**Univerzita Karlova v Praze**

**1. lékařská fakulta**

Autoreferát disertační práce

# Diagnostický příspěvek k hodnocení intervenčních modelů léčby diabetu mellitu 1. typu

Diagnostic contribution to the evaluation of intervention models in the treatment of type 1 diabetes mellitus

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

**CONTENT**

**ABSTRAKT**

Při imunointervenční či transplantační léčbě diabetu je nutné monitorovat ukazatele imunitní nebo rejekční destrukce přežívající inzulín produkující tkáně. Disertační práce si klade za cíl zlepšit možnosti sledování průběhu autoimunitního procesu a in vivo průkazu přežívání transplantovaných Langerhansových ostrůvků.

Dílčí úkoly zahrnovaly průkaz vitality izolovaných ostrůvků určených k transplantaci měřením respirační aktivity, objasnění průběhu in vitro značení izolovaných ostrůvků superparamagnetickou kontrastní látkou pro následné zobrazení pomocí magnetické rezonance a sledování transportu této látky po transplantaci. Pro identifikaci kontrastních částic v histologickém materiálu jsme studovali nově připravenou paramagnetickou kontranstní látku s navázaným fluoresceinem. Dále jsme se zaměřili na hodnocení autoimunitního procesu pomocí sledování cytokinové odpovědi na specifickou stimulaci autoantigenem. Autoimunitní poškození beta buněk v experimentu u myší jsme se snažili ovlivnit polyklonálními anti-thymocytárními protilátkami.

Nová metoda testování respirační aktivity ostrůvků dobře korelovala s ostatními metodami testování kvality ostrůvků a byla navržena do diagnostického schématu před klinickou transplantací. Při studiu intercelulárního transportu železitých kontrastních látek jsme získali důležité výsledky, které ukázaly, že se nanočástice železa během značení dostávají endocytózou do všech typů ostrůvkových buněk. Po transplantaci ostrůvků jsou částice přemístěny do tkáňových makrofágů, kde v případě tolerance zůstávají, ale při rejekci jsou z jater eliminovány. Prokázali jsme, že nová bimodální kontrastní látka dovoluje zobrazení ostrůvků pomocí magnetické rezonance i ověření jejich lokalizace fluorescenční mikroskopií.

Při vyšetření profilů cytokinové reakce lymfocytů po stimulaci potenciálními autoantigeny in vitro u nově zjištěných diabetiků a jejich zdravých příbuzných jsme zjistili rozdíly svědčící o aktivaci zánětlivého procesu ve skupině pacientů. Pomocí průtokové cytometrie jsme zjistili zvýšení populace regulačních T-lymfocytů po léčbě antithymocytární globulinem.

Nové nálezy přispěly k řešení dlouhodobých projektů na řešitelském pracovišti a byly publikovány v časopisech s faktorem impaktu.

**Klíčová slova:** diabetes mellitus 1. typu, Langerhansovy ostrůvky, transplantace, magnetická rezonance, autoreaktivní lymfocyty, imunointervenční terapie, anti-thymocytární globulin

**ABSTRACT**

During treatment of diabetes mellitus by immunointervention or transplantation, it is necessary to monitor the markers of immune destruction or rejection of surviving insulin producing cells. An aim of this thesis is to improve the possibilities of following autoimmunity and to detect the survival of transplanted pancreatic islet in vivo.

Partial aims included vitality testing of isolated islets for transplantation by measurement of respiration activity, observing the process of in vitro labeling of isolated islets with superparamagnetic iron oxide (SPIO) contrast agent for subsequent magnetic resonance imaging (MRI) of islets and observing SPIO particles transport after transplantation. We also studied a new dual paramagnetic contrast agent combined with fluorescein intended for identification of the MRI contrast agent in samples for histology. Further, we assessed autoimmune reaction by evaluation of cytokine response to specific stimulation with auto-antigens. We tried to affect beta-cells destruction by polyclonal anti-thymocyte antibodies in a mouse experimental model.

A new method of the islet respiration measurement correlated with other methods of islet quality testing and it was suggested as a diagnostic test before clinical transplantation. Results obtained studying the intercellular transport of SPIO particles showed that the particles are incorporated into all islet cell types during islet labeling. After transplantation, the particles were translocated into tissue macrophages, they persisted there in the case of immune tolerance but they were eliminated from the liver during rejection. We showed that a new dual contrast agent enables both islet imaging by MRI and localization by fluorescence microscopy. We found differences in cytokine reaction profiles after stimulation of lymphocytes with specific auto-antigens in groups of recent onset diabetic patients and their healthy relatives. This pointed to activation of inflammatory reaction in this group of patients. By flow cytometry we found an increase of regulatory T-lymphocytes population after the anti-thymocyte globulin treatment.

The new findings helped to solve long-term projects of the working group and were published in impacted journals.

Keywords: type 1 diabetes mellitus, islets of Langerhans, transplantation, magnetic resonance imaging, auto-reactive lymphocytes, immunointervention therapy, anti-thymocyte globulin

## INTRODUCTION

Type 1 diabetes mellitus (T1D) is characterized by destruction of the insulin-producing beta cells of the islets of Langerhans in the pancreas by autoimmune process. The reduced number of beta cells is insufficient for required insulin production and leads to high blood glucose levels that can cause serious health complications.

