UNIVERZITA KARLOVA V PRAZE
FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

Diplomová práce

2011 Zuzana Kadová

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Katedra biochemických věd

ZKOUMÁNÍ ÚČINKU CTA1R7K-ChrA-DD ZPŮSOBUJÍCÍHO TOLERANCI NA BDC2.5 CD4 BUŇKY VYVOLÁVAJÍCÍ DIABETES

INVESTIGATIONS OF TOLEROGENIC EFFECTS OF CTA1R7K-ChrA-DD ON DIABETOGENIC BDC2.5 CD4 CELLS

Diplomová práce

Vedoucí diplomové práce: Prof. Ing. Vladimír Wsól, Ph.D.

Hradec Králové 2011 Zuzana Kadová

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„I declare that my diploma thesis is my original publication. All used literature and other sources are named in References and cited properly."

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V Hradci Králové 6.5. 2011 Zuzana Kadová

Acknowledgements

I would like to give thanks to:

My tutor in Gothenburg Linda Mark and my working group (Linda Löfbom, Sara Rhost, Saikiran Sedimbi and Susanna Cardell) for their help.

Prof. Ing. Vladimír Wsól, Ph.D. for being my supervisor in the Czech Republic.

My family for financial support.

Abstrakt

Univerzita Karlova v Praze
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Katedra biochemických věd

Kandidát: Zuzana Kadová
Školitel: Prof. Ing. Vladimír Wsól, Ph.D.
Název diplomové práce: Zkoumání účinku CTA1R7K-ChrA-DD způsobujícího toleranci na BDC2.5 CD4 buňky vyvolávající diabetes

Tento projekt byl zaměřen na studium CTA1R7K-ChrA-DD proteinu, navozujícího toleranci. Bylo zkoumáno, jestli tento protein může inhibovat autoimunitní diabetes a zda-li léčba CTA1R7K-ChrA-DD proteinem může ovlivňovat proliferaci a produkci cytokinů BDC2.5 CD4 buněk. Léčba CTA1R7K-ChrA-DD proteinem byla účinná, když myši byly léčeny vícekrát (ale pouze jednou ze dvou opakovaných pokusů s použitím stejného protokolu), na druhou stranu, když obdržely pouze jednu dávku, léčba byla bez efektu. Po *in vitro* restimulaci PS3 peptidem by se dalo očekávat, že u myší, které byly léčeny CTA1R7K-ChrA-DD proteinem bude produkce INF gamma a proliferace snížena, ale ve většině případů, jsme dostali opačný výsledek. Výsledky této studie naznačují, že CTA1R7K-ChrA-DD protein by mohl v budoucnu být jednou z možností léčby autoimunitního diabetu.

Abstract

Charles University in Prague
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Candidate: Zuzana Kadová
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Title of diploma thesis: Investigations of tolerogenic effects of CTA1R7K-ChrA-DD on diabetogenic BDC2.5 CD4 cells

This project was focused on the study of a novel tolerance inducing vaccine, CTA1R7K-ChrA-DD. It was investigated if this construct can inhibit autoimmune diabetes and if the CTA1R7K-ChrA-DD treatment can affect proliferation and cytokine production of BDC2.5 CD4 cells. The treatment with CTA1R7K-ChrA-DD was effective only one time of two repeated experiments (using the same protocol) when the mice received the dose of construct more times on the other hand when they were treated only once the treatment was without effect. After in vitro restimulation with the PS3 peptide it would be expected less INF gamma production and less proliferation in CTA1R7K-ChrA-DD treated mice but in most cases, we got the opposite result. This study incites hope for that CTA1R7K-ChrA-DD construct actually has ability to induce protection against diabetes and is a good start for further studies.

Content

1 INTRODUCTION

Type 1 diabetes is an autoimmune disease where the insulin producing beta cells in pancreas are destroyed. Autoreactive CD4 cells are key mediators of disease progress (Verdaguer et al. 1997). Thus, a vaccine that could inhibit the autoreactive activities of the CD4 cells is an attractive way of preventing or curing this disease.

