration routes, tolDCs modification by diverse drugs or a short-term depletion of autoreactive T cells (to reinforce the stability and semi-mature phenotype of tolDCs) (Creusot, Giannoukakis et al. 2014).

Table 1a: Representative completed clinical trials with tolDCs

Diseas	Trial ID	Generation, modification	App.	Cell dose	Scheme	Results	Ref.
T1D	NCT00445913	antisense oligonucleotides targeting CD40, CD80, CD86	i. d.	1×10^6	four times (2 weeks apart)	well tolerated and safe; ↑ B220^{+} CD11c^{-} B cells	Giannoukakis et al. 2011
RA	CRiS KCT0000035	pulsed with recombinant PAD4, RA33,citrullinated-filaggrin and vimentin	s. c.	$0{,}5 \times 10^7$ $1{,}5 \times 10^7$	five times (2 or 4 weeks apart)	well tolerated and safe; ↓ IFN-γ-secreting T cells; ↓ Ag-specific autoantibodies	Joo et al. 2014
	NCT00396812	NF-κB inhibitor BAY 11-7082 citrullinated peptides	i. d.	1×10^6 5×10^6	once	well tolerated and safe; ↓ T effector cells, IL-15, IL-29; ↑ T regulatory cells	Benham, Nel et al. 2015
IA	NCT01352858	dexamethasone, vitamin D3, MPLA loaded with autologous synovial fluid as Ag	athr.	1×10^6 3×10^6 10×10^6	once	well tolerated and safe	Bell, Anderson et al. 2017
CD	2007-003469-42	dexamethasone, vitamin A, cytokines	i. p.	2×10^6 5×10^6 10×10^6	once or three times (2 weeks apart)	well tolerated and safe; ↓ Crohn's disease Activity Index; ↓ Crohn's disease Endoscopic Index of Severity	Jauregui-Amezaga et al. 2015

Table 1b: Representative ongoing clinical trials with tolDCs

Diseas	Trial ID	Generation, modification	App.	Cell dose	Scheme	Results	Ref.
T1D	NCT02354911	antisense oligonucleotides targeting CD40, CD80, CD86	i. d.	unpublished	four times (2 weeks apart)	unpublished	web 1
	NCT03895996	AVT001 (autologous DC therapy)	i. v.	$7 – 10 \times 10^6$	3 times (monthly)	unpublished	web 2
MS	NCT02283671	dexamethasone pulsed with myelin peptides	i. v.	unpublished	three times (2 weeks apart)	unpublished	web 3
	NCT02611902	vitamin D3 pulsed with myelin peptides	i. d.	5×10^6 10×10^6 15×10^6	5 times (dose number unpublished)	unpublished	web 4
	NCT02903537	vitamin D3 pulsed with myelin peptides	i. n.	5×10^6 10×10^6 15×10^6	6 times (2 and 4 weeks apart)	unpublished	web 5

↑ Increase; ↓ Decrease; T1D, type 1 diabetes; RA, rheumatoid arthritis; IA, inflammatory arthritis; CD, Crohn's disease; MS, multiple sclerosis; app. r., application route; i. d., intradermal; s. c., subcutaneous; athr., arthroscopically; i. p., intraperitoneal; i. v., intravenous; i. n., intranodal.

1.1.2 Generation of tolerogenic dendritic cells in T1D

1.1.2.1 Culture conditions

In humans, tolDCs for T1D cell-based therapy could be propagate *in vitro* from T1D patient's peripheral blood monocytes. On the other side, in mouse experimental models, tolDCs are cultivated *in vitro* from bone marrow progenitors (BMDCs) commonly in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) together with interleukin 4 (IL-4). A simplified overview with example protocols of tolDCs in T1D is attached in **Table 2**. BMDCs propagated in the absence of IL-4, especially under serum-free conditions, express suboptimal levels of MHC class II, CD40, CD80 and CD86 (Wells, Darling et al. 2005). Propagation with IL-4 significantly improves the *in vivo* migratory capacity of GM-CSF-induced BMDCs to target lymph nodes and the spleen (Yin, Wang et al. 2011).

For cell cultivation, fetal bovine serum (FBS) is most frequently used for tolDCs propagation in T1D protocols (see also **Table 2**) despite the fact that clinical testing is always carried out with cells prepared in serum-free conditions. Looney et al. have showed that tolDCs generated under serum-free conditions achieve stronger tolerogenic properties, increase levels of CD4$^+$Foxp3$^+$ regulatory T cells and are more effective in T1D prevention in NOD mice compared to FBS-tolDCs (Looney, Chernatynskaya et al. 2014).

1.1.2.2 Tolerizing environment

Necessary tolerogenic environment during tolDCs generation is successfully created commonly by vitamin D together with dexamethasone. This is a frequently used combination of tolerogenic agents for tolDCs generation which was verified in many protocols worldwide (Funda, Palova-Jelinkova et al. 2019).

An active and lipophilic compound of vitamin D is able to cross biological membranes and bind to the transcription factor VDR located in the nucleus. A relevant number of nuclear vitamin D receptors (nVDR) is detected in parathyroid gland, intestine, kidneys, skin but also in cell types of innate and adaptive immune system in humans (web 6). Activation of this transcriptional factor leads to expression of hundreds of vitamin D target genes, namely 189 primary target genes playing roles as enzymes, receptors or transporters in human monocytes (Seuter, Neme et al. 2016, Neme, Seuter et al. 2017, Nurminen, Neme et al. 2019; cit. as per Nurminen, Seuter et al. 2019).

The total amount of accessible glucose is a very important parameter for tolDCs development, specifically at the beginning of tolDCs generation process. Vitamin D influences an early transcriptional reprogramming of intracellular metabolic pathways (e.g. PI3K/Akt/mTOR) which control glycolysis and glucose availability. Induction and also maintenance of tolDCs tolerogenic character are strongly dependent on the glucose metabolism (Ferreira, Vanherwegen et al. 2015).

Molecular mechanism of vitamin D on tolDCs development is clarified in Vanherwegen's study (Vanherwegen, Eelen et al. 2019). Vitamin D, specifically the active compound 1,25-dihydroxyvitamin D3 (calcitriol; $1\alpha,25(OH)_2D_3$), positively regulates the activity of glycolytic enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB) 4 on gene expression level. PFKFB4 is probably the primary regulator in the tolerogenicity of $1\alpha,25(OH)_2D_3$-induced DCs. Its expression is significantly increased upon the exposure to $1\alpha,25(OH)_2D_3$ and its activity also raises the glycolytic flux rate (Vanherwegen, Eelen et al. 2019). Some protocols of tolDCs generation use a synthetic analogue vitamin D2 (paricalcitol; 19-nor-1,25-dihydroxyvitamin) instead of vitamin D3. It was documented that both D3 and D2 induce comparable tolerogenic properties in *in vitro* generated tolDCs (Sochorova, Budinsky et al. 2009).

Dexamethasone, with comparable and common anti-inflammatory and immunosuppressive effects as other corticosteroids, also contributes to the generation of the tolerogenic phenotype of tolDCs and is used in many protocols (Kim, Jung et al. 2018). Moreover, tolDCs cultivated with dexamethasone maintain their tolerogenic properties for several days even after dexamethasone is eliminated (Xia, Peng et al. 2005; cit. as per Kim, Jung et al. 2018).

Another tolerogenic agent with an ability to successfully induce a stable tolerogenic character of tolDCs is rapamycin. In comparison with vitamin D-tolDCs or dexamethasone-tolDCs, in cultures of rapamycin-tolDCs no production of IL-10 was detected (Naranjo-Gomez, Raich-Regue et al. 2011). Most likely, this fact reflects that rapamycin induces tolerogenicity by another mechanism compared to vitamins D and dexamethasone.

Some protocols utilized in T1D research for tolDCs propagation used immunosuppressive cytokine IL-10, usually in combination with GM-CSF (Haase, Yu et al. 2010, Tai, Yasuda et al. 2011). Additionally, Tai et al. reported a diabetes-preventive effect of GM-CSF+IL-10 induced tolDCs in NOD mice and in "humanized" transgenic model for T1D, the HLA-DQ8/RIP-B7.1 mouse.

1.1.2.3 TolDCs stability

Barring tolerogenic properties of tolDCs, a necessary and very crucial parameter for this cell-based therapy in T1D (and also in other autoimmune diseases) is to retain their stable tolerogenic phenotype. An undesirable risk of tolDCs consists in their potential ability to change their properties from tolerogenic towards immunostimulatory activity, e.g. typically under proinflammatory conditions; in case of T1D under hyperglycemia which is associated with higher oxidative stress (Grohová, Dáňová et al. 2019).

For final tolDCs differentiation lipopolysaccharide (LPS) or its non-toxic synthetic and well-tolerated analog monophosphoryl lipid A (MPLA) are often used. Anderson et al. detected changes in tolDCs phenotype and function after LPS stimulation, specifically in chemokines expressions (Anderson, Swan et al. 2009). TolDCs treated with MPLA are able to maintain their tolerogenic features, to exhibit high amounts of CCR7 and CXCR4, to induce production of anti-inflammatory cytokines and have a decreased capacity to stimulate CD4+ T cell propagation (Garcia-Gonzalez, Morales et al. 2013).

Nevertheless, still many of the animal protocols test tolDCs without their terminal activation (see **Table 2**). For the clinical translation to humans it is really necessary to take this issue into consideration and include the stabilization in tolDCs protocols.

Table 2: Example protocols of tolDCs in T1D: Generation

TolDC generation	Modification	Culture condition	Stabilization	Ref.
GM-CSF + IL-4		FCS		Lo, Xia et al. 2018
GM-CSF + IL-4	Vit D2/Dex	FBS/ /serum-free	MPLA	Funda, Golias et al. 2018
GM-CSF + IL-4	Microspheres with antisense oligos. CD40/CD80/CD86	FBS		Engman, Wen et al. 2015
GM-CSF + IL-4	Antisense oligos. CD40/CD80/CD86	FBS	LPS	Di Caro, Phillips et al. 2014
GM-CSF + IL-4		FBS/ /serum-free		Looney, Chernatynskaya et al. 2014
GM-CSF		FBS		Pujol-Autonell, Ampudia et al. 2013
GM-CSF GM-CSF + IL-4 GM-GSF + IL-10		FCS		Tai, Yasuda et al. 2011
GM-CSF + IL-4	IL-4 transduced DCs (electroporated)	FCS		Creusot, Chang et al. 2010
GM-CSF + IL-10		FBS/normal mouse serum		Haase, Yu et al. 2010
GM-CSF		FBS		Marin-Gallen, Clemente-Casares et al. 2010
GM-CSF + IL-4		FCS		Lo, Peng et al. 2006
GM-CSF + IL-4	Antisense oligos. CD40/CD80/CD86	FBS	LPS	Machen, Harnaha et al. 2004
GM-CSF + IL-4	IL-4 transduced DCs (adenoviral vector)	FBS		Feili-Hariri et al. 2003

1.1.2.4 Antigen-loading

As shown in **Table 3**, many protocols use loaded tolDCs, but several studies achieved even better diabetes prevention with unloaded tolDCs cultivated without an autoantigen. This issue is discussed with the respect to the most effective and also safe properties of tolDCs in T1D prevention (or eventually in T1D treatment).

A general expectation about tolDCs clinical usage was that loaded tolDCs achieve a more effective and specific qualities. Nevertheless, this common opinion is not supported

by experimental data (Funda, Palova-Jelinkova et al. 2019), also in our study (Funda, Golias et al. 2018). The main requirement for clinical testing is naturally the safety of this cell-based therapy. Contrary to loaded-tolDCs tested *in vitro*, a major risk *in vivo* consists in subsequent alloantigen processing and presentation by endogenous APCs leading to sensitization and priming of CD4$^+$ T cells (Smyth, Ratnasothy et al. 2013). The other unknown parameter of loaded tolDCs is the antigen dose (**Table 3**). Some studies documented that low peptide doses positively correlate with induction of CD4$^+$Foxp3$^+$ T regulatory cells, specifically via a weak TCR signaling pathway (Akt/mTOR pathway). In the opposite case, higher antigen doses provoked strong signaling through the Akt/mTOR pathway resulting in expansion of Foxp3$^-$ Th cells and considerable production of IL-6 by T cells regardless of DC phenotype (Turner, Kane et al. 2009, Long, Rieck et al. 2011).

Table 3: Example protocols of tolDCs in T1D: Ag-specific vs. Ag-unspecific, Ag dose

Loading	Ag dose	Ref.
insulin B9-23 GAD65$_{78–97}$ GAD65$_{260–279}$	3μM	Lo et al.
GAD65 OVA GAD65 peptide no. 35	1 μg or 2 μg/ml	Funda et al.
insulin B9-23	5 μg	Engman et al.
GAD65$_{217–236}$	10 μg/ml	Looney et al.
NIT-1 apoptotic bodies	3 x 10^5 cells	Pujol-Autonell et al.
insulin B9-23 insulin B15-23	10 μg/ml	Haase et al.
NIT-1 apoptotic bodies SV-T2 apoptotic bodies	3 x 10^5 cells	Marin-Gallen et al.
insulin B9-23 proinsulin C19-A3 GAD65$_{78–97}$	3μM	Lo et al.
NIT-1 lyzate	n. a.	Machen et al.

1.1.3 Migration and administration routes

In animal experiments application routes of tolDCs include the intraperitoneal, intravenous, subcutaneous and also intradermal administration (**Table 4**). The preferred target organ of tolDCs in T1D therapy represents pancreatic lymph node which plays a key role in induction of β cell specific effector or tolerogenic immune responses (Clare-Salzler, Brooks et al. 1992,

Gagnerault, Luan et al. 2002, Jaakkola, Jalkanen et al. 2003) and serves a pivotal function in this potential cell-based therapy. Older but very elegant study by Creusot et al. has documented *in vivo* homing of BMDCs after intravenous and intraperitoneal applications. The intraperitoneal delivery led to a predominant accumulation of BMDCs in pancreatic lymph nodes in comparison with the intravenous injection which resulted in an accumulation not only in pancreatic lymph nodes but also in spleen (Creusot, Yaghoubi et al. 2009). The two clinical trials with tolDCs in T1D have employed the intradermal administration route (Roep et al. 2015, Giannoukakis, Phillips et al. 2011), other current human trials with tolDCs have also tested the intravenous and even the intraperitoneal administrations (Phillips, Garciafigueroa et al. 2017).