The autoimmune process may be undetected for many years and the first clinical symptoms become apparent after a majority of the beta cells have been destroyed. The destruction of pancreatic beta cells is T-cell dependent. CD8+ T cell-mediated beta cell killing is likely a major mechanism of beta-cell destruction. CD4+ T cells mostly provide help to both B cells and CD8+ T cells by providing cytokines. The balance between proinflamatory and suppressive immune reaction can be crucial in the initiation and progression of diabetes.

In our study, we detected cytokines produced by peripheral blood mononuclear cells (PBMCs) after stimulation with diabetogenic auto-antigens using a protein microarray.

This method enables an immediate semiquantitative analysis of the entire cytokine spectrum so that the character of immune response can be distinguished.

Several immunointervention studies have been focused on how to inhibit the beta cell destruction and to treat newly established T1D. The most prominent effects were achieved after administration of immunosuppressive agents against T cells as anti-thymocyte globulin (ATG) [1], anti-lymphocyte serum [2] or antibodies against CD3 antigen [3]. The positive effects of immunointervention have been demonstrated in experimental models. However, in clinical studies, only partial preservation of insulin secretion and partial reduction of T1D progression have been achieved. We investigated the effects of ATG therapy on peripheral lymphoid tissues that may be important to re-establish immune tolerance in diabetes. We utilized the model of T1D non-obese diabetic (NOD) mice.

The T1D is most commonly treated with exogenous insulin that, despite great efforts, is still not sufficiently effective in blood glucose correction. The only treatment that can maintain long-term normoglycemia is transplantation of the pancreas or pancreatic islets. The transplantation of isolated pancreatic islets is a therapeutic option for patients with brittle T1D who are at risk of life due to events of hypoglycemia [4-5].

Effective islet transplantation requires a sufficient mass and quality of isolated islets. In the presented study, we utilized high-resolution oxymetry for islet quality testing.

It is known that a significant loss of islets takes place immediately after transplantation and is caused by immunological and nonimmunological events [6]. Currently, the only markers of islet survival are indicators of islet function such as glycemia and C-peptide levels. However, these markers do not reflect early changes in islet mass. As a result, the fate of islets after transplantation is mostly unknown. Accordingly, a method that would make it possible to follow the transplanted islets in vivo is needed.

Magnetic resonance imaging (MRI), after previous labeling with superparamagnetic iron oxide (SPIO) nanoparticles, is still the only direct method for the long-term visualization of transplanted islets with sufficient spatial resolution [7] and applicable in clinical transplantation [8-9].

For further improvement of the labeling technique as well as for safety reasons, a more detailed study of iron particle uptake by the islet cells and its functional impact is needed.

In the presented study, we investigated in detail the time behavior of the labeling process and iron accumulation in different islet cell types which is important for labeling optimization and for the evaluation of its safety for the islet cells in vitro as well as in vivo.

Islets labeled in vitro with SPIO particles can be detected in vivo by MRI as hypointense spots [10-12]. The MRI signal can be detected at the site of transplantation for up to six months and the number of spots correlates with the number of transplanted islets despite the fact that the hypointense signal decreases gradually over time [13]. Moreover, in allogeneic models of transplantation, a reduction of the number of islet-related spots correlates with islet rejection [11, 14].

It is still unclear whether the loss of the islet-related hypointense spots is caused only by islet destruction (due to rejection, ischemia, and other factors) or if the iron particles are cleared out of surviving cells by natural mechanisms. Iron particles from necrotic islets may also be incorporated into the surrounding host tissue and may remain detectable despite the loss of the original insulin producing transplanted tissue, as Marzola et al. recently suggested based on experiments with an allogeneic rat model [15].

To determine whether translocation and elimination of the SPIO particles is caused by tissue rejection or represents a natural process, we studied the fate of SPIO particles in labeled pancreatic islets in both syngeneic and allogeneic models.

MRI shows excellent spatial resolution. Then again, the sensitivities of other techniques, such as optical methods, single-photon emission computed tomography (SPECT) or positron

emission tomography (PET), are much higher. Thus, the design and synthesis of so called dual or multimodal probes is an important field. The combination of both approaches utilizing a dual probe, e.g. magnetic nanoparticles tagged with fluorescent compound, establishes a very useful method for bioimaging. Such application enables efficient cellular labeling in vitro followed by in vivo tracking of the cells implanted into the organism. Moreover, the newly utilized nanostructural materials can comprise a higher amount of magnetic particles so that it shows a more intensive effect on MRI [16]. Dual MRI contrasts have been already used for islet labeling and monitoring [12, 17].

The presented study reports about the preparation and properties of new fluorescent magnetic nanoparticles based on the perovskite manganite La1-xSrxMnO3 (LSMO) and tested among others the suitability of the nanoparticles for islet labeling.

## AIMS OF THE THESIS

Replacement of insulin producing cells and the influence of diabetogenic autoimmune reaction represent perspective approaches of T1D treatment. The aim of this thesis was to find out methods that allow diagnosis of effects of two treatment models: 1) transplantation of isolated pancreatic islets and 2) immune intervention.