In this study, we aim at studying a novel tolerance inducing vaccine, CTA1R7K-peptide-DD. This protein construct is composed of enzymatically inactive A1 subunit of CT and two D subunits of *Staphylococcus aureus* protein A. BDC2.5 CD4 cells are transgenic cells specific against the peptide part of this construct (PS3). It has previously been established by researchers at the department of Microbiology and Immunology (Gothenburg University) that this type of protein construct indeed is tolerogenic (Hasselberg et al. 2010) and can inhibit CIA (Hasselberg et al. 2009). The goal of this study is to investigate if and how this type of construct can also inhibit type 1 diabetes and how treatment with CTA1R7K-ChrA-DD construct can affect proliferation and cytokine production (IFN gamma) of BDC2.5 cells.

In this project, diabetes will be induced by the transfer of BDC2.5 CD4 cells into NOD-scid mice (Peterson and Haskins 1996). The NOD-scid mice will be treated with CTAR7K-ChrA-DD to induce tolerance of CD4 cells (that are specific against the peptide part of the construct) and protect the mice against type 1 diabetes. The glucose levels in the urine will be measured and proliferation by ^{3}H thymidine incorporation and cytokine production (IFN gamma) by ELISA will be assessed.

2 REVIEW OF LITERATURE

2.1 Type 1 diabetes

Diabetes is a disease which occurs when the beta cells of pancreatic islets cannot produce enough insulin or when the body cannot effectively use the insulin. This causes that the concentration of glucose in the blood to increase. It leads to a persistent hyperglycemia.

In this study we investigate the prevention and treatment of type 1 diabetes. Type-1 diabetes is known as insulin dependent diabetes and typically occurs in children, adolescent and young adults. Type 1 diabetes is an autoimmune disease and is characterized by the destruction of insulin-producing beta cells of pancreatic islets. Insulin is the main hormone, which is necessary to move glucose into cells, where it is stored and later used for energy. Without enough insulin, the level of glucose in the bloodstream increases instead of going into the cells. The body can not use this glucose for energy. Beta cell destruction results in persistent hyperglycemia and long term complication. Long-term complications of type 1 diabetes develop gradually, over years. The most frequently occurring complications include heart and blood vessel disease, nerve damage (neuropathy), kidney damage (nephropathy), diabetic retinopathy, foot damage, skin and mouth conditions, osteoporosis, pregnancy complications. Diabetes contributes to the development of cardiovascular disease, such as: angina pectoris, atherosclerosis, heart attack, stroke and high blood pressure. A high level of sugar can cause damage of the walls of the capillaries that nourish the nerves, especially in the legs. This can lead to tingling, burning and pain usually begins at the tips of the toes or fingers and gradually spreads upward. It can result in loss of sense of feeling in the affected limbs. When the nerves that control digestion are injured, it can lead to problems with nausea, vomiting, constipation or diarrhea. The people with diabetes are more susceptible to skin problems, the bacterial and fungal infections occur more often. Kidney serves as a filter of the waste from our blood and serve damage of this filtering system can result in kidney failure or irreversible end-stage kidney disease. Diabetes increases the risk of retinopathy caused by damage of blood vessels of the retina. When around 70% of the beta cells are destroyed and the pancreas can no longer produce insulin, the typical symptoms can occur. The most alarming symptoms include dehydration, feeling tired,

dry skin and mouth, urinating more often (to try to clear the sugar from the body), nausea, vomiting, stomach pain, weight loss, losing the feeling in the feet and etc. The treatment of type-1 diabetes needs to start as soon as possible. The goal is to keep blood glucose level as closely as possible to normal level of sugar in bloodstream. Daytime blood sugar level should be between 4.4 to 6.7 mmol/l and the bedtime numbers between 5.6 to 7.8 mmol/l. Everyone who has type-1 diabetes needs insulin therapy to survive. A very important part of treatment is a healthy lifestyle (including healthy eating, counting carbohydrates and physical activity) and blood sugar monitoring. Sometimes short-term complications such as hypoglycemia, hyperglycemia and diabetic ketoacidosis can appear and may lead to seizures or to the loss of consciousness (Alemzadeh et al. 2007, American Diabetes Association 2010, American Diabetes Association 2010)