As seen in **Table 4**, numbers of cell doses in example protocols are very different and vary from 1 x 10^5 to 3 x 10^6 by either one or several applications at weekly intervals.

Regarding the *in vivo* survival of tolDCs, in the already mentioned study, tolDCs were reported to survive for 1–2 weeks *in vivo* after injection (Creusot, Yaghoubi et al. 2009). Certainly, administration routes may significantly affect tolDCs survival.

As described above, tolerogenic properties are commonly induced by vitamin D. It was documented that 1,25D$_3$-modulated DCs maintain their migratory capacity and are able to drift into pancreatic lymph nodes and decrease T cell proliferation *in vivo* (Ferreira, Gysemans et al. 2014). The important role of PLNs in T1D was revealed when these lymph nodes were surgically removed at the age of three weeks which led to the prevention of T1D development in NOD mice (Gagnerault, Luan et al. 2002).

Expression of the C-C chemokine receptor 7 (CCR7), a member of G protein-coupled receptor family, has been reported as an important parameter for tolDCs trafficking and migration into mucosal immune compartment (including pancreatic lymph node) (Jang, Sougawa et al. 2006, Worbs, Hammerschmidt et al. 2016).

This migration pattern is decreased by tolerogenic agents (e.g. dexamethasone and vitamin D3) and also by IL-10. On the other hand, MPLA terminal differentiation increases expression of CCR7 and CXCR4 (Garcia-Gonzalez, Morales et al. 2013). The functional migratory properties are also enhanced after IL-4 exposure toward the end of GM-CSF cultivation of tolDCs (Yin, Wang et al. 2011).

1.1.4 Prevention and treatment

Most of tolDCs protocols monitored an impact of tolDCs on diabetes prevention but some of them tested a potential influence of tolDCs on advanced insulitis in NOD mice. Furthermore, some studies also tested the ability of tolDCs to cure T1D or restore normoglycaemia in already diabetic NOD mice. Representative studies and data are attached in **Table 4**.

Table 4: Example protocols of tolDCs in T1D: Routes of administration, cell dose, prevention vs. treatment

App. route	Scheme	Cell dose	Model	Age	Prevention	Pre-diabetic	Cure	References
s. c. (footpad)	3 times, weekly or 3 times, weekly + every other week	1×10^5	NOD	9 weeks	yes	yes		1
i. p.		3×10^6	NOD NOD-SCID	7–8 weeks	yes			2
s. c.	8 times, weekly	2×10^6	NOD	newly-diagnosed diabetes confirmed*			yes	3
s. c. (abdominal)	single or 8 times, weekly	2×10^6	NOD	8–18 weeks diabetes confirmed*			yes	4
i. v.	5 times, weekly	1×10^5	NOD	8–9 weeks	yes			5
i. p.		1×10^6	NOD	diabetic**			no	6
i. p., i. v. i. v.	3 times, weekly	1×10^6 3×10^6	NOD-DQ8/RIP-B7.1 NOD-SCID	5–8 weeks	yes			7
i. v.		1×10^6	NOD	12 weeks (prevention experiments) 11–30 weeks		yes	yes	8
i. p.		1×10^6	NOD	5–12 weeks	yes			9
i. p.		1×10^6	NOD RIP-IFN.β	2 weeks	yes			10
s. c. (footpad)	3 times, weekly	1×10^5	NOD	9 weeks	yes			11
i. p.		2×10^6	NOD	7 weeks	yes			12
i. v.	single or 2 times, weekly	$4\text{-}5 \times 10^5$	NOD	5–8, 10 or 15 weeks	yes	yes		13

*two consecutive readings of tail vein blood glucose >300 mg/dL;
**blood glucose level >200 mg/dL, or when a measure >360 mg/dL

Importantly, there is a very limited amount of tolDCs interventions which successfully cured T1D in NOD mice (except a strong immunosuppression). Thus, tolDCs represent a very promising tool also for early T1D therapy (**Table 4**).

However, numerous questions in this area remain unanswered and it is truly necessary to optimize tolDCs protocols first of all in animal models for a successful and most effective application in clinical settings.

1.2 Type 1 diabetes

Type 1 diabetes is an autoimmune disease caused by a specific destruction of pancreatic β cells by infiltrating autoreactive CD4^{+} and CD8^{+} T cells. The primary trigger of this pathological process is still unknown but a key role in activation of T lymphocytes during T1D development may play probably B lymphocytes (Egia-Mendikute, Arpa et al. 2019). This pathological cellular invasion leads in reduction of β cell mass and results in a relative or absolute insulin deficiency.

At this moment, a typical classification of diabetes in the two main types – type 1 diabetes and type 2 diabetes – is slightly broken, moreover, type 2 diabetes represents a highly heterogeneous disease. Because of distinct disease progression and risk of diabetic complications, individuals with diabetes can be clustered in five subgroups and T1D is defined as GADA (glutamic acid decarboxylase antibodies) positive and C-peptide concentrations <0,3 nmol/L (Ahlqvist, Storm et al. 2018). At time of writing, last updated data indicated a steady increase in cases of T1D (3%–5% annually) (IDF Diabetes Atlas 8th Edition).

Genetic background of T1D is not fully clarified, however, there are currently identified more than 50 genetic loci that relate with onset of this disease (Storling and Pociot 2017). An appreciable part that accounts for T1D development is the HLA locus located on chromosome 6, specifically alleles HLA-DRB1*03 or HLA-DRB1*04 with DQB1*03:02 (Nguyen, Varney et al. 2013). Other discussed genetic variabilities barring the HLA polymorphism are polymorphisms within the insulin gene located on chromosome 2, CTLA-4 gene located on chromosome 2 (Wang, Liu et al. 2014), and PTPN22 (protein tyrosine phosphatase non-receptor type 22) gene located on chromosome 1 (Prezioso, Comegna et al. 2017).

The tendency of constantly increased number of T1D diagnoses, specifically in developed countries, in last decades cannot be completely attributed to genetics. Discussed are many factors, briefly e.g. relationship with gut microbiome (Siljander, Honkanen et al. 2019) and gut microbial metabolites (Marino, Richards et al. 2017), exposure to environmental chemicals (Howard 2019) or perinatal risk factors (Waernbaum, Dahlquist et al. 2019).
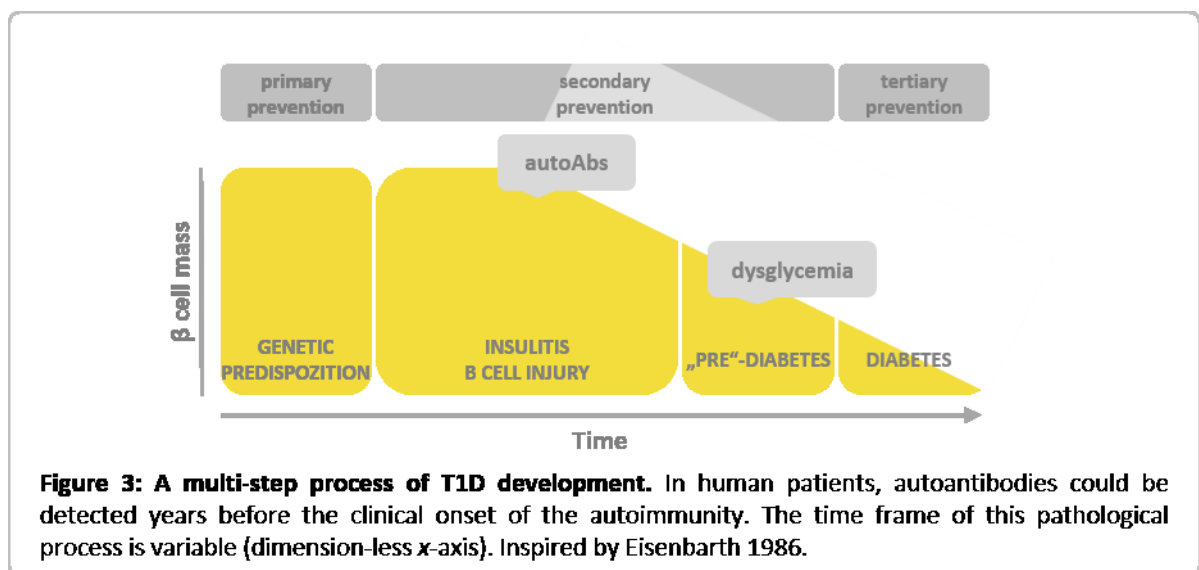
1.2.1 Other drivers of T1D

A quite newly discussed field in autoimmune diseases, specifically in the initiation of autoimmunity, are the post-translationally modified epitopes (McGinty, Marre et al. 2015). An increasing evidence suggests that recognition of neo-epitopes by autoreactive T cells contributes to the β cell destruction in T1D. Specifically in T1D, many modifications are described for generation of neo-epitopes, e.g. modifications created by antigen processing and presentation, deamination or aberrant mRNA translation (Kracht, van Lummel et al. 2017, Gonzalez-Duque, Azoury et al. 2018, Mannering, Di Carluccio et al. 2019). For T1D therapy, neo-epitopes as the drivers of autoimmunity may be an interesting and promising sphere of interest for Ag-specific tolDCs therapy.

In a recent paper from Ahmed et al. are as the potential drivers of autoimmune T1D discussed the unique lymphocytes that are called dual expressers (DEs) because of their co-expression of both BCR and TCR and lineage markers of B and T cells. In addition to that these cells disprove the elementary compartmentalization of adaptive immune cells into T and B cells, these cells are significantly more frequently found in peripheral blood of T1D patients compared to healthy controls. Moreover, DEs encode an effective autoantigen which can form functional complexes with DQ8 molecule and stimulate autoreactive diabetic CD4 T cells. Finally, cytotoxic CD4 T cells could also be activated by idiotypic mAbs produced evidently by DEs (these observations were also described in other autoimmune diseases (Aas-Hanssen, Funderud et al. 2014, Ahmed, Omidian et al. 2019).

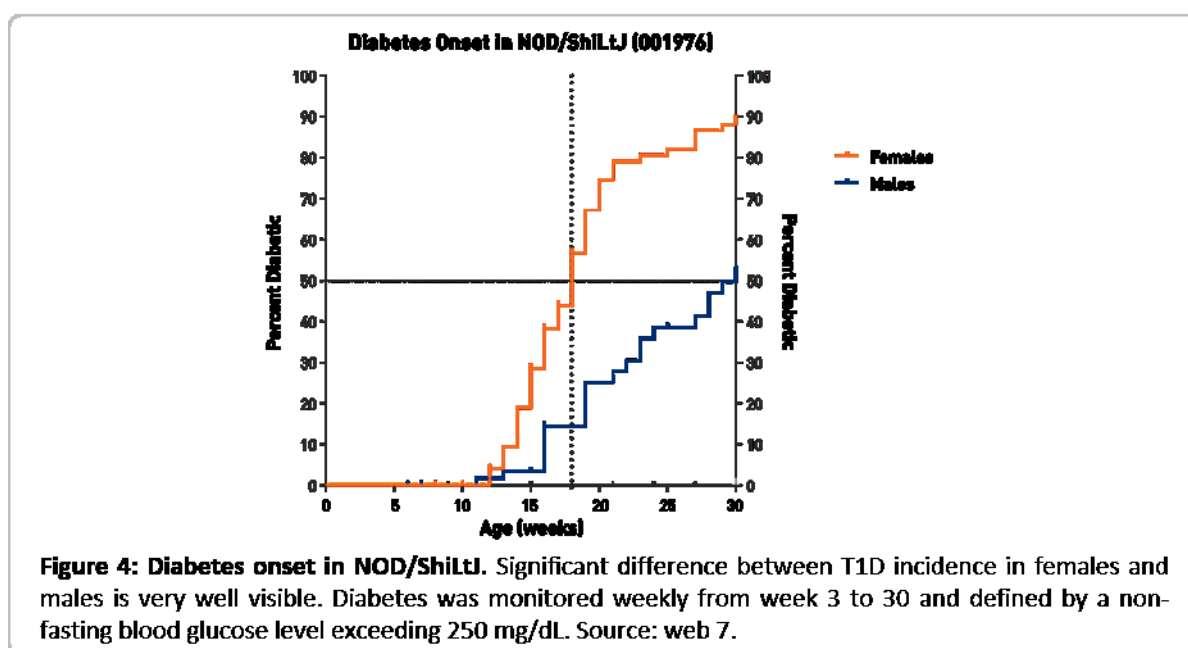### 1.2.2 Current therapy and future perspectives

T1D development is a multi-step pathological process (**Figure 3**) and the model was at first fully described in 1986 by Eisenbarth (Eisenbarth 1986). At this moment, the standard T1D therapy is an exogenous insulin replacement that is used as the first-line therapeutic option (Pathak, Pathak et al. 2019). In general, it is well known that the autoantibodies in T1D can be detected years before the clinical onset. Since the recent advancement to identify the *at-risk* individuals to progress to the clinical onset of T1D, the cell-based tolerogenic therapy is a suitable candidate for prevention of the disease in such high-risk individuals.

Figure 3: A multi-step process of T1D development. In human patients, autoantibodies could be detected years before the clinical onset of the autoimmunity. The time frame of this pathological process is variable (dimension-less *x*-axis). Inspired by Eisenbarth 1986.

Excepting tolDCs, as the other potential cell-based therapies in T1D is discussed the usage of T regulatory cells and stem cells. At time of writing, two clinical trials with autologous Tregs (Bluestone, Buckner et al. 2015, Marek-Trzonkowska, Myśliwiec et al. 2016), autologous umbilical cord blood as a source of immunomodulatory cells (Haller, Wasserfall et al. 2011, Zhao, Jiang et al. 2012, Delgado, Perez-Basterrechea et al. 2015) are completed with data describing their safety and good tolerance. The other alternative for exogenous insulin replacement is the islet transplantation which is unfortunately related with negative side effects (Pathak, Pathak et al. 2019). There is also a promising evidence that the tolerance in new-onset T1D patients may be reestablished by autologous hematopoietic stem cell transplantation with primary end-point an insulin-independency, not a delayed loss of insulin (van Megen, van 't Wout et al. 2018).