**Part 1**

The first aim of this thesis was to assess a new method of islets quality testing based on an oxygen consumption measurement.

The second aim of this thesis was to describe an ultrastructural localization of SPIO nanoparticles in islet cells after in vitro labeling and to follow the particles after transplantation of the labeled islets in syngenic and allogenic transplantation in rats.

The third aim of this thesis was to prove the efficiency of islet labeling with a new dual MRI contrast agent based on fluorescein and Mn perovskite and to describe a distribution of the particles in the islet cells.

**Part 2**

The fourth aim of this thesis was to find out a test of reactivity of immune cells in diabetic patients that could be applicable for following of the outcomes of intervention in T1D and to reveal differences in cytokine secretion profiles after stimulation with auto-antigens in groups of T1D patients, their relatives and healthy controls.

The fifth aim of this thesis was to test effects of immunointervention treatment with anti-thymocyte globulin on T1D progression and immune cells populations in non-obese diabetic mice.

## METHODS

### Part 1

#### Islet isolation

Pancreatic islets were isolated from male Wistar or Lewis rats by collagenase digestion and separation on a discontinuous Ficoll gradient. Isolated islets were cultivated in CMRL-1066 medium supplemented with 10 % fetal calf serum, 1 % HEPES, and 1 % penicillin/streptomycin/L-glutamine (all reagents from Sigma-Aldrich, USA).

#### Islet cells vitality test

Measurement of islet vitality was based on staining with propidium iodide and acridine orange. The islets were inspected under fluorescence microscope and vitality scores were assigned according to the percentage of cells stained green (live) versus red (death).

#### In vitro insulin secretion testing

The secretion of insulin in vitro was evaluated by static incubation. Islets were incubated in Krebs' solution with 3 mmol/L glucose (basal solution), subsequently with 22 mmol/L glucose and finally again with 3 mmol/L glucose. Results were expressed as stimulatory index (SI, stimulatory to basal insulin release ratio).

#### Oxymetry

Samples of fresh or 24-hour cultivated islets from Wistar rats were used for measurement of respiration which was performed by respirometer Oxygraph 2K (Oroboros Instruments, Austria). Oxygen consumption (pmol/mL · s) was counted as a negative time derivative of oxygen concentration (nmol/mL). The influence of islet number on oxygen consumption was tested on 1000 and 2000 islets obtained from the same rat donors.

#### Islet labeling with SPIO particles

Islet labeling was performed by a culture in medium supplemented with the SPIO contrast agent ferucarbotran (Schering AG, Germany) at a dose of 5 μL/mL. The islets were cultivated with ferucarbotran for 1, 4, 8, 12, and 24 hours. From each labeling period, samples of islets were taken for MRI, light and electron microscopy, assessment of iron content and relaxometry. For transplantation, islets were labeled for 48 hours.

#### Islet transplantation

Labeled islets were transplanted under the kidney capsule or into the portal vein. The syngeneic recipients were sacrificed on days 1, 7, 30, or 3 months after transplantation. Allogeneic recipients were sacrificed 9 or 14 days after transplantation; two recipients were kept for two months for persistent MRI signal in the kidney. The tissue with transplanted

islets was dissected and processed for immunohistological examination and for transmission electron microscopy.

**Transplantation of double labeled islets**

Islets labeled with ferucarbotran were subsequently labeled with CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) (Invitrogen, USA) according to manufacturer´s instructions. Double labeled islets were transplanted into syngeneic recipients under the kidney capsule and into the portal vein. Recipients were sacrificed seven days after transplantation.

**Magnetic resonance imaging**

MRI examinations of labeled islet and transplanted animals were performed by 4.7 T spectrometer Biospec equipped with a resonator coil (Bruker, Germany). Isolated islets were scanned as aliquots in 4 % gelatin. Animals were scanned one week after islet transplantation and in the week before sacrifice. Animals with allogeneic islets were examined by MRI on days 2, 8, and 13 after transplantation or after 2 months.

**Histological examination and immunofluorescence antigen detection**

Islet and tissue samples were fixed 4 % formaldehyde, dehydrated and embedded in paraffin. Immunofluorescence antigen detection was performed on the tissue sections. Following primary antibodies were used: rabbit anti-C-peptide; mouse anti-human glucagon; mouse anti-rat monocytes/macrophages - CD68; rabbit anti-human S100A4; mouse anti-α smooth muscle actin and goat anti-collagen VI. Subsequently secondary antibody conjugated with Alexa Fluor 488 or Alexa Fluor 555 were used (Molecular Probes, Invitrogen, USA). Tissue sections were counterstained with DAPI (Fluka, Sigma-Aldrich, USA), mounted in DABCO®-Mowiol® solution (Sigma-Aldrich, USA), examined using an Olympus BX41 fluorescence microscope and recorded by Olympus DP71 digital camera (Olympus, Japan).