It is not exactly known what causes type-1 diabetes but the contributors of the development of this autoimmune disease are environmental and genetic risk factors.

2.2 Immune system

The main function of the immune system is to defend the organism against attack by foreign invaders (such as bacteria, parasites, viruses, fungi and etc.). The other activities of the immune system are identification and destruction of abnormal or mutant cells. The immune system needs to be able to distinguish between the body´s own cells and foreign cells and etc.

The immune system is divided into two basic subdivisions, the innate immune system (non-specific) and the acquired immune system (specific) (Fig. 1).

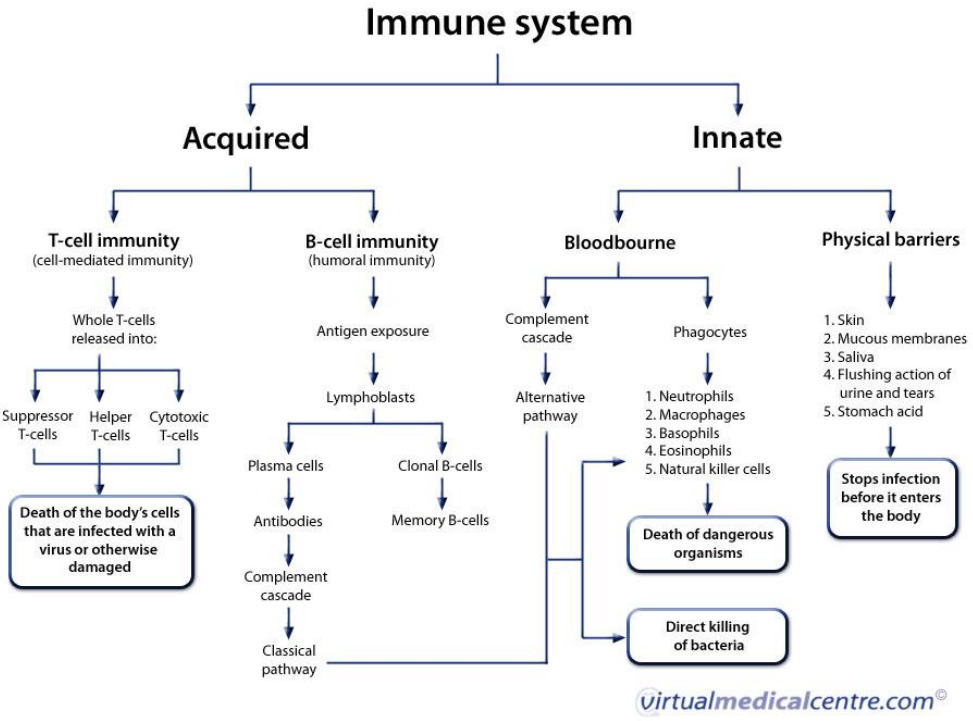

Figure 1: Parts of immune systém

Source: http://www.virtualmedicalcentre.com/uploads/VMC/Anatomy/Immune_system_large.jpg