1.2.3 Animal models

The NOD mouse represent a spontaneous and at the same time a dominant experimental animal model in T1D. It displays many important similarities with human T1D but also some differences. The main advantages are the spontaneous development of T1D and relevant genetic background to the human disease. Comparable to the human parameters of T1D, NOD mice also display an increased level of circulating autoreactive T cells and develop autoantibodies against analogous β cell autoantigens. Nevertheless, the murine initiating autoantigen seems to be insulin compared to several initiating human autoantigens. Other distinct parameter is the histological character of insulitis which is more severe and frequent compared to humans. T1D in NOD model manifests with an incomplete penetrance that well corresponds with the contribution of environmental factors in T1D (Funda, Palova-Jelinkova et al. 2019).

Figure 4: Diabetes onset in NOD/ShiLtJ. Significant difference between T1D incidence in females and males is very well visible. Diabetes was monitored weekly from week 3 to 30 and defined by a non-fasting blood glucose level exceeding 250 mg/dL. Source: web 7.

While in human patients, autoantibodies can be detected in the blood years before the clinical onset of the autoimmunity, in NOD the onset of T1D is well predictable based on the age of mice and mouse autoantibodies are commonly detected around 8–10 weeks and the earliest onset of disease around 12 weeks. Besides, incidence of T1D in this experimental model depends on various factors, e.g. on the microbiome composition, diet or gender as shown in **Figure 4** (90–100% incidence in females by 30 weeks of age, 40–60% in males by 30–40 weeks of age) (web 7).

Other experimental animal model used in T1D research is the NOD-SCID mouse (homozygous for the severe combined immune deficiency spontaneous mutation $Prkdc^{scid}$) employed for adoptive co-transfer experiments because of very good xeno- or allogenic accepting. Although this model is artificial, an indisputable advantage is much shorter time for diabetes incidence monitoring compared to NOD mouse.

Finally, the other spontaneous model of T1D is the lymphopenic BB rat. Compared to NOD mice, BB rats are significantly more sensitive to ketoacidosis and diabetes onset occurs at 7–14 weeks of age (Pearson, Wong et al. 2016). Several other transgenic and knock-out mice, often on the NOD background, have also been developed. To bring testing closer to clinical trials, transgenic humanized mice have been employed, e.g. HLA-DQ8/RIP-B7.1 or HLA-DR4 mice (Tai, Yasuda et al. 2011, Gibson, Nikolic et al. 2015).

2. Aims

In this thesis, we focus on studying tolerogenic dendritic cells, more specifically to optimize some of their features as a promising cell-based intervention strategy in type 1 diabetes using NOD and NOD-SCID mouse models. For this purpose, we have been investigating:

- phenotype features of tolDCs,
- migratory capacity of tolDCs and their capability to target the critically important pancreatic lymph nodes,
- comparison of application routes,
- diabetes-preventive effect of tolDCs with respect to application routes.

3. Methods

1.2.4 Animal models

Female NOD and NOD-SCID mice were purchased from Taconic (Albany, NY, USA), whereas female BALB/c mice were obtained from the animal facility of the Institute of Microbiology, Czech Academy of Science, Prague, Czech Republic and used in experiments as described below. The mice were maintained in the specific pathogen-free animal facilities under standard light- and climate-controlled conditions, fed standard Altromin 1414 diet, and water was provided *ad libitum*.

1.2.5 DCs generation

The femur and tibia removed from 8–12-week old female NOD mice were cleaned of muscle and connective tissues. The bone marrow was irrigated with a syringe/needle combination by a saline solution (Ardeapharma). A minor part of isolated cells was labeled with Trypan Blue solution (0,4%; Sigma-Aldrich) and cells were counted for absolute live cells quantity. Subsequently, cells were cultured (37°C, 5% CO_2) in Petri cell-culture dishes in complete L-glutamine containing RPMI-1640 (Lonza) supplemented with heat-inactivated fetal bovine serum (FBS; 10%; Gibco-Life Technologies), NEM non-essential amino acid solution (100x; Sigma-Aldrich), sodium pyruvate (100 mM; Sigma-Aldrich), penicillin-streptomycin (100x; Sigma-Aldrich) and 2-β-mercaptoethanol (50μM; Serva). Cells were plated at a density of 4 x 10^6 cells/10 mL in the above described medium. To promote differentiation into DCs, BMDCs were proliferated in the presence of GM-CSF (20 ng/mL; PeproTech) and IL-4 (4,5 ng/mL; PeproTech). Fresh medium (10 mL) was added on day 3. At day 6, half (10 mL) of the medium was replaced with fresh medium with cytokines, collected cells were counted and resuspended in 10 mL of fresh medium and added back into the culture.

Tolerogenic DCs were induced by dexamethasone (1μM; Medochemie) and vitamin D2 (1,5 ng/mL; Zemplar) on day 0, 3, 6 and 7 (**Table 5**). On the contrary, immature and control DCs were generated without these tolerogenic agents. At day 7, nonadherent cells were collected, washed, counted and plated at a density of 1x10^6 cells/mL in fresh medium on 6-well plates. After 4 hours, cells were finally stimulated with MPLA (1 μg/mL; InvivoGen) for 22 hours. At the end of the cell cultivation, the nonadherent cells were collected, counted for absolute live cells, and in the same culture medium immediately processed for follow-up experiments.

Table 5: Scheme of distinct tolDCs protocols

	day 0	day 3	day 6	day 7
iDC				
cDC				MPLA
tDC short			VIT D2, DEX	VIT D2, DEX, MPLA
tDC middle		DEX	VIT D2, DEX	VIT D2, DEX, MPLA
tDC long	VIT D2, DEX	VIT D2, DEX	VIT D2, DEX	VIT D2, DEX, MPLA

1.2.6 Flow cytometry

Cells were stained with the following fluorochrome-conjugated antibodies:

Table 6: List of fluorochrome-conjugated antibodies for flow cytometry

Maturation markers					
Marker	CD11c	CD40	CD80	CD86	MHC II
Antibody	APC	PerCP-eFluor 710	FITC	PE	PE-cyanine7
Clone	N418	1C10	16-10A1	GL1	M5/114.15.2
Manufacturer	eBioscience	eBioscience	eBioscience	eBioscience	eBioscience
DC migration					
Marker	CD11c	CD3			
Antibody	Alexa Fluor 700	Alexa Fluor 488	Hoechst33342	PKH26	
Clone	N418	145-2C11			
Manufacturer	Invitrogen	eBioscience	Sigma-Aldrich	Sigma-Aldrich	
CCR7 expression					
Marker	CD11c	CD40	CD83	CD86	CCR7
Antibody	PE-cyanine7	PerCP-eFluor 710	eFluor 660	FITC	PE
Clone	N418	1C10	Michel-17	GL1	4B12
Manufacturer	eBioscience	eBioscience	eBioscience	eBioscience	Invitrogen

Hoechst was used for exclusion of dead cells. Data were acquired by LSR II flow cytometer (BD Bioscience) and samples were analysed by FlowJo software (Tree Star).
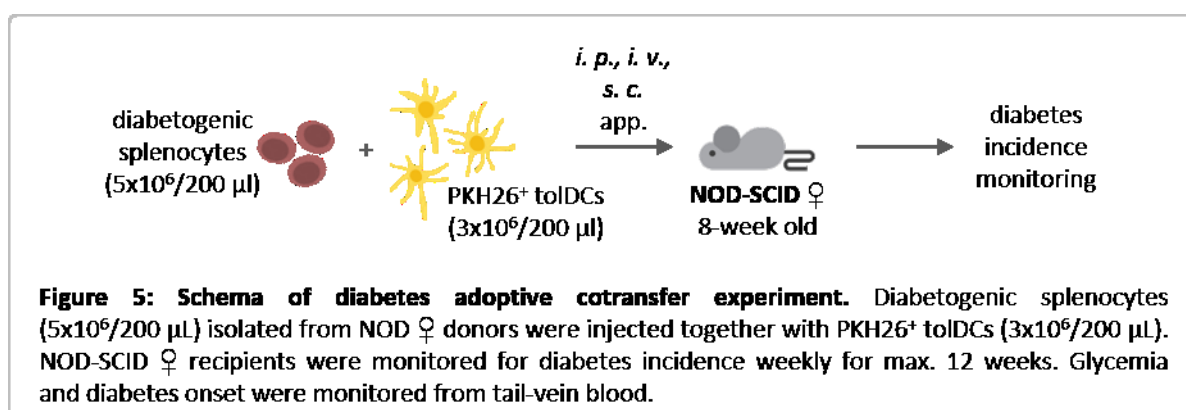
1.2.7 DCs migration

Generated tolDCs were labelled with PKH26 Red Fluorescent Cell Linker kit (Sidma-Aldrich) for *in vivo* migration monitoring according to the official manufacturer's protocol. Briefly, cells generated as described above were firstly washed using medium without serum and subsequently gently resuspended in Diluent Solution C (1mL/1x10^7 cells) and then stained with PKH26 (4µl PKH26/1 mL diluent C) for 5 min at room temperature with periodic mixing leading to successful dispersion. The staining reaction was stopped by addiction of medium containing FBS. After centrifugation, cells were washed and centrifugated three times to ensure the removal of unbound dye. Labelled tolDCs were applied i. p. and s. c. (both right and left sides of the body) to 4–6-week-old NOD females at dose of 4x10^6/200 µL. The control group was injected with the same dose of unlabelled tolDCs.

Cell suspensions from spleen (free of red blood cells by Red Blood Cell Lysing Buffer Hybri-Max [Sigma-Aldrich]), mesenteric lymph nodes, pancreatic lymph nodes and systemic inguinal lymph nodes were prepared after 1, 3, 5, 7 and 9 days of injection, and live PKH26$^+$CD11$^+$ were detected by flow cytometry.

1.2.8 Adoptive co-transfer, diabetes monitoring

Eight-week-old NOD-SCID females were used as recipients in the adoptive co-transfer experiment for monitoring of diabetes incidence (**Figure 5**).

Figure 5: Schema of diabetes adoptive cotransfer experiment. Diabetogenic splenocytes (5x10^6/200 µL) isolated from NOD ♀ donors were injected together with PKH26$^+$ tolDCs (3x10^6/200 µL). NOD-SCID ♀ recipients were monitored for diabetes incidence weekly for max. 12 weeks. Glycemia and diabetes onset were monitored from tail-vein blood.

Diabetogenic splenocytes were isolated from 12-week-old prediabetic NOD female donors. After 8 days tolDCs cultivation, live tolDCs resuspended in medium in the final concentration of 3x10^6/200 µL were injected i. p., s. c. (both right and left sides of the body) and i. v., and diabetogenic splenocytes (free from red blood cells) were injected i. p. in the final concentration of 5x10^6/200 µL. The control group was only injected with 5x10^6/200 µL diabetogenic

splenocytes. All NOD-SCID recipients were monitored for diabetes incidence weekly for max. 12 weeks. Glycemia and diabetes onset were monitored from tail-vein blood with the glucometer Freestyle Lite (Abbott Diabetes Care). Diabetes diagnosis was based on two consecutive blood glucose readings >12 mM in three days (the first reading was then used as the date of diabetes onset).

1.2.9 Microscopy

At day 8, unlabelled DCs generated as described above were observed by confocal microscope (OLYMPUS FV-1000) in complete medium in 6-well plate with magnification 20x.

For the visualization of migrated tolDCs, generated tolDCs (on the basis of "middle" protocol) were labelled with PKH26$^+$ and injected intraperitoneal ($10 \times 10^6/200$ μl) in NOD ♀ mice. At day 3 of tolDCs application, pancreatic lymph node was gently removed from mice and placed to formaldehyde (3,6%). After that, tissue was transferred to saccharose (30%; 24 hours) and then putted in freezer. Organ was sliced on 10μm sheets, labelled with DAPI (Sigma-Aldrich) and scanned with slide scanner Axio Scan.Z1 (Zeiss). Samples were analysed by ZEN software (Zeiss).