**Iron detection**

On the same tissue sections, subsequent detection of iron ($Fe^{3+}$) was performed by Prussian blue staining (2% hydrochloric acid mixed with 2% potassium ferrocyanide). The areas of sections matching with previously recorded areas were scanned and co-localization of iron particles and immuno-detected cells was performed.

**Iron removal**

To uncover hidden cell antigens, iron was removed from tissue sections by 1% sodium dithionite (Sigma-Aldrich, USA) in water. After that, the sections were processed for immunofluorescent detection of macrophages and compared to untreated sections stained with Prussian blue.

**Transmission electron microscopy (TEM)**

Selected samples were fixed in a mixture of 4 % formaldehyde and 2.5 % glutaraldehyde, post-fixed in 1 % OsO4, dehydrated, and embedded in Epon resin (all from Polysciences, Inc, USA). Ultrathin sections were counterstained with uranyl acetate and lead citrate and examined in a JEOL 1010 transmission electron microscope (JEOL Tokyo, Japan) equipped with Mega View III camera (SIS, Muenster, Germany).

**Labeling of isolated islets with LSMO nanoparticles**

Fluorescent magnetic nanoparticles based on the perovskite manganite La1–xSrxMnO3 (LSMO) were synthesized at Department of Inorganic Chemistry, Faculty of Science, Charles University. Rat pancreatic islets were incubated for 24 hours in CMRL-1066 medium containing product at concentration corresponding to 0.11 mmol(Mn)·L–1. Vitality of labeled islets and secretion of insulin was tested. The uptake of nanoparticles into islets was investigated by means of immunofluorescence detection for distinguishing different cell types within the islet. Gel samples of labeled islets were prepared for visualization by MRI.

**Part 2**

**Patients and tested subjects**

Patient with recent onset T1D were included into the studies. Blood samples were collected within a week after the diagnosis and eventually retested 6 and 12 months later. The high-risk first-degree relatives of the T1D patients and healthy controls were included as well.

Cytokine secretion of stimulated peripheral blood mononuclear cells (PBMC)

PBMCs isolated by Ficoll gradient centrifugation were cultivated with a mixture of following diabetogenic auto-antigens: GAD65 peptide amino acids (a.a.) 247–279; a.a. 509–528; a.a. 524–543; IA-2 a.a. 853–872 and beta proinsulin chain a.a. 9–23. All experiments were completed with positive control stimulated with phytohemagglutinin as well as with a negative control. The medium was harvested after 72-h stimulation (37°C, 5% CO2) and the production of selected cytokines was assessed with protein microarray (RayBiotech, USA). The results were expressed as a percentage of signal intensity of integral positive controls.

Non-obese diabetic (NOD) mice and Anti-mouse thymocyte globulin (mATG) administration

Female NOD mice (strain NOD/ShiLtJ) were utilized for immunointervetion study. Blood glucose levels of mice were measured twice a week. Diabetic mice were randomized into four groups with termination at days 0, 7, 14, and 28 after the initiation of the study. NOD mice were treated with two doses of intraperitoneally administrated mATG (Genzyme, Framingham, MA) at days 0 and 4 (1 mg totally) or maintained without treatment as controls. IPGTT was performed on day 24. After overnight fasting, glucose (1 mg/g body weight) was

provided by intraperitoneal injection. Blood glucose values were obtained at 0, 10, 20, 30, 40, 50, and 60 minutes. We determined the coefficient of glucose assimilation.

Flow Cytometry

Mouse splenocytes isolated by mild dissociation were separated by gradient centrifugation in Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). Mouse lymph node cells were isolated by dissociation of nodes through 60-□m mesh. Isolated cells were labeled for flow cytometric analysis. We used fluorescent antibodies against surface antigens CD3, CD4, CD8, CD25. Subsequently, the fixed cells were permeabilized and the intracellular antigen FoxP3 was labeled. The labeled cells were analyzed using the flow cytometer LSR II (BD, USA). CD4+ and CD8+ T-lymphocyte ratio were determined and FoxP3+ regulatory T-cells (Tregs) were analyzed.

## RESULTS

### Islet quality testing

In vitro islet cells viability, insulin secretion, and oxygen consumption data are summarized in Table 1.

Table 1. Results of islet quality tests

| | Viability (%) | Stimulation index | Oxygen consumption (pmol/mL*s) |
|---|---|---|---|
| Fresh Islets | 91.1 ± 2.1 (n = 10) | 12.1 ± 8.2 (n = 5) | 18.8 ± 3.3 (n = 5) |
| Cultivated Islets | 92.1 ± 4.4 (n = 10) | 10.0 ± 3.7 (n = 8) | 15.2 ± 6.4 (n = 10) |

Data are mean value ± SD. Data are not significantly different: $P > 0.05$.

Oxygen consumption was proportional to islet number, as oxygen consumption increased 1.92-times after doubling of the islet number (20.7 ± 1.7 vs. 39.7 ± 3.9 pmol/mL · s ($n = 3$; $P = 0.01$).