The innate immunity represents the first line of defense against invasion of pathogens. The innate immunity is antigen-nonspecific and does not demonstrate immunological memory and lasting protective immunity. The non-specific immune system includes both humoral immunity and cell mediated immunity. The cell-mediated immunity is represented by the phagocytic cells (such as neutrophils, eosinophils, basophils, monocytes, macrophages) and natural killer. The humoral immunity involves complement cascade, cytokines, interferon and acute phase protein. In addition, crucial in protection against infections are anatomical barriers (intact skin surface, mucous membranes- ciliated epithelial cells, saliva, tears, mucous secretions), temperature (the temperature of body inhibits microbial growth), low pH (stomach, skin, vagina), chemical factors (such as fatty acids, pepsin, lysozyme- hydrolytic enzyme and etc.) (Hořejší and Bartůňková 2005)

Acquired immune system is the second line of defense. The acquired immune response is antigen- dependent. There exists lag time between exposure and maximal response unlike the innate immune system. The characteristic feature of these reactions is immunological memory, so the second time the adaptive immune system reacts more rapidly on subsequent exposure to the same organism. All agents that are foreign to the body have on their surface unique patterns called antigen (immunogen- is an antigen

recognized by the body as nonself). This part (antigen) allows the cells of acquired immune system to detect them. After the detection of the antigen, the agents are recognized as foreign and the adaptive immune response is stimulated and the acquired immune system can mount the attack. The adaptive immune system includes humoral (mediated by B-cells) and cell-mediated immunity (based mainly on T- lymphocytes) (Hořejší and Bartůňková 2005, Peakman and Vergani 2009)

2.2.1 The main parts of immune system (cells, tissue and organs of IS)

The immune system is made up of many different organs and tissues (widely dispersed throughout the body), cells and molecules. Immune responses are provided by many types of cells and molecules and by their mutual interactions (Hořejší and Bartůňková 2005)

2.2.1.1 Organs/tissues of the immune system

The lymphoid organs are divided into two types: primary and secondary lymphoid organs. The primary (central) lymphoid organs include bone marrow and thymus. These organs provide the environment for birth, differentiation, maturation and central selection of immunocompetent cells. As for the secondary (peripheral) lymphoid organs, spleen, lymph nodes, Peyer´s patches in the intestine, tonsils and appendix and mucosa-associated lymphoid tissues (MALT) in respiratory, gastrointestinal and reproductive system belong to this group. In these organs the antigen-specific immune reactions take place. They are the site where lymphocytes are exposed to antigen and where the development of adaptive immune response occurs (Goldsby et al. 2003, Stewart and Cooley 2009)

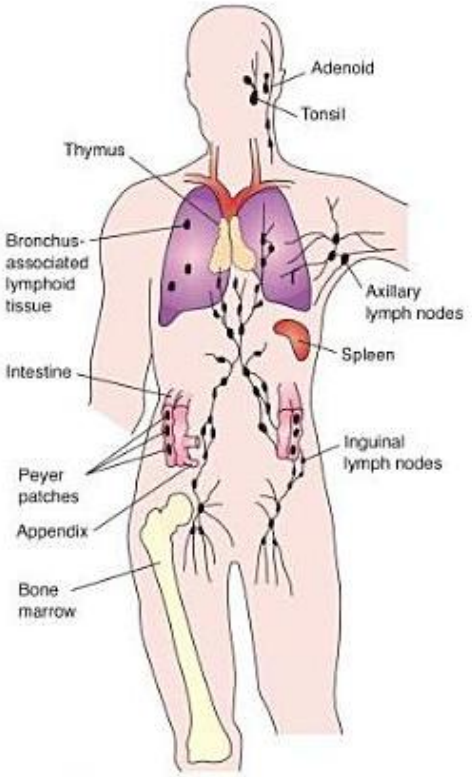

Figure 2: Central and peripheral lymphoid organs and tissues

Source: Essentials of Pathophysiology: Concepts of Atered Health States (Porth 2010)