1.2.10 Statistical analyses

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). The one-way ANOVA followed by Tukey's multiple comparison post-test were used for assessment of data from multiple measurements from multiple groups. Differences were considered statistically significant when p-value was < 0.05. The cumulative diabetes incidence was assessed using the Kaplan–Meier estimation and contingency tables. The p-values were compensated for multiple comparison (Bonferroni) of survival curves for each experiment.
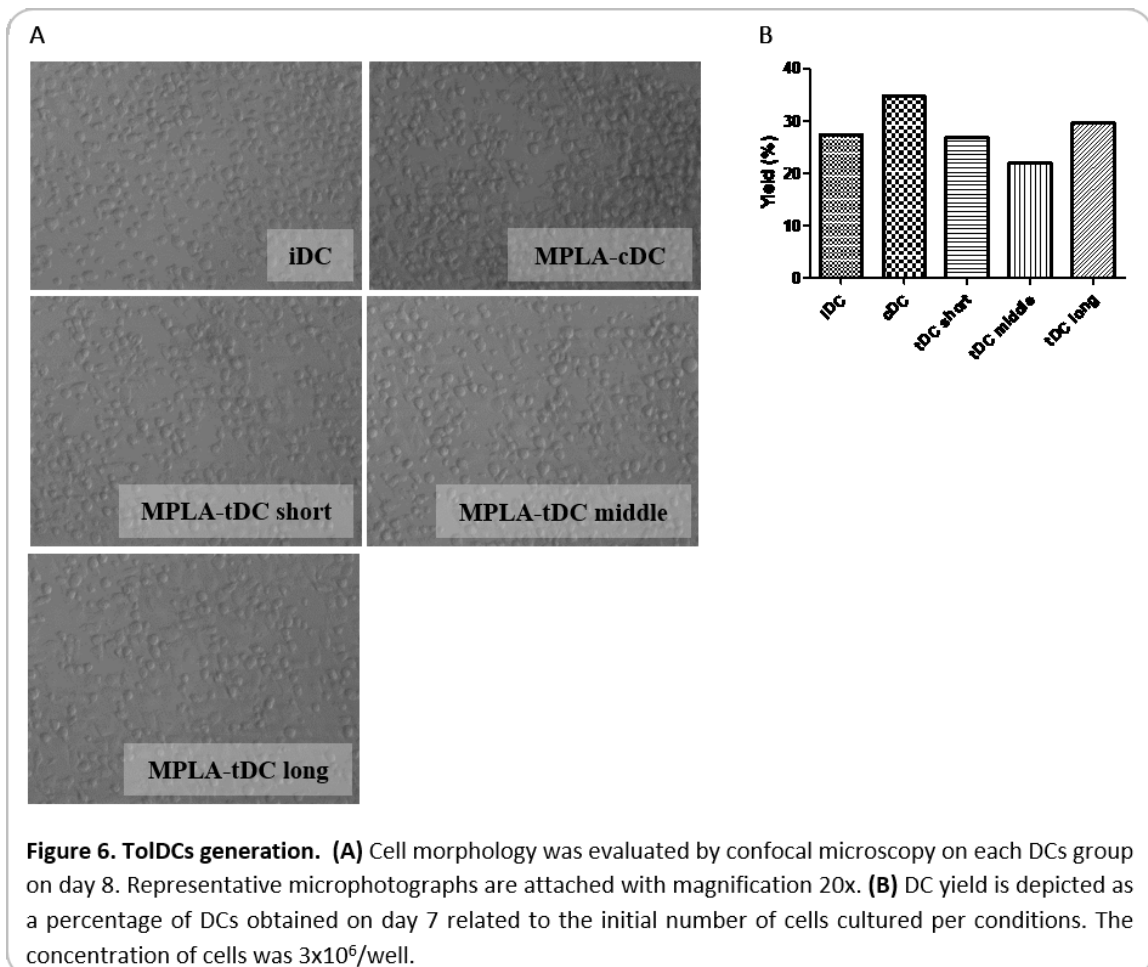
4. Results

1.3 TolDCs generation

The level of expression of co-stimulatory and co-inhibitory molecules plays a crucial role in tolerogenic effects of tolDCs. Therefore, the first aim of our experiments was to compare three distinct protocols of tolDCs generation with regard to tolDCs tolerogenic properties and tolerogenic phenotype. As described above, tolDCs were generated from bone marrow progenitors from NOD or BALB/c mice under IL-4 and GM-CSF conditions. For the induction of tolerogenic character of tolDCs, we used vitamin D2 together with dexamethasone on day 0, 3, 6 and 7. Cells were stabilized by terminal activation by MPLA on day 7 for 22 hours. (**Table 5**)

1.3.1 Cell morphology

After DCs propagation on day 8, cells displayed a round shape without any visible phenotypic variations (**Figure 6A**). DCs cellular yield was not substantially affected by distinct differentiation conditions (example shown in **Figure 6B**).

Figure 6. TolDCs generation. **(A)** Cell morphology was evaluated by confocal microscopy on each DCs group on day 8. Representative microphotographs are attached with magnification 20x. **(B)** DC yield is depicted as a percentage of DCs obtained on day 7 related to the initial number of cells cultured per conditions. The concentration of cells was 3×10^6/well.

1.3.2 Maturation markers

A phenotypic characterization of distinct DCs groups was analyzed by flow cytometry with respect to distinct culture protocols and the resulting expression of co-stimulatory (CD80, CD86), antigen presenting (MHC II) and functional activator (CD40) molecules. Because of a present baseline expression of the maturation markers, data were expressed and assessed as histograms and changes in mean fluorescence intensity.

As shown in **Figure 7**, diverse DCs groups exhibited distinct patterns of maturation markers reflecting their immature, mature or semi-mature tolerogenic character. Specifically, tolDCs expressed in general a lower level of CD40, CD80 and CD86 compared to control mature DCs regardless of the culture protocol.

As regards the CD40 expression, it was substantially increased on mature cDCs in comparison to both immature and all groups of tolerogenic DCs. Cells propagation with a short exposure to tolerogenic agents led to a less effective downregulation of CD40 compared to the longer-lasting protocols. In parallel, CD80 expression achieved a similar level in both middle and long exposure of tolDCs to tolerogenic inducers, while on mature and as well short-exposed tolDCs was the CD80 expression upregulated. There were no considerable differences in CD86 expression on all types of tolDCs, only it was upregulated on mature DCs. A comparable pattern was detected in expression of MHC II in all groups of DCs regardless of the immature, semi-mature or mature character.

Taken together, these data show that maturation markers are in general decreased on tolDCs compared to cDCs, and it corresponds with the semi-mature stage of their phenotypic differentiation. Moreover, changes in fluorescence intensity show that a short exposure to tolerogenic agents, specifically to vitamin D and dexamethasone, leads to less optimal downregulation of t expression of maturation markers on tolDCs.
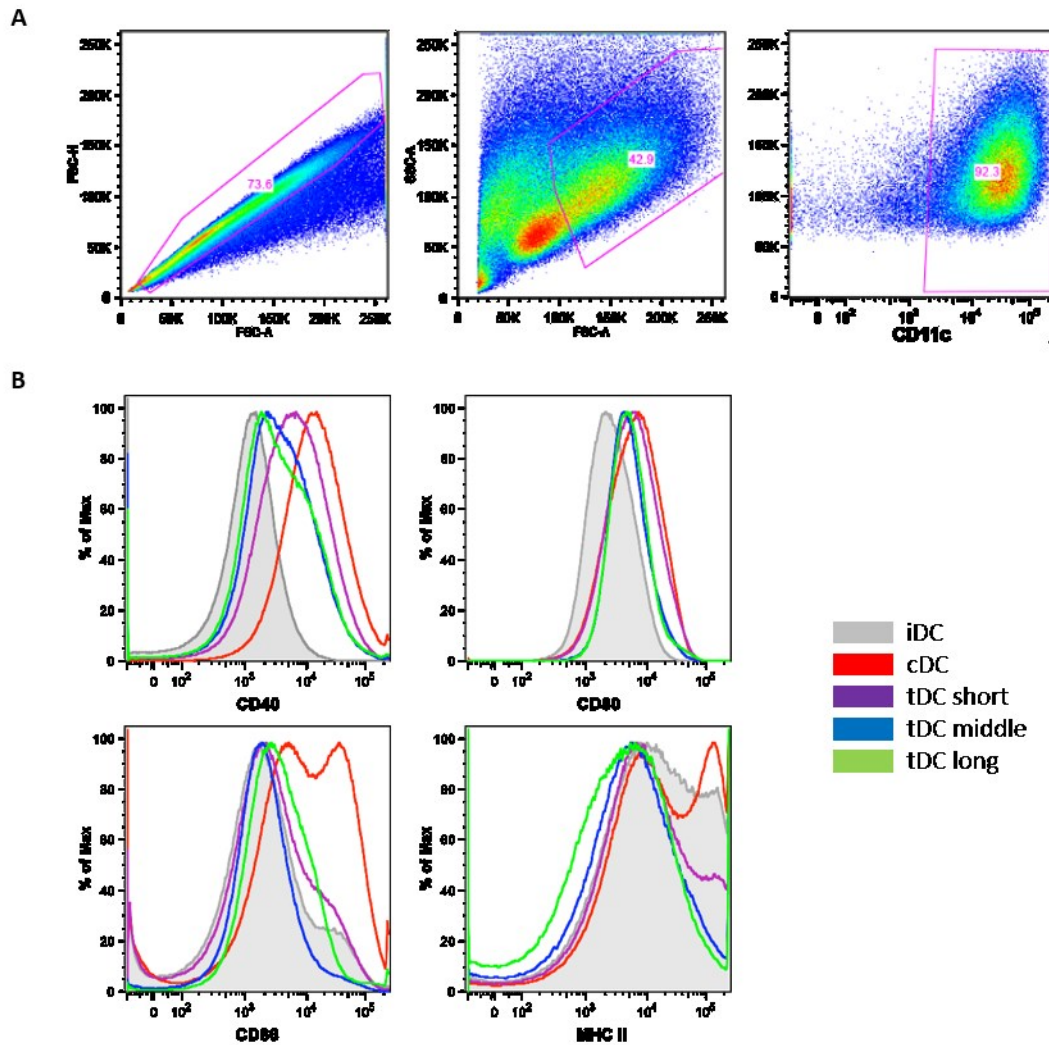
Figure 7: Expression of maturation markers in immature, semi-mature and mature DCs. Cells were generated from bone marrow of 6-months-old BALB/c ♀ mice on the basis of three distinct culture protocols (short, middle, long). After generation, the level of maturation markers (CD80, CD86, CD40 and MHC II) expression was determined by surface staining of live cells and flow cytometry.
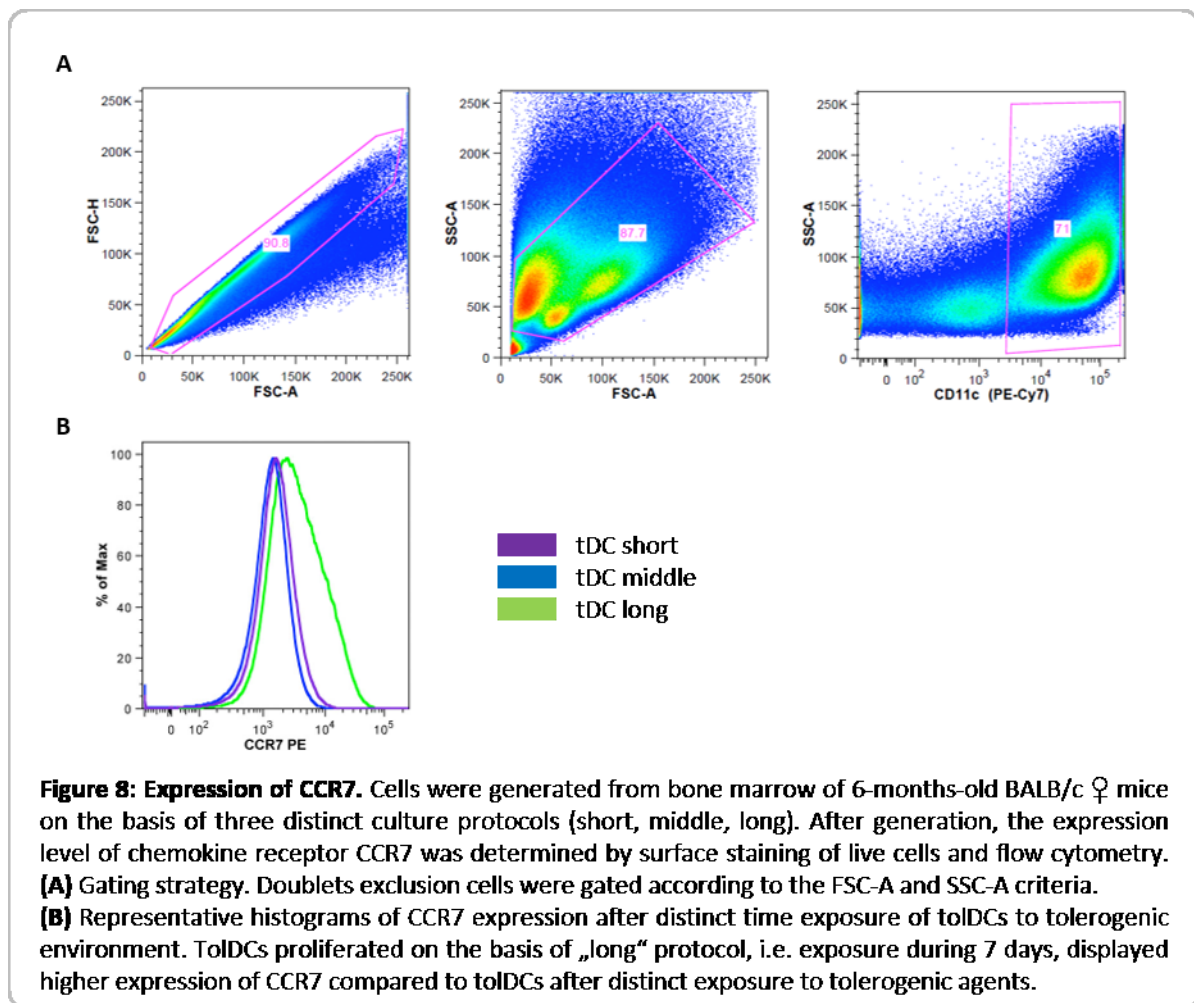
(A) Gating strategy. Doublets exclusion cells were gated according to the FSC-A and SSC-A criteria.

(B) Representative histograms of each marker show decreased expression level of cellular markers on tolerogenic DCs compared to mature DCs. Untreated DCs were used as immature controls.

1.3.3 CCR7 expression

One of the advantageous and promising properties of tolDCs for cell-based therapy is their capability to migrate more specifically to secondary lymphoid organs. Because tolDCs trafficking depends on chemokines gradients and expression of chemokines receptors, we investigated if distinct culture protocols affect expression of chemokine receptor 7, that is critical for migration to the mucosal lymphoid compartment, including pancreatic lymph nodes.

While both "short" and "middle" culture protocols displayed comparable levels of CCR7 expression, the "long" culture protocol affected surface expression of this molecule (**Figure 8**). TolDCs generated by the longest time exposure to tolerogenic agents during the 7- day protocol displayed remarkably higher expression of CCR7. Finally, these data indicate that distinct culture protocols influence not only the expression of tolDCs surface markers (CD80, CD86, CD40 and MHC II) but also chemokine receptor CCR7 that is important for tolDCs homing to mucosal lymphoid organs.

Figure 8: Expression of CCR7. Cells were generated from bone marrow of 6-months-old BALB/c ♀ mice on the basis of three distinct culture protocols (short, middle, long). After generation, the expression level of chemokine receptor CCR7 was determined by surface staining of live cells and flow cytometry.
(A) Gating strategy. Doublets exclusion cells were gated according to the FSC-A and SSC-A criteria.
(B) Representative histograms of CCR7 expression after distinct time exposure of tolDCs to tolerogenic environment. TolDCs proliferated on the basis of „long" protocol, i.e. exposure during 7 days, displayed higher expression of CCR7 compared to tolDCs after distinct exposure to tolerogenic agents.