### Labeling of pancreatic islets with SPIO particles for in vivo detection

On MRI images, the labeled islets were demonstrated as hypointense spots which were visible immediately after a 1-hr culture period, and their intensity and iron content increased with the prolonged labeling time. By immunohistology and electron microscopy, iron was detected in the islets as early as after a 1-hr culture period. Initially, ferucarbotran was found adhered on the endocrine cell surface, whereas few particles were already found in the endocytic structures of macrophages. After a 4-hr culture period, the particles were localized also in the beta-cell vesicles. With the prolonged labeling time, the amount of incorporated particles

further increased and formed huge electrodense clusters (Fig.1). Ultrastructure and function of the labeled islet cells was not affected.

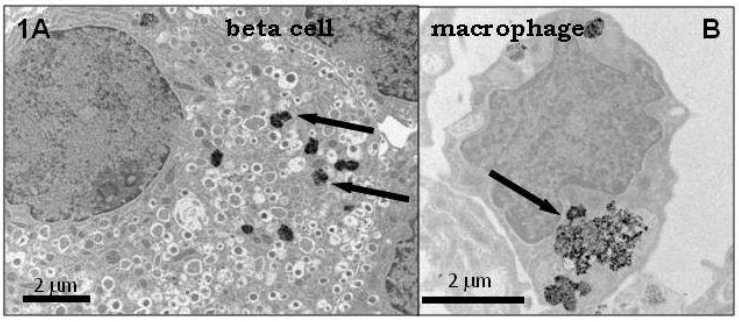


Fig. 1: Electron microscopy of labeled islet cells A) beta cell, B) macrophage. Arrows show cluster of SPIO particles.

Using light microscopy and MRI, we were able to detect the iron particles in islet cells and islet macrophages for as long as 120 days after transplantation into the liver.

SPIO particles in transplanted islets

In case of syngeneic transplantation into the liver, the SPIO labeled islets were imaged as hypointense dispersed spots on MRI (Fig. 2A). In syngeneic transplantation beneath the renal capsule, the labeled islets were detected as a confluent hypointense area at the edge of the kidney (Fig. 2B).

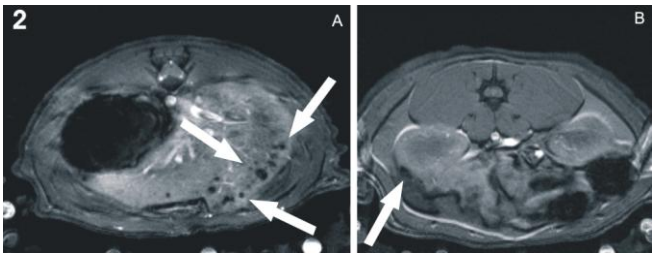


Fig. 2. MRI of labeled islets transplanted A) into the liver and B) beneath the kidney capsule.

After transplantation, we observed considerable changes in localization of the SPIO particles. The particles were gradually moved from endocrine cells and were detected in the tissue surrounding the transplanted islets. Some particles were found in myofibroblasts and fibroblasts. However, most of the iron particles persisted in large quantities in macrophages surrounding the syngeneic islets for whole study period (Fig. 3A).

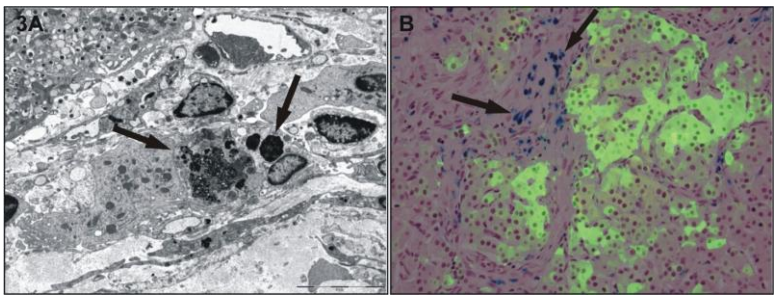


Fig. 3: SPIO particles (arrows) accumulated in macrophages in the vicinity of transplanted islets A) in TEM image, B) in model of islet labeled with CMFDA – green fluorescence.

In case of transplantation of double labeled islets, the iron particles were found preferentially in CMFDA-negative macrophages. This revealed the host origin of iron storing cells (Fig. 3B).

After allogeneic transplantation of the SPIO labeled islets, the endocrine cells were rejected up to 9 days after transplantation in the liver and under the kidney capsule as well. However, under the kidney capsule, confluent hypointense signals on MRI at the edge of the kidney remained apparent up to two month later. The concentrated SPIO particles were found in situ in the infiltrating macrophages. In contrast, the spots related to the labeled allogeneic islets transplanted into the liver gradually disappeared and were almost absent on day 13 and SPIO particles released from rejected islets did not persist in the transplant area. (Fig. 4)

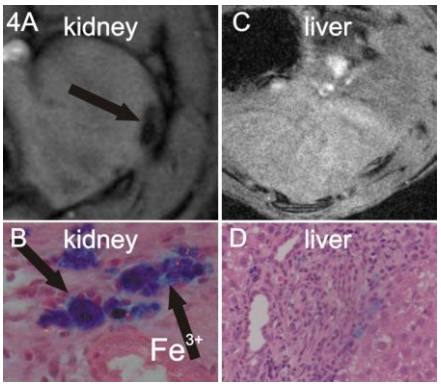


Fig. 4: Transplantation site following rejection of allogeneic islets labeled with SPIO particles. A) Persistent MRI signal and B) iron particles beneath the kidney capsule; C) liver without hypointens MRI signal and D) with reduced amount of iron particles.