The thymus is a flat, bilobed and elongated organ situated in the lower part of the neck above the heart. T-cell progenitors migrate from the bone marrow to enter the thymus. Thymus is the place where the process of maturation of T lymphocytes is located. Pre-T cells that enter the thymus are functionally and phenotypically immature. As the T-cells move through the cortex into medulla, they undergo the process of proliferation and selection. Cell multiplication, maturation and selection are under the influence of thymic hormones and cytokines. During the maturation the T cells receptors and antigens (that distinguish them from nonself) start to be developed. In thymus, two types of selection take place. T cells are first positively selected, which means that those T lymphocytes which do not have adequate affinity to self- antigen bound to MHC molecules expressed on cortical thymic epithelial cells die by apoptosis. Then they are negatively selected. During this process T cells that are able to bind strongly with self-peptide MHC complexes that are presented on APCs (such as dendritic cells and macrophages) are eliminated and destroyed. The mature, immunocompetent T cells (called naive) leave the thymus and migrate to peripheral lymphoid tissues through the bloodstream (Moran et al. 2008, Porth 2010)

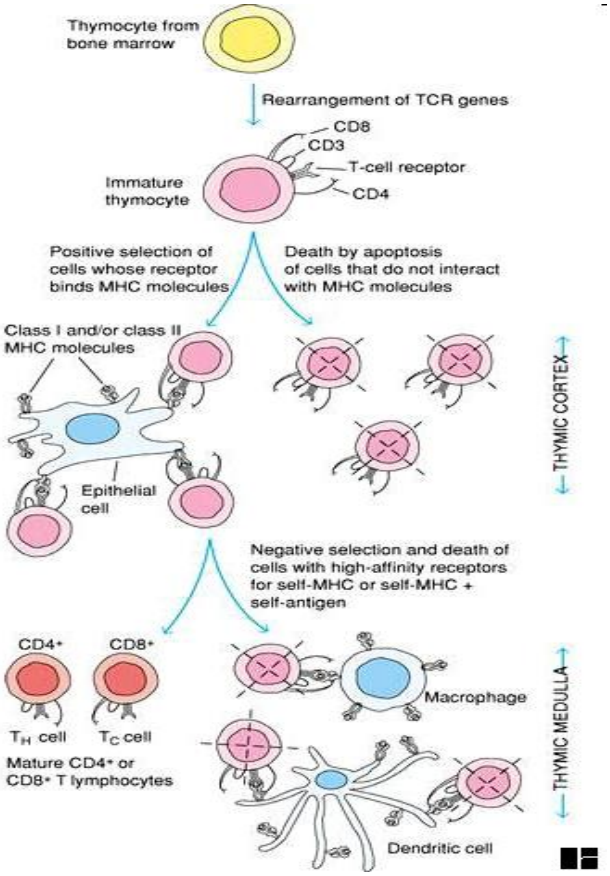

Figure 3: The processes of maturation,proliferation and selection of T lymphocytes in thymus

Source:

http://www.cartage.org.lb/en/themes/sciences/LifeScience/GeneralBiology/Immunology/ImmunityInfection/CellActivation/TCellMaturation/TCellMaturation.htm

Bone marrow is the spongy tissue found inside the bones. All cells of the immune system have a common origin. All these cells (white blood cells, red blood cells and platelets) arise from pluripotent stem cell in bone marrow. The process of B cell maturation takes place in this organ in opposite to T cells, where the process of maturation occurs in the thymus.

Lymph nodes are small, oval, bean-shaped and encapsulated structures that are spread in the body, especially in the axillae and groin, and along the great vessels of the neck and abdomen and thorax. The lymphoid organs are connected with other organs and tissues by networks of lymph vessels, blood vessels and capillaries. The lymph vessels called afferent bring the lymph from tissues into the lymph nodes. The lymph leaves the lymph nodes via efferent lymph vessels. The lymph nodes serve as a filter and their

function is the elimination of foreign materials from lymph before it enters the bloodstream. Naive lymphocytes migrate from the primary lymphoid organs (bone marrow and thymus) to the nodes via bloodstream. Lymphocytes and antigen presenting cells flow through the node and antigen is displayed to lymphocytes. It causes that the lymphocytes can interact with antigen (Hořejší and Bartůňkova 2005, Porth 2010)