1.4 TolDCs migration

1.4.1 Preferential accumulation of tolDCs in lymphoid organs

Our next experiments aimed to compare distinct administration routes of tolDCs, specifically the intraperitoneal and subcutaneous (on the both right and left sides of the belly) routes in NOD female recipients and to follow trafficking of tolDCs to preferential lymphoid organs. For this purpose, we used the "middle" cultivation protocol, i.e. tolerizing environment was created at day 3 of cells generation (**Table 5**), that has been used in other projects, including human tolDCs. (Dáňová, Grohová et al. 2017)

Before staining for flow cytometry, samples of homogenized tissues were counted for total number of cells recovered from relevant organs after intraperitoneal (**Figure 9A**), subcutaneous left (**Figure 9B**) and subcutaneous right (**Figure 9C**) applications. The increase in total number of cells recovered from spleen, mucosal (MLN and PLN) and systemic (ILN and ALN) lymphoid organs was used to asses DC-mediated local development of immune responses.
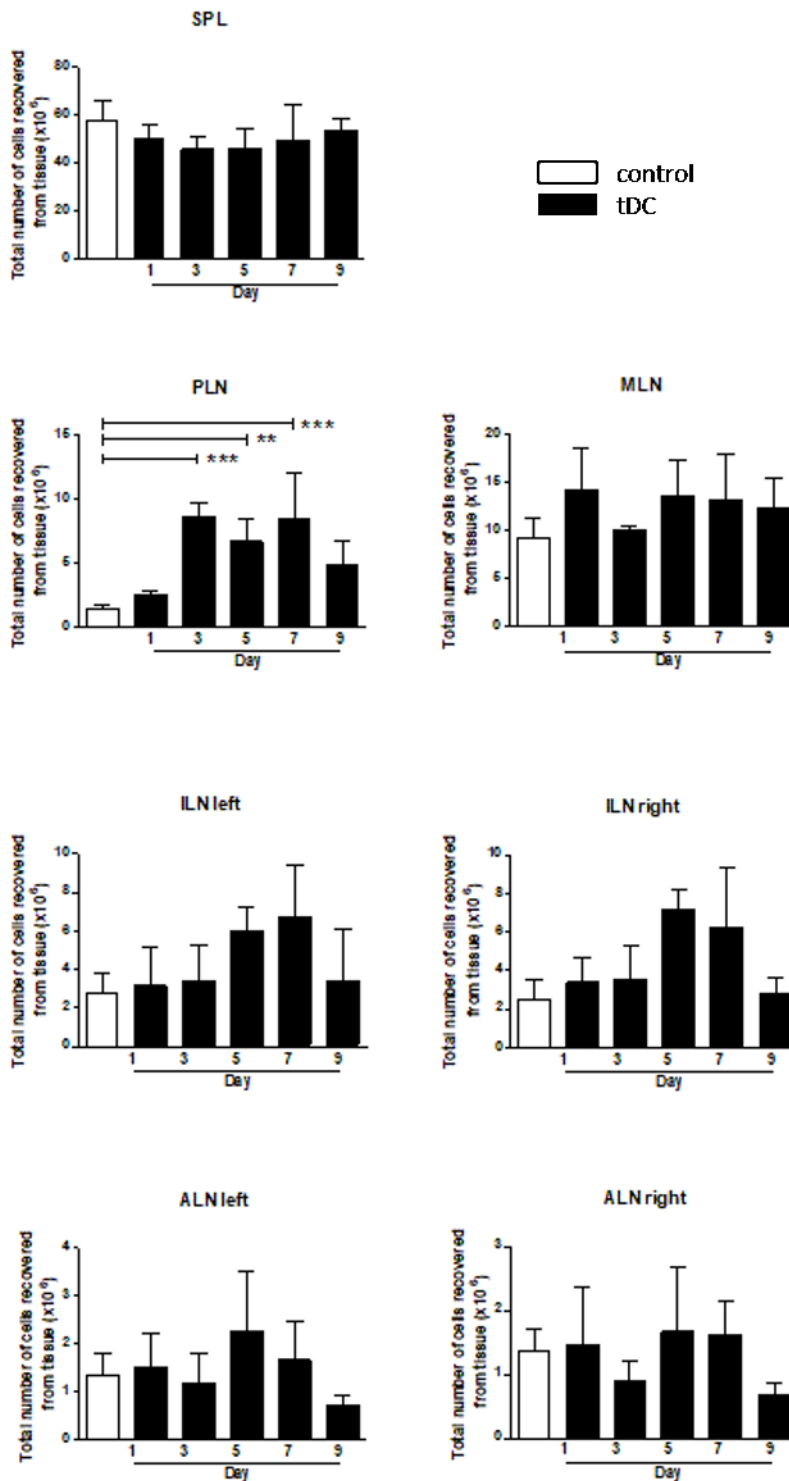
Figure 9A: Total number of cells recovered from tissues after intraperitoneal application.
TolDCs generated on the basis of „middle" protocol were injected i. p. right in NOD ♀ recipients. At day 1, 3, 5, 7, and 9 after tolDCs application, SPL, PLNs, MLNs, ILNs left, ILNs right, ALNs left and ALNs right (5 mice per group) were removed and homogenized. After that, cells recovered from relevant tissues were counted. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.