**In vitro labeling of pancreatic islets with Dual Imaging Probes for Magnetic Resonance Imaging and Fluorescence Microscopy LSMO**

The vitality and insulin secretion of islets was not impaired by labeling with LSMO probes. Microscopic investigation after immunofluorescent staining of the labeled PIs revealed that the nanoparticles were present inside the peripheral islet cells. They were internalized in various cell types, including insulin producing beta-cells (Figure 5). The signal of the labeled islets was strong; islets were observable in MRI images acquired even with one acquisition.

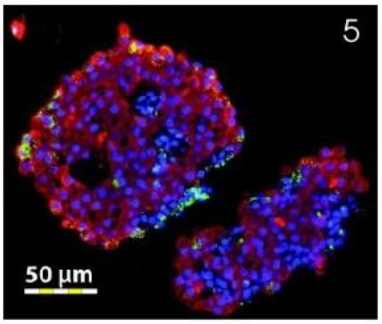


Fig. 5: Immunofluorescent staining of the labeled PIs:
red spots – immunostained c-peptide indicating beta-cells, green spots – LSMO, blue spots – DAPI stained cell nuclei.

**Monitoring of cellular autoreactivity in T1D patients and their relatives**

Stimulated and spontaneous secretion of cytokines by PBMC showed differences between the groups of T1D patients, their relatives and control healthy persons.

In the first study, the higher basal secretion of IL-2, IL-4, IL-5, IL-13 cytokines and GCSF was observed in control group and lower secretion in the group of relatives. After stimulation in controls, there was a significant decrease in IL-2, IL-13, GCSF, and IFN-gamma. The stimulated cytokine secretion in group of T1D patients was very variable. Only 12 months after T1D diagnosis, we observed decrease of secretion of IL-2 and IL-6, Th1 cytokines INF-gamma and TNF-beta, Th2 cytokine IL-4 and Th3 cytokine TGF-beta.

In the second study, the secretion of cytokines INF-gamma and TNF-beta showed the main Th1 profile in T1D high- risk children. In contrast, TNF-α and IL-6, classified as inflammatory cytokines, the chemokines RANTES, MCP-1 and IL-7 as well as the Th3 cytokines TGF-β and IL-10 were elevated in T1D children compared to high-risk children. The results revealed relation of higher secretion of Th1 cytokines and lower secretion of inflammatory cytokenes in high-risk persons and inversely lower secretion of Th1 cytokines and higher secretion of inflammatory cytokenes in T1D patients (Fig. 6).

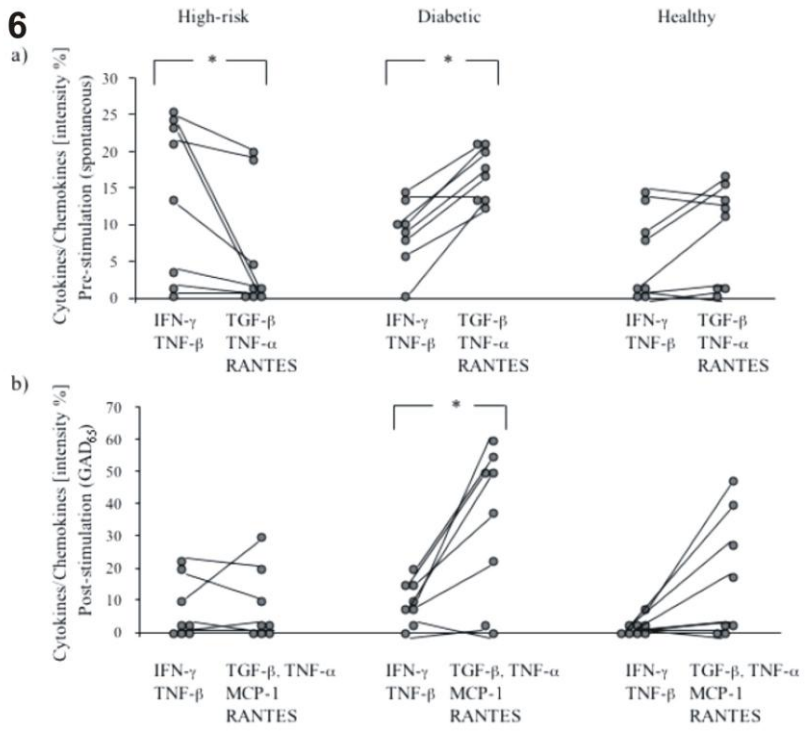


Fig. 6: Comparison of secretion of Th1 (IFN-γ, TNF-β) versus Th3 (TGF-β)/inflammatory marker (TNF-α)/chemokine (RANTES) on individual basis in high-risk, T1D and healthy children.