SPL, spleen; PLNs, pancreatic lymph nodes; MLNs, mesenteric lymph nodes; ILNs, inguinal lymph nodes; ALNs, axillary lymph nodes.
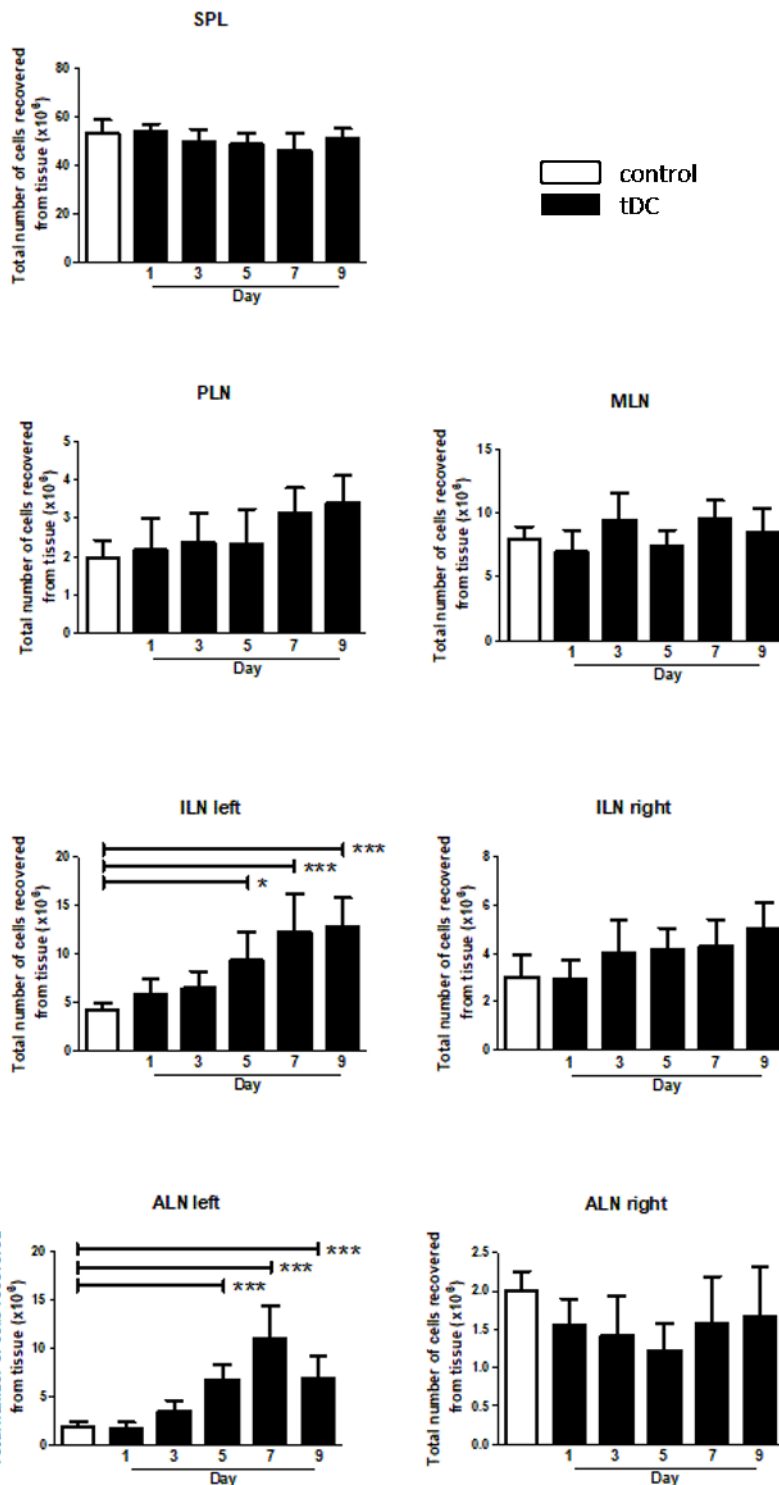
Figure 9B: Total number of cells recovered from tissues after subcutaneous application (left).
TolDCs generated on the basis of „middle" protocol were injected s. c. left in NOD ♀ recipients. At day 1, 3, 5, 7, and 9 after tolDCs application, SPL, PLNs, MLNs, ILNs left, ILNs right, ALNs left and ALNs right (5 mice per group) were removed and homogenized. After that, cells recovered from relevant tissues were counted. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
SPL, spleen; PLNs, pancreatic lymph nodes; MLNs, mesenteric lymph nodes; ILNs, inguinal lymph nodes; ALNs, axillary lymph nodes.
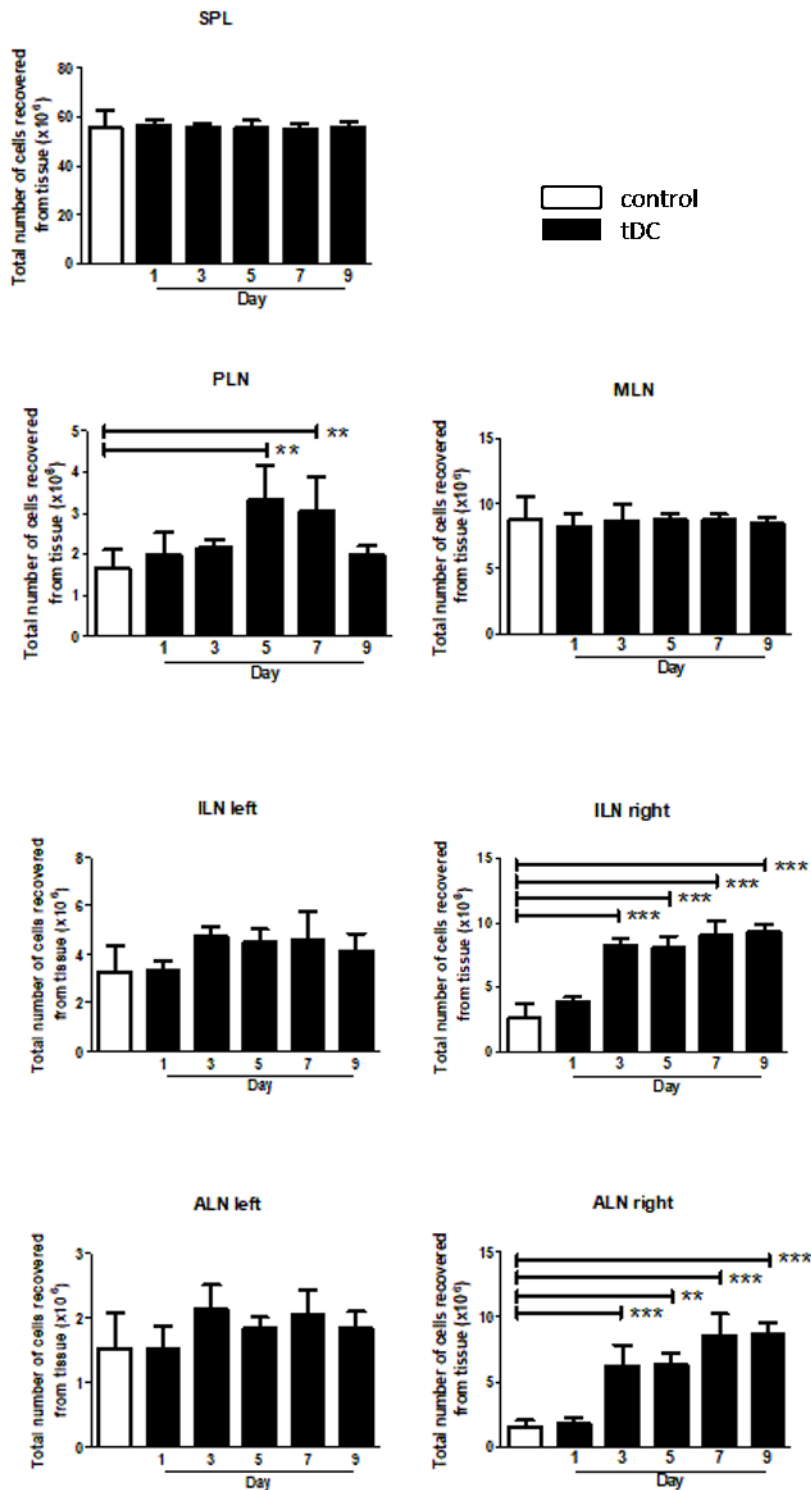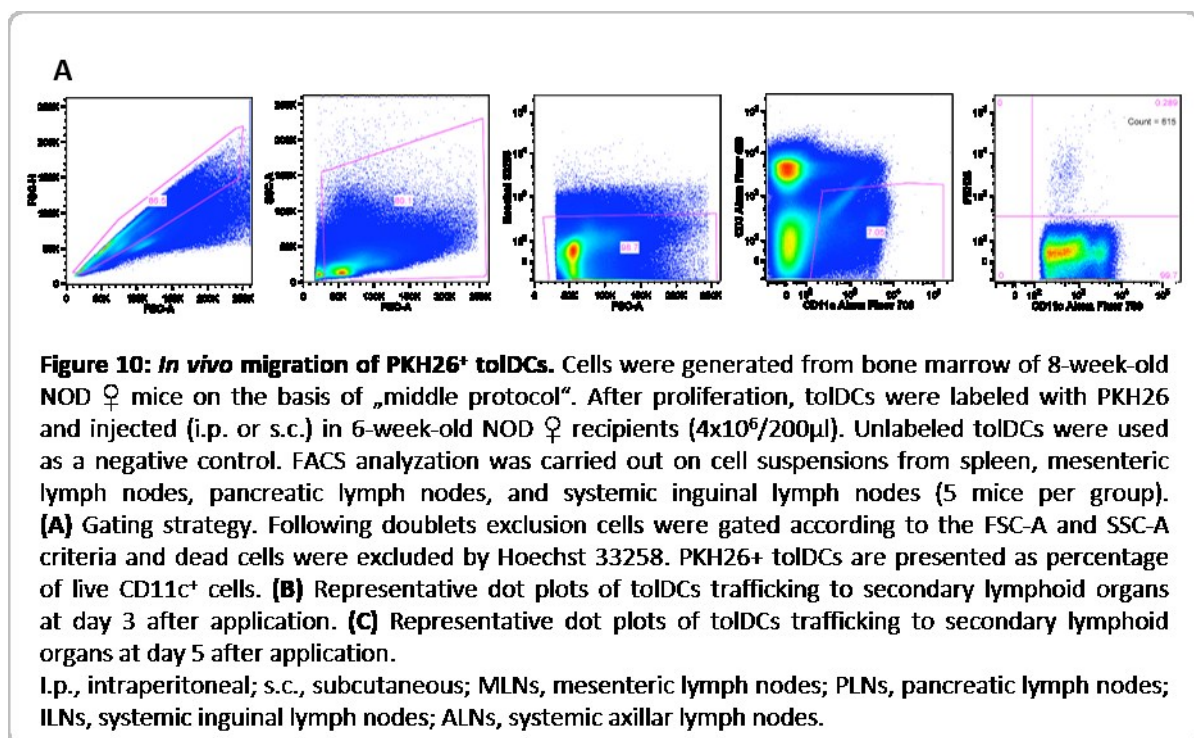
Figure 9C: Total number of cells recovered from tissues after subcutaneous application (right).
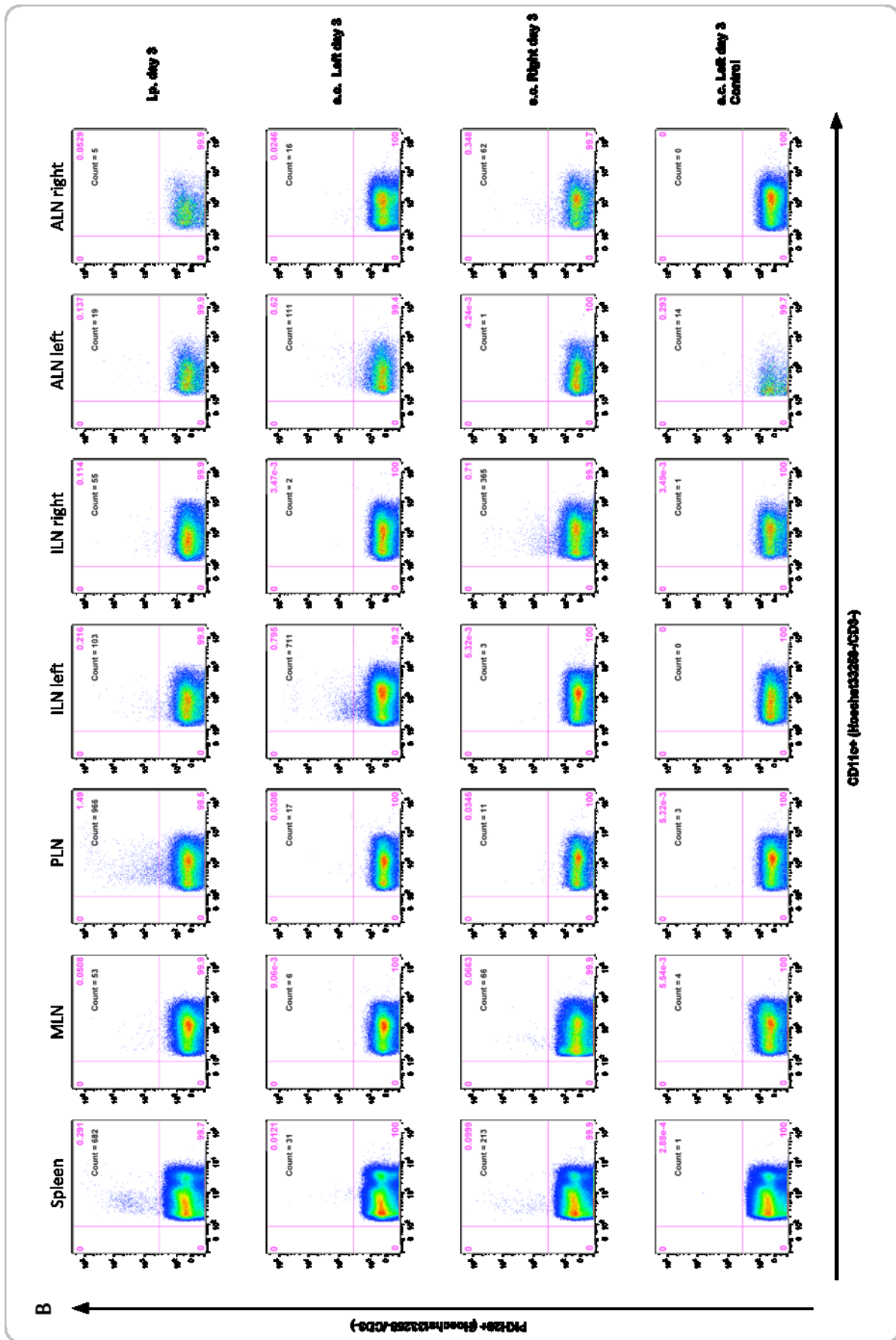TolDCs generated on the basis of „middle" protocol were injected s. c. right in NOD ♀ recipients. At day 1, 3, 5, 7, and 9 after tolDCs application, SPL, PLNs, MLNs, ILNs left, ILNs right, ALNs left and ALNs right (5 mice per group) were removed and homogenized. After that, cells recovered from relevant tissues were counted. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
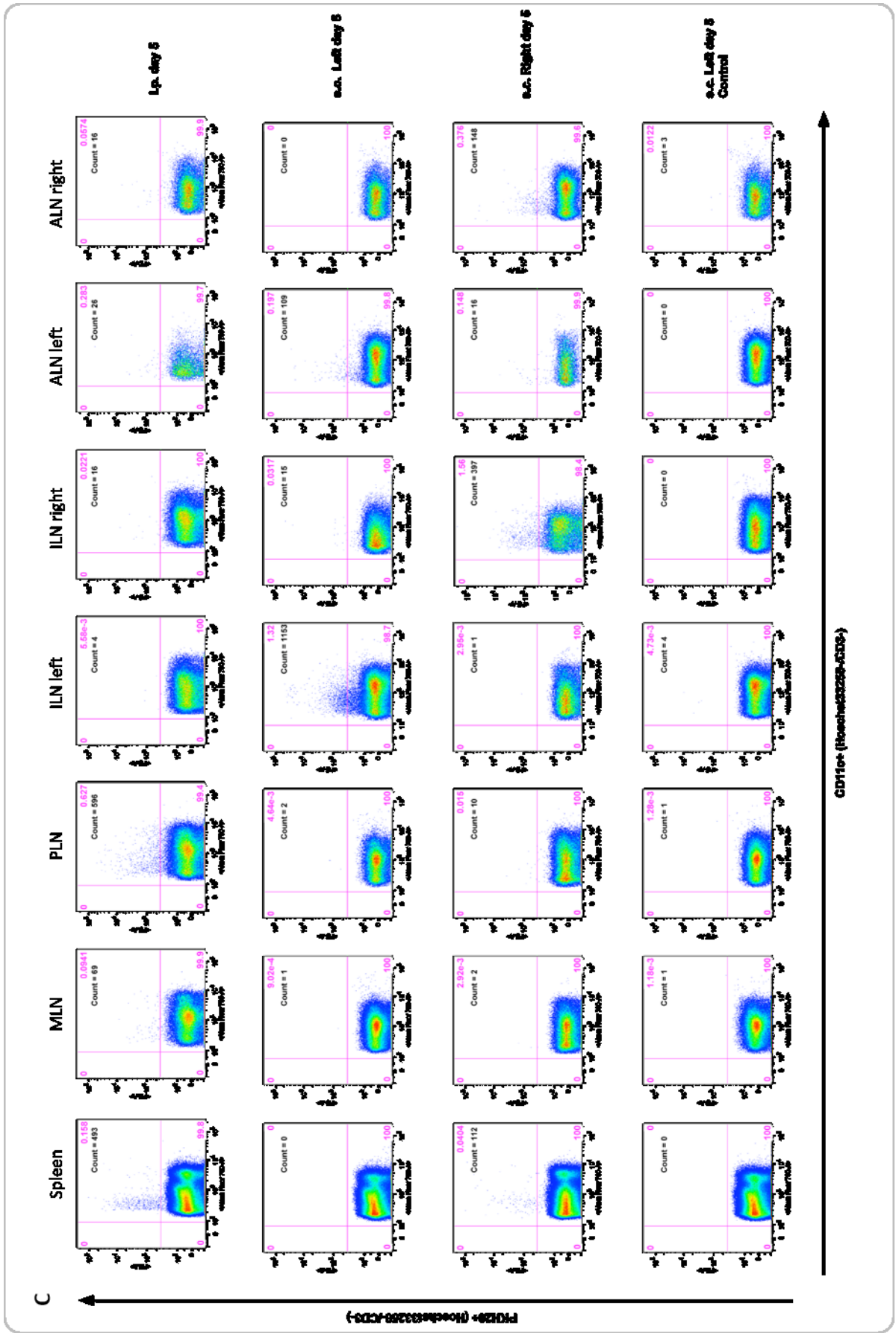SPL, spleen; PLNs, pancreatic lymph nodes; MLNs, mesenteric lymph nodes; ILNs, inguinal lymph nodes; ALNs, axillary lymph nodes.

As seen in above attached graphs (**Figure 9A, 9B, 9C**), the total amount of cells recovered from tissues was significantly increased in pancreatic lymph nodes after i. p. application and in subcutaneous lymph nodes after s. c. application on the corresponding side of the body. Subcutaneous (s. c. right and s. c. left) administrations led only to smaller or insignificant increases of isolated immune cells in the pancreatic lymph node (**Figure 9B, 9C**).

Next, we analyzed cell suspensions of relevant lymphoid organs by flow cytometry. Cells were labelled with fluorochrome-conjugated antibodies (**Table 6**) and live Hoechst$^-$CD11c$^+$CD3$^-$PKH26$^+$ tolDCs were detected in cell suspensions from spleen, mesenteric lymph nodes, pancreatic lymph nodes, and systemic inguinal and axillary lymph nodes after 1, 3, 5, 7 and 9 days of administration. Following attached dot plots (**Figure 10**) are examples illustrating *in vivo* trafficking of live PKH26-labelled tolDCs from the most representative days, i.e. from the day 3 and 5.

Figure 10: *In vivo* migration of PKH26$^+$ tolDCs. Cells were generated from bone marrow of 8-week-old NOD ♀ mice on the basis of „middle protocol". After proliferation, tolDCs were labeled with PKH26 and injected (i.p. or s.c.) in 6-week-old NOD ♀ recipients (4x10^6/200μl). Unlabeled tolDCs were used as a negative control. FACS analyzation was carried out on cell suspensions from spleen, mesenteric lymph nodes, pancreatic lymph nodes, and systemic inguinal lymph nodes (5 mice per group). **(A)** Gating strategy. Following doublets exclusion cells were gated according to the FSC-A and SSC-A criteria and dead cells were excluded by Hoechst 33258. PKH26+ tolDCs are presented as percentage of live CD11c$^+$ cells. **(B)** Representative dot plots of tolDCs trafficking to secondary lymphoid organs at day 3 after application. **(C)** Representative dot plots of tolDCs trafficking to secondary lymphoid organs at day 5 after application.
I.p., intraperitoneal; s.c., subcutaneous; MLNs, mesenteric lymph nodes; PLNs, pancreatic lymph nodes; ILNs, systemic inguinal lymph nodes; ALNs, systemic axillar lymph nodes.
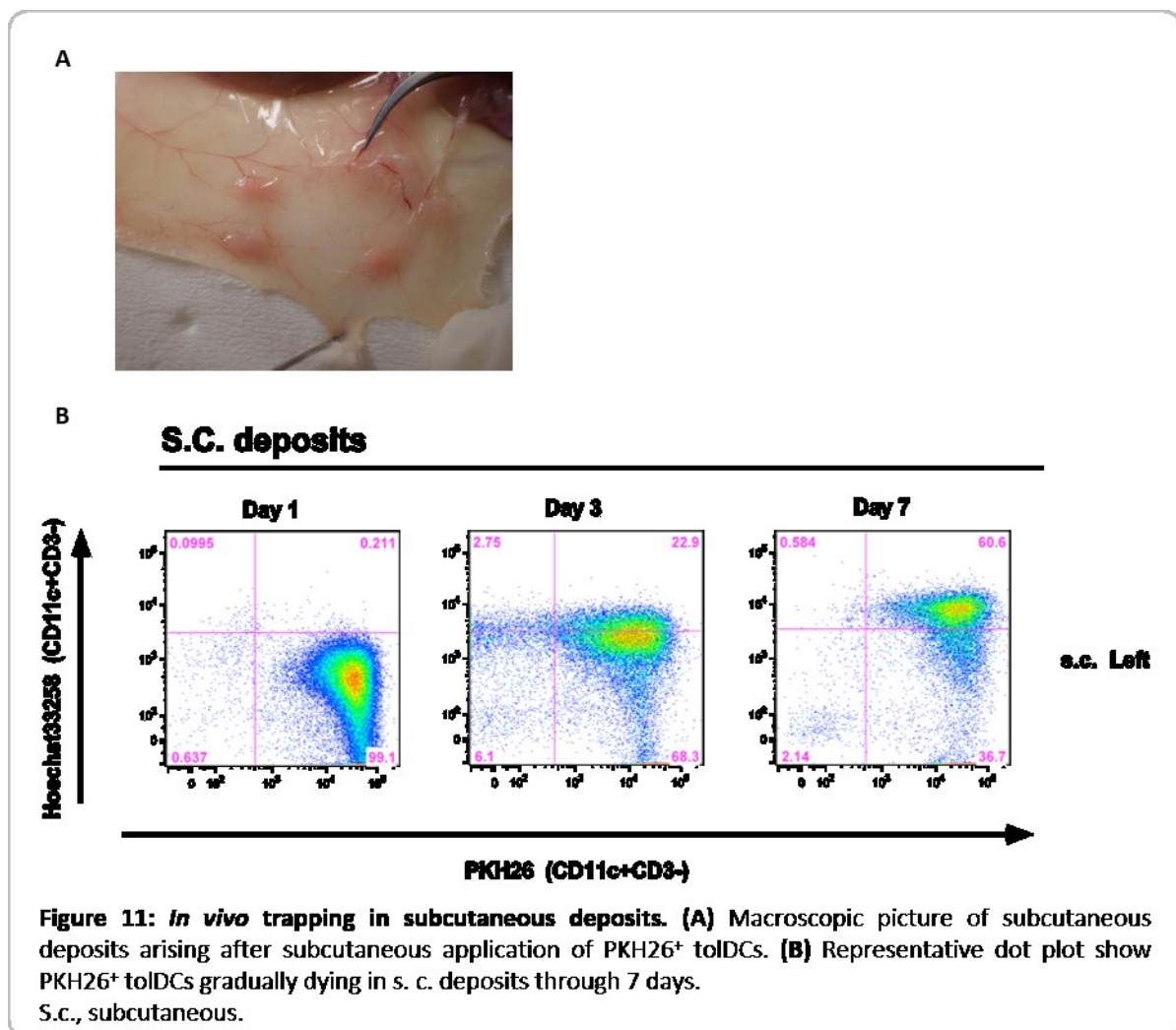
As seen in above attached dot plots, the preferential migratory patterns of tolDCs cannot be ignored. There is a strong evidence that after i. p. administration tolDCs preferentially accumulated in pancreatic lymph nodes and spleen, while after s. c. right or left administration tolDCs clustered in relevant subcutaneous lymph nodes, i.e. in inguinal and to a lesser extent (due to the s. c. injections being situated in the lower portion of the belly) also axillary draining lymph nodes. Thus, these data parallel data obtained from the total cell recovery (**Figure 9A, 9B, 9C**) and indicate that for trafficking and induction of immune responses in pancreatic lymph nodes the i. p. administration route of tolDCs is superior.
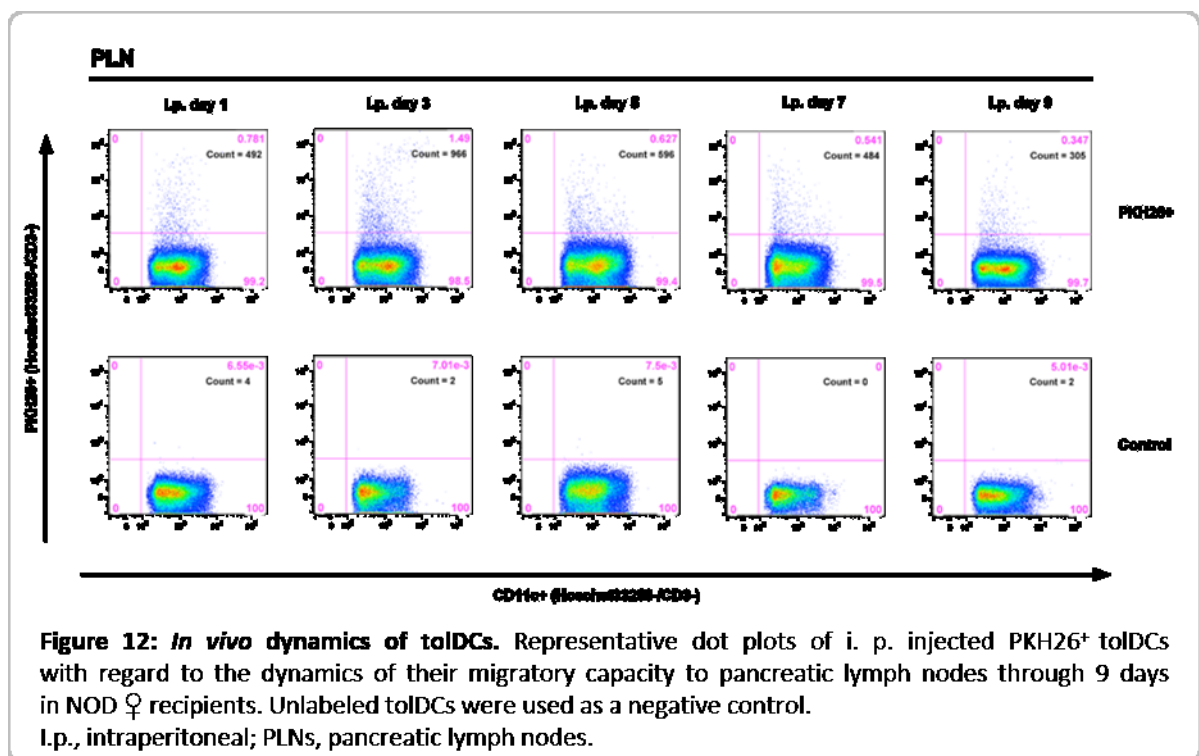
After subcutaneous injection, we noted macroscopic structures, that were generated by s. c. injections and consisted of deposits of PKH26$^+$ tolDCs (**Figure 11A**). To find out their destinies, we analysed cell suspensions of these recovered deposits by flow cytometry. As shown in **Figure 11B**, a substantial proportion of s. c. injected PKH26$^+$ tolDCs was trapped "*in situ*" and formed these s. c. deposits in which they were found alive after 24 hours with gradually dying (Hoechst33258$^+$).

Figure 11: *In vivo* trapping in subcutaneous deposits. **(A)** Macroscopic picture of subcutaneous deposits arising after subcutaneous application of PKH26$^+$ tolDCs. **(B)** Representative dot plot show PKH26$^+$ tolDCs gradually dying in s. c. deposits through 7 days.
S.c., subcutaneous.

1.4.2 Dynamics of tolDCs homing

Besides the migratory capability, we analysed also the dynamics of tolDCs homing-trafficking. Bellow, there is an example of tolDCs migration to pancreatic lymph nodes at days 1, 3, 5, 7, and 9 after intraperitoneal administration.
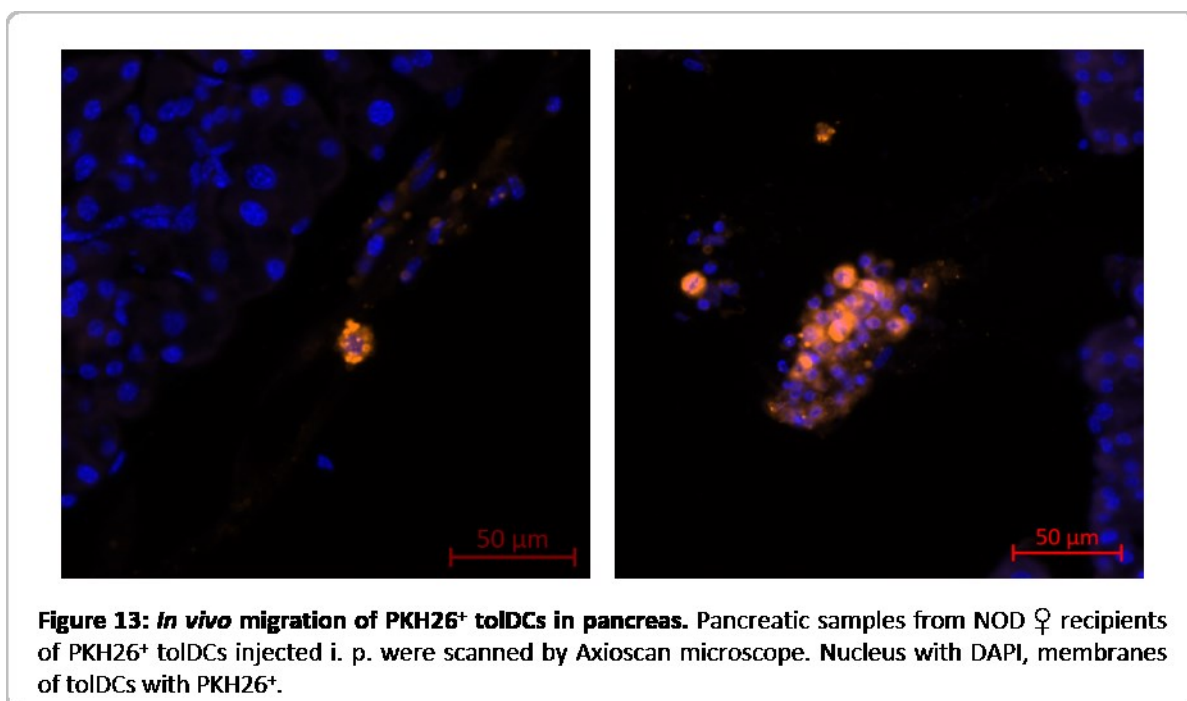
As seen in **Figure 12**, the highest accumulation of live PKH26$^+$CD11c$^+$ tolDCs in PLNs was detected at day 3, while high but progressively decreasing numbers of tolDCs were detected also on day 5, 7, and 9. These data also document that tolDCs survive *in vivo* for relatively long time, with a significant portion of tolDCs being detected in the lymph node of interest even 9 days past their injection.

Figure 12: *In vivo* **dynamics of tolDCs.** Representative dot plots of i. p. injected PKH26$^+$ tolDCs with regard to the dynamics of their migratory capacity to pancreatic lymph nodes through 9 days in NOD ♀ recipients. Unlabeled tolDCs were used as a negative control.
I.p., intraperitoneal; PLNs, pancreatic lymph nodes.

1.4.3 Microscopic detection

To evaluate the migratory capacity of tolDCs, we used slide scanner Axio Scan with possibility to detect PKH26$^+$ tolDCs directly within organs of interest, i.e. pancreas. Organs were removed from NOD mice on day 3 after the intraperitoneal application.

In figure below (**Figure 13**) are shown representative pictures of migrated PKH26$^+$ tolDCs. These preliminary data are attached for an illustration and other experiments for detection of PKH26$^+$ tolDCs combined with insulin staining of islets or in pancreatic lymph nodes are in progress.

Figure 13: *In vivo* **migration of PKH26$^+$ tolDCs in pancreas.** Pancreatic samples from NOD ♀ recipients of PKH26$^+$ tolDCs injected i. p. were scanned by Axioscan microscope. Nucleus with DAPI, membranes of tolDCs with PKH26$^+$.

1.5 T1D prevention

Many studies described the preventive effect of tolDCs or even their therapeutic potential but no protocol was tested for its efficacy as regards a side by side comparison of administration routes in a search for the optimal or strongest effect on the T1D prevention. For this reason, we compared various application routes, more specifically the intraperitoneal, subcutaneous (on both side of the body) and intravenous for their efficacy to prevent T1D.

The NOD-SCID model, a mouse model with severe combined deficiency affecting both T and B lymphocytes on NOD background, allows quick induction of the disease (diabetes onset in ± two weeks) by transfer of diabetogenic splenocytes from prediabetic NOD mice and also much shorter disease-observation period compared to the spontaneous NOD model. Thus, we are using the NOD-

SCID model of an adoptive co-transfer of diabetes, as it substantially shortens observation period for diabetes onset (data presented are from the time-point of 70 days of diabetes monitoring).

As seen in **Figure 14**, the tested administration routes achieved different effects on T1D development. The most efficient application routes were the intravenous and intraperitoneal, while subcutaneous routes regardless which side of the body were less efficient. Nevertheless, they all were sufficient to statistically significantly prevent diabetes transfer. Taken together, intraperitoneal and intravenous application of tolDCs together with diabetogenic splenocytes led to more efficient, reduction of diabetes onset in the NOD-SCID model of adoptive co-transfer of diabetes.
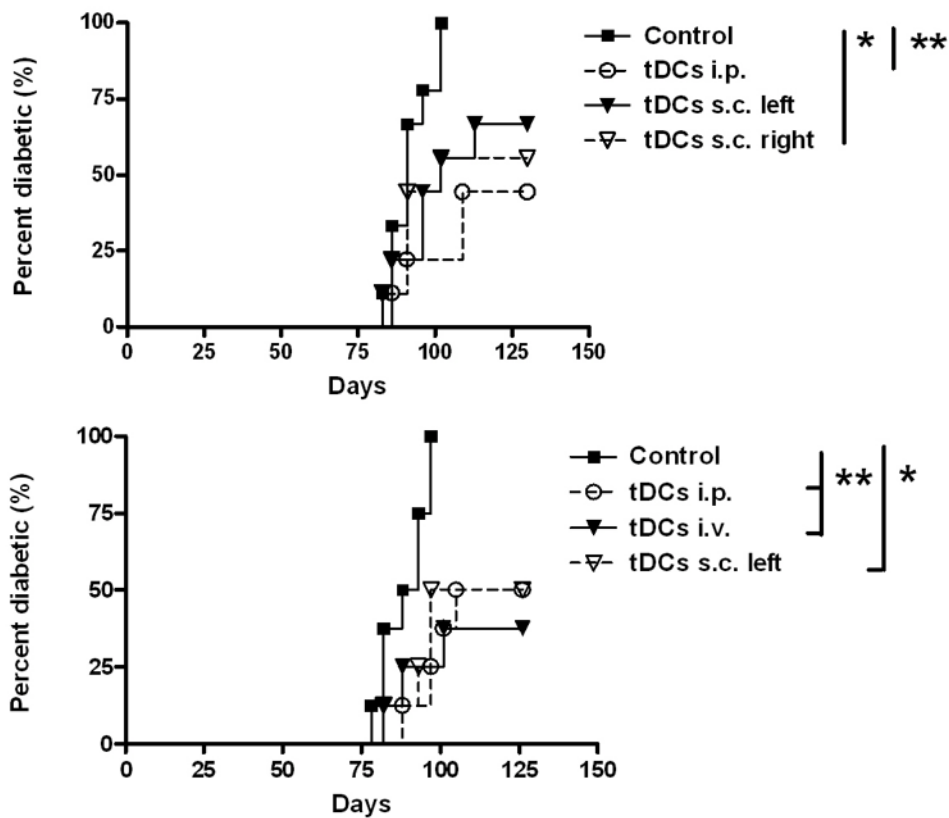
Figure 14: Effect of application routes on diabetes prevention by tolDCs in the NOD-SCID mouse model. 8-week-old NOD-SCID ♀ recipients received 3×10^6/200 µl tolDCs (generated from 8–10-week-old NOD ♀) together with 5×10^6/200 µl diabetogenic splenocytes (isolated from 12–13-week-old prediabetic NOD ♀), controls were injected with diabetogenic splenocytes in PBS only. Diabetes incidence shown for 70 days of diabetes monitoring. Although all tested application routes led to statistically significant disease prevention, compared to s. c. applications, the i. v., and i. p. application routes displayed slightly superior effects.