* $p < 0.05$

**Intervention of T1D with mATG in NOD mice model**

In the group of mice administered with mATG, diabetes remission (sustained glycemia at levels less than 13 mmol/L) occurred in 16% (3/19) of mice. In all of them, the pre-treatment blood glucose level was 15.6 mmol/L and less. Only one case of remission was observed in the control group (6%; 1/16). Although the IPGTT was out of normal range in all diabetic

animals, the coefficient of glucose assimilation was significantly higher among the mATG group (0.36 versus 0.12 mmol/L/min; $P < 0.05$).

mATG therapy led to a significant decrease in CD8+/CD4+ T-lymphocyte ratio compared with the control group. Among splenocytes, the changes were detected on day 7; whereas lymph node cells showed a decrease even on day 28 (Fig. 7).

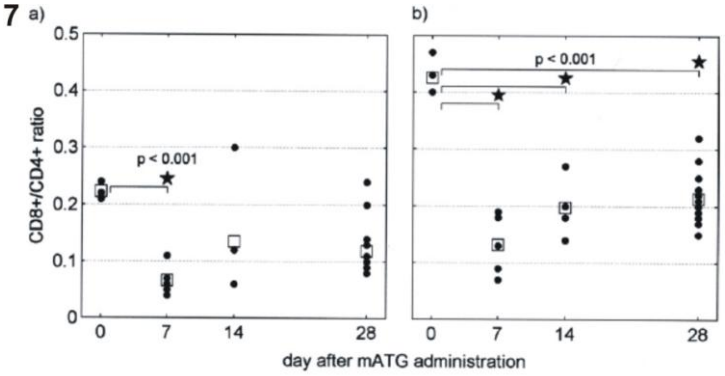


Fig. 7: CD8+/CD4+ T-lyphocyte ratio after mATG administration A) in splenocytes and B) in lymph nodes. represents mean value, * represents P value 0.05.

The population of Tregs was significantly increased among splenocytes 7 and 14 days after ATG administration but at day 28 the Tregs decreased to 12.2% (Fig. 8A). In the control group, the Treg population among splenocytes was constant (Fig. 8C). However, the difference between the mATG and the control group was significant only on day 14 ($P = 0.02$).

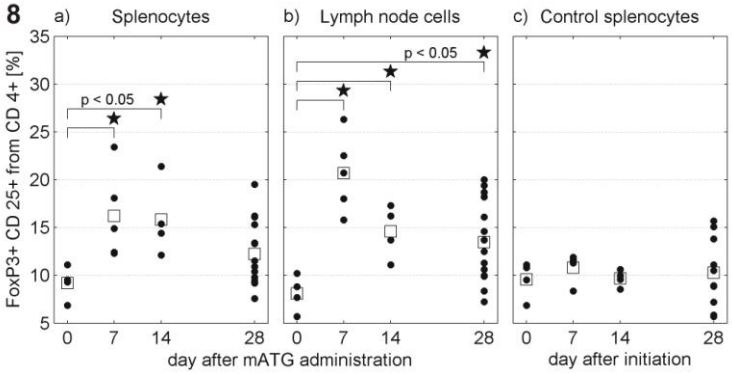


Fig. 8: Regulatory T-cell population after mATG administration A) in splenocytes, B) in lymph node cells and C) in splenocytes of control group.

Among lymph node cells, the Treg population was also increased after mATG administration at day 7 ($P = 0.0008$). The gradual decrease in the Treg population during the time was lower than among splenocytes and Treg population kept higher from day 0 to day 28 (Fig. 8B). However, because the mild increase in the Treg population among the control group, a significant difference between groups was only detected at day 7 ($P = 0.001$). The Treg population in controls was 8.2% at day 0, 10.3% at day 7, 11.2% at day 14, and 12.1% at day 28.

## DISCUSSION

The success of islet transplantation is, in part, based on the quality of transplanted islets. For islet quality testing, fast, sensitive and reproducible methods are needed. As respirometry represents a very sensitive method for the evaluation of tissue or cell metabolic condition [18-19] we applied it for islet vitality testing. The rate of oxygen consumption by isolated islets was tested also by other authors and results show that it correlates with the islet cells number and cellular membrane integrity [20]. Moreover, the method could be extended with detection of specific increase of metabolism due to glucose stimulated insulin secretion [21-22]. A special set of chambers with fluorescent sensors was invented for islet respiration testing (Instech Lab) [23]. Subsequently, the islet oxygen consumption represents the easiest and fastest method that shows islet quality and their capability for transplantation [24].

A long-term monitoring of transplanted islets with sufficient resolution and applicability for clinical use is still possible only with islet SPIO labeling and their imaging by MRI [25]. We described the process of islet labeling in time, we optimized the labeling and we proved the safety of the method. Our results are in accordance with other authors that showed the incorporation of the SPIO particles into all types of islet cells with preferences of macrophages [14, 26-27]. Our method of islet labeling did not influence islet function. Similarly, other authors described the labeling as safe and with no influence on either rodent or human islets [26-29].

The following of the labeled islets after transplantation showed the translocation of iron particles mainly into host macrophages. Translocation of the SPIO particles from labeled islet cells into other cells has been described in studies previously [27, 30-31]. Accumulation of iron particles in cells surrounding the islets was demonstrated by Marzola et al. [15]. Nevertheless, in a syngeneic model, the stored iron remained in the vicinity of the transplanted islets for a long period of time and enabled the detection of site of transplanted islets. In an allogeneic model, most of the iron particles were also translocated into macrophages after islet transplantation. To our surprise, under the kidney capsule, we did not observe any difference between MRI of functioning syngeneic islets and islets which were completely rejected. Furthermore, the iron particles were detected in the macrophages at the transplantation site even after islet rejection. To our knowledge, an MRI of islet rejection beneath the renal capsule in immunocompetent animals has not yet been studied. In the liver, the decrease of amount of iron at the transplantation site after islet rejection corresponded with the decrease of the number of islet-related MRI signals as was previously described [11, 14, 32]. In our model, almost all iron was eliminated from the liver after completion of islet

rejection. This could be due to emigration of the macrophages from the site of rejection in the resolution-phase of inflammation, presumably through the lymphatic system [33].

New bimodal contrast agents could be used for more sensitive imaging of transplanted islets as they enable simultaneous detection by MRI and luminescence or fluorescence. The SPIO contrast agent conjugated with fluorophore Cy5.5 was utilized for islet imaging by Evgenov et al. [12] and Kotkova et al. [34] labeled isolated islets with Gadollinium based contrast agent conjugated with fluorescein. In accordance with our results, the bimodal contrast agent was easily detected inside the islet cells and the labeling did not influenced quality of the islets.

Multiparameric methods have recently been employed to monitor autoimmune reaction against islet antigens during the onset of T1D or immunointervention treatment. The antigen stimulation of PBMC and semiquantitative cytokine microarray used in our studies showed some cytokine responses specific to diabetic patients and their relatives. The lower secretion of proinflamatory cytokine and higher secretion of Th1 cytokines in group of relatives observed in our study corresponded to the results of Karlsonn et al. [35]. In diabetic patients, the response varied over time. In recent onset of T1D, we observed rather proinflamatory cytokine profile. Similarly, Pfleger et al. [36] observed an increase of INF-gamma after diagnosis and decrease of reactivity during later period. The time period from diabetes onset seems to be an important factor in diabetogenic reactivity monitoring. In addition, the choice of stimulation antigens may play a crucial role in this type of tests.

The most effective immunointervention studies in diabetes were based on administration of anti-lymphocyte preparations like ATG or the anti-CD3 antibody. In our study, mATG treatment of diabetic NOD mice showed only partial metabolic improvement among previously hyperglycemic NOD mice. Most of the successful studies were performed formerly on pre- diabetic mice. Of the 463 agents evaluated in the intervention diabetic studies, only 23 protocols were initiated in diabetic NOD mice with only 16 considered successful [37]. Although mATG treatment alone did not cure diabetes in our study, there were metabolic ad immune effects. The higher rate of remission in mATG group seems to be dependent also on the level of initial glycemia. A further relevant aspect for treatment efficiency is the time between manifestation of diabetes and treatment initiation [2]. The main effect of mATG treatment was evident in T-lymphocyte subpopulations. We observed that mATG administration decreased the CD8+/CD4+ T-lymphocyte ratio in splenocytes and pancreatic lymph node cells, favoring Tregs. In contrast, an increase of CD8+/CD4+ ratio

was described in clinical studies [38-39] but peripheral blood populations were monitored there and in our study we monitored the lymphocytes in the spleen and lymph nodes. The influence of anti T-cell therapy on the regulatory T-cell population has been shown in clinical and experimental studies in vitro and in vivo [40-41]. Better results may be obtained with more prolonged mATG treatment [42].

**CONCLUSIONS**

I. The oxygen consumption measurement correlated with the other methods of islet quality testing. In comparison to the others, this is a fast, simple and reproducible method.

II. We described that the SPIO particles were incorporated into all islet cell types, preferentially in macrophages during islet in vitro labeling. The Labeling did not influence function and quality of the islets. After transplantation of labeled islets, the SPIO particles translocated from islet cells mainly into host macrophages. In a syngeneic model, the particles stayed in the vicinity of the islets for long period. In an allogeneic model of transplantation under the kidney capsule, the SPIO particles remained at the site of transplantation even after islet rejection. The MRI is therefore of no value for following their function. On the contrary, in the liver, the SPIO-rich macrophages moved gradually out of the transplant area after islet rejection. The MRI monitoring has the potential to provide a timely diagnosis of rejection of islet transplanted in to the liver.

III. The dual MRI contrast agent based on fluorescein and Mn perovskite is applicable for islet labeling. The contrast agent provided sufficient signal on MRI and fluorescence as well.

IV. The multiparametric cytokine secretion analysis allowed following the reactivity of immune cells in diabetic patients. The method revealed the Th1-like cytokines secretion profile in group of relatives with high risk of T1D and secretion of inflammatory and Th3-like cytokines in group of T1D patients.

V. The immunointervention treatment of NOD mice with anti-thymocyte globuline was not sufficient to cure established overt diabetes. However, the treatment led to increase of Treg population and proportional decrease of CD8+ lymphocytes in the spleen and pancreatic lymph nodes.

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**PUBLICATION ACTIVITY:**

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