5. Discussion

TolDCs have become a promising strategy for prevention or even treatment of autoimmune diseases, including T1D. Many animal studies used tolDCs as a modulator of immune response mainly in NOD mice, but generation of these cells is quite challenging and it is necessary to optimize the culture protocols for both efficacy and safety before their translation to humans and clinical testing. The main aim of optimization strategies in tolDCs-based therapies is generation of tolDCs with potent tolerogenic properties, their optimal migration/location and indeed a stable and robust tolerogenic phenotype.

1.6 Distinct culture protocols affect tolerogenic properties of tolDCs

Our first experiments described in this thesis compared three distinct protocols of tolDCs propagation with regard to final tolDCs phenotype features. Necessary tolerogenic environment was achieved by exposure to dexamethasone and vitamin D. Dexamethasone is commonly used as an immunomodulator with anti-inflammatory and immunosuppressive impact (Kim, Jung et al. 2018) and it has been also utilized in clinical trials with tolDCs (Jauregui-Amezaga, Cabezón et al. 2015, Bell, Anderson et al. 2017). Secondly, vitamin D influences glucose metabolism, thereby contributes to maintenance of tolerogenic character of tolDCs and is specifically important in the beginning phase of tolDCs development (Ferreira, Vanherwegen et al. 2015, Vanherwegen, Eelen et al. 2019). Thirdly, MPLA used as a synthetic analog of LPS promotes the final differentiation of tolDCs. More importantly, it also secures a stable phenotype and chemokines expressions (Garcia-Gonzalez, Morales et al. 2013).

Frequently used vitamin D3 was in our experiments replaced by its analog vitamin D2 with comparably potent immunomodulatory capabilities (Sochorova, Budinsky et al. 2009). Stability of tolDCs generated in above described culture conditions was not clarified only in animal studies but also in cell cultures of human tolDCs (Dáňová, Grohová et al. 2017).

As documented in this thesis, different culture protocols, more specifically the length of exposure to tolerogenic agents, influence expression of maturation markers (CD80, CD86, CD40) and also an important homing molecule, the chemokine receptor CCR7. Reduced expression of maturation markers and on the other hand, increased expression of inhibitory molecules are in general the typical phenotypic features of tolDCs compared to mature DCs. In addition, similar yields of MPLA stabilized tolDCs were obtained (**Figure 6B**). These data are in an accord with results from a previous study by Garcia-Gonzalez et al. (Garcia-Gonzalez,

Morales et al. 2013) but differ from another one which reported decreased yields in dexamethasone-induced tolDCs (Naranjo-Gomez, Raich-Regue et al. 2011).

Apart from barring expressions of maturation markers, distinct tolDCs protocols also affect expression of chemokine receptor CCR7 (**Figure 8**) that is critically important for tolDCs` lymph nodes-homing capacity (Jang, Sougawa et al. 2006). Higher expression of CCR7 is critical for the trafficking efficacy of tolDCs to secondary (mucosal) lymph nodes (Worbs, Hammerschmidt et al. 2016), thus in the future it is important to test also other molecules, mainly of the chemokine family, that might improve migratory capabilities of tolDCs.

1.7 TolDCs migrate to preferential lymphoid organs according to the application route

The migration of tolDCs to the disease-critical lymph nodes is a strong and promising argument for cell-based therapy and induction of specific immunosuppression. Since pancreatic lymph nodes in T1D were shown as unique and important in the disease prevention (Clare-Salzler, Brooks et al. 1992), they are equally involved in pathogenesis of T1D as the site of priming of diabetogenic T cells or the site of initial interaction between islet autoantigens and autoreactive lymphocytes (Jaakkola, Jalkanen et al. 2003). This fact was also confirmed e.g. by chirurgical removal of these lymphoid organs that in 3-week-old NOD mice protected later development of T1D (Gagnerault, Luan et al. 2002). Similarly, Ferreira et al. documented preferential drifting of vitamin D3-induced tolDCs to PLNs and suppression of T-cell proliferation *in vivo* (Ferreira, Gysemans et al. 2014). Thus, it is highly desirable to increase migratory capacity of therapeutic tolDCs to the T1D-relevant, pancreatic lymph nodes.

Very important and still unparalleled study by Creusot et al. showed different preferential accumulation of tolDCs *in vivo* when comparing distinct application routes (Creusot, Yaghoubi et al. 2009). While the intravenous administration led to tolDCs accumulation in pancreatic lymph nodes and also in spleen, similar to our data, the intraperitoneal injections resulted in major aggregation in pancreatic lymph nodes and to a lesser extent also in the spleen. Nevertheless, s. c. applications that are claimed by some authors as optimal and safe (Creusot, Yaghoubi et al. 2009, Nikolic, Welzen-Coppens et al. 2009) were not tested and compared in this study.

On the basis of our results (**Figure 10B, C**) the preferential tolDCs home-trafficking patterns cannot be ignored. With the need to maximize the effect of minimum dose of tolDCs an idea of remarkably improved migration or a direct injection of therapeutic tolDCs into pancreatic lymph nodes may seem highly attractive for future testing in the field of T1D.

Apart from animal studies, clinical trials with tolDCs carried out in patients with T1D or rheumatoid arthritis were based on the subcutaneous (Joo et al. 2014) or intradermal (Giannoukakis, Phillips et al. 2011, Benham, Nel et al. 2015) injections (**Table 1**). The subcutaneous administration route preferably close to the abdominal location of pancreas was first mentioned in the review by Creusot et al. (Creusot, Giannoukakis et al. 2014), and then re-cited in follow-up studies but not tested experimentally in comparison with other application routes. Nevertheless, at least from anatomical point of view such a claim seems a bit suspicious. Subcutaneous application may be preferred as a least invasive and safe by regulatory agencies. However, there is already one clinical trial employing i. p. application of tolDCs in the Cronh`s disease (Jauregui-Amezaga, Cabezón et al. 2015). Another explanation may be that dendritic cells and/or tolDCs are so potent immune cells, that even low number is sufficient to change the course of developing or already developed immune responses. This fact may be supported by initial experiments in which tolDCs were applied s. c. into the mouse footpad – a rather distant place from PLNs and pancreas (Lo, Peng et al. 2006). Although s. c. application does not lead to frequent presence of injected cells in mucosal lymphoid organs e.g. PLN (**Figure 10B, C**), other papers also documented T1D prevention in NOD mice by subcutaneous tolDCs (Lo, Peng et al. 2006, Lo, Xia et al. 2018).

Besides the promising advantages of unloaded tolDCs, there is evidence of surprisingly less effective Ag-loaded tolDCs (Funda, Golias et al. 2018) that in addition possesses a potential risk of promoting autoantigen-specific effector immune responses. Thus, loaded antigens from dying Ag-specific tolDCs may get processed and presented by recipient APCs with potential danger of T cells activation (Smyth, Ratnasothy et al. 2013). Moreover, as we show in **Figure 11**, subcutaneous application of tolDCs is characterized by formation of subcutaneous deposits (**Figure 11A**) where they gradually die *in situ* (**Figure 11B**). Although s. c. tolDCs were well documented to prevent development of T1D, this phenomenon of the *in situ* s. c. accumulations of dying tolDCs may represents a potential risk for induction of effector immune responses.

Our data in **Figure 10B** and **10C** show also one disbalance between the left and right s. c. applications. Besides the expectable preferential accumulation of s. c. administered PKH26⁺ tolDCs in inguinal and axillary lymph nodes on the relevant side of the body, there is evidence of distinct tolDCs accumulation in spleen after subcutaneous administration on the right side but not after s. c. administration on the left side.

The mechanisms of tolDCs migration are still not fully investigated. Very interesting for these purposes may be the *in vivo* monitoring of trafficking patterns of injected tolDCs by modern microscopic technics, e. g. the light-sheet microscopy. At time of the writing, these experiments are in progress thanks to the kind support and collaboration with the Faculty of Science, Charles University. Our preliminary data from light-sheet microscope are in progress and we hope that we will be able to detect the tolDCs accumulation in e.g. undissected pancreatic lymph nodes and with captured fluorescent signals of a whole organ. The other promising microscopic method is based on slide scanner microscope (**Figure 13)** and potential to acquire multiple Z-stacks tiles. This technique should provide us with information on distribution of tolDCs within the whole pancreas and more specifically in relation to insulin-positive beta cells (islets) and blood vessels.

1.8 Dynamics of tolDCs migration

The other unclear parameters in tolDCs therapies is the tolDCs dose, repetitive vaccination schemes and also tolDCs survival *in vivo*. As mentioned above, *in vivo* survival of injected tolDCs was reported for up to 1–2 weeks after i. p. or i. v. tolDCs administrations (Creusot, Yaghoubi et al. 2009). We focused on dynamics of tolDCs accumulation in pancreatic lymph nodes after intraperitoneal application (**Figure 12**). The highest number of immigrated and accumulated tolDCs was detected at day 3 with slightly decreasing number in following days (5, 7, and 9). This pattern shows relatively high and over one-week-stabile presence of tolDCs in the most important lymph node as regards T1D.

1.9 Application routes affect diabetes-preventive capability

Routes of administration and their potential impact on T1D prevention have not yet been sufficiently compared in animal models. First experiments using s. c. application for prevention of T1D were performed with pulsed tolDCs, specifically in footpad of NOD mice and resulted in the disease prevention (Lo, Peng et al. 2006).

Our diabetes incidence data show that administration routes affect not only the preferential accumulation of tolDCs in targeted lymphoid organs but also influence the efficacy of T1D prevention in the NOD-SCID mouse model of adoptive co-transfer of diabetes.

As showed in **Figure 14**, compared to control NOD-SCID mice receiving only diabetogenic splenocytes, all other groups with co-transferred tolDCs were more or less protected from the diabetes onset. Moreover, differences in efficacy of application routes are noticeable. Thus, our two repeated experiments showed diminished preventive effect of s. c. applications (irrespective of the body side) compared to i. v. and i. p. injections (**Figure 14**). As regards the effect of Ag-loading, our previous experiments documented that Ag-loaded tolDCs were slightly less effective in T1D prevention compared to unloaded tolDCs (Funda, Golias et al. 2018).

Experiments presented in **Figure 14** are carried out in NOD-SCID mouse model of adoptive co-transfer of diabetes, however, we hope to repeat and extend these experiments also in the spontaneous, less artificial NOD mouse model, in which diabetes incidence is monitored for period of 300 days. These experiments are made possible due to a long-lasting and effective collaboration with the Bartholin Institute in Copenhagen. Another goal of our project in this field is testing of other, highly experimental application routes. At present we are monitoring diabetes incidence in NOD-SCID mice after direct injections of tolDCs into the pancreas.

1.10 Summary and future prospects

Tolerogenic DCs immunotherapy is one of a few approaches that besides the prevention also led to the cure of diabetes or reversal of hyperglycemia in already diabetogenic NOD mice. (Creusot, Chang et al. 2010, Di Caro, Phillips et al. 2014, Engman, Wen et al. 2015). In addition, this cell-based therapy represents a very promising approach also as an early pre-onset diabetes intervention, since it is nowadays possible (albeit expensive) to identify individuals with a high risk of progression towards clinical onset of the disease.

Quite a few not yet well explored parameters might change or modulate the potential efficacy of therapeutic tolDCs in human patients, e.g. diabetic hyperglycemia or metabolic pathways (Dáňová, Grohová et al. 2017, Grohová, Dáňová et al. 2019). Other parameters in need of an attention are e.g. tolDCs doses (Turner, Kane et al. 2009), single versus multiple

doses (Di Caro, Phillips et al. 2014) or effect of serum-free conditions (Haase, Ejrnaes et al. 2005, Looney, Chernatynskaya et al. 2014).

Besides approaches based on long-term immunosuppression in T1D such as cyclosporine (Feutren, Papoz et al. 1986) or anti-CD3 mAb (Sherr, Ghazi et al. 2014), tolDCs represent a unique strategy that has been even capable of reverting diabetes and thus it is a hot candidate for further development and/or for combination therapy approaches. Examples of combination approaches may be inspired by e.g. prevention of T1D by acetylated dextran microparticles with rapamycin and pancreatic peptide P31 (Pujol-Autonell, Ampudia et al. 2013, Chen, Kroger et al. 2018), low-dose IL-2 (Dwyer, Ward et al. 2016), tolerogenic mucosal (oral or intranasal) administration of autoantigens (Hanninen and Harrison 2004), or a non-ablative autologous hematopoietic stem cell transplantation (van Megen, van 't Wout et al. 2018).

Taken together, this thesis aimed to address only a few but important aspects of the wide area of preclinical tolDCs research in T1D. In the near future, it is necessary to optimize the other discussed parameters in animal models in order to bring this promising cell-based therapy to the patients.

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