The spleen is a large and ovoid organ found in the left abdominal part. This organ is divided into two parts: the red pulp and the white pulp. The red pulp serves as a filter of the blood (red blood cells). This is the part where old (senescent) or demaged red blood cells are removed. The red pulp is well supplied with arteries. The white pulp is the part of spleen filled with B and T cells that are permeated by macrophages and dendritic cells. The antibodies are synthesized in white pulp (Porth 2010)

2.2.1.2 Cells of the immune system

Very important parts of immune system are different types of white blood cells (leucocytes). These cells of immune system have the origin in bone marrow (they arise from pluripotent stem cells). There exist two basic lines of cells of immune system: myeloid and lymphoid. They differentiate along distinct pathways (Fig. 4) (Hořejší and Bartůňková 2005)

The cells derived of myeloid line are the part of non-specific immunity. The myeloid line includes:

- Monocytes: Circulate in the bloodstream and they migrate to the tissue and here they differentiate into different types of macrophages. The main functions of these cells are phagocytosis, cytokine production and antigen presentation.

- Macrophages: The precursors of these cells are monocytes. Macrophages have specific names determined by their location in the body. As well as monocytes their main function is phagocytosis. They serve as an antigen presenting cells and it results in activation of T lmphocytes. So, macrophages are not only the part of innate immune system, but these cells play very important role in adaptive immunity because they stimulate lymphocytes to respond to pathogens.

- Dendritic cells: Denritic cells present antigen (APCs).

- Neutrophils: These cells circulate in the blood and they are the most common cells of white blood cells (make up about 50 to 75 %). Neutrophils are the first type of

immune cells that arrive to the areas of infection and injury tissue through the process called chemotaxis (often within an hour). Their main function is phagocytosis.

- Eosinophils: As other granulocytes, they are able to phagocyte larger particles. These cells participate in allergic reactions and parasitic infections.

- Basophils: Basophils circulate in the bloodstream and they migrate into the tissue. These cells in tissue are called mast cells and they have many similar characteristics. The granulocytes participate in inflammatory reaction. They store in their granules histamine and other mediators and enzymes. On their surface they have the receptor with high affinity to Fc fragment of IgE. IgE is an immunoglobulin which plays a very important role in macroparasite defense and allergy. When IgE bind to the receptor on their surface, the cells start to secret inflammatory chemicals such as histamine, leucotrienes, serotonin and etc (Eales 2003)

The lymphoid line is made up of NK cells, T lymphocytes and B lymphocytes. The cells of the adaptive immune system are lymphocytes. Lymphocytes come in two major groups: B cells and T cells. The maturation of B cells takes place in bone marrow and they mediate humoral immunity, whereas T cells are produced by bone marrow but they maturate in thymus. T cells are involved in cell-mediated immune response. On their surface they have a receptor (B cell receptor/T cell receptor). These receptors are specific for particular antigen. So, each B cell and T cell is able to bind only to a particular molecule structure.

B cells play a key role in humoral immune response. They produce antibodies in response to antigen. As it was mentioned above, they develop in the bone marrow and then they migrate through the bloodstream to various part of the body. The immune reaction mediated by B cells is based on detection of antigen via B cell receptor on the surface of B cell. After the binding of antigen and BCR, the B cells engulf the antigen and digest it into fragments. These antigen fragments are then presented on the B- cell class II MHC (this structure is specific for helper T cells). Helper T cells then bind the B cells. Cytokines secreted by the Th cells help the B cells to differentiate into plasma cells that produce antibodies and into the B memory cells that are formed during the primary immune response.

T cells are responsible for cell-mediated immunity. On the surface of T cells there are T cell receptors (TCRs) which recognize only the peptide fragments part of antigens that are bound to specialized glycoprotein molecules called MHC on the surface antigen presenting cells. There exist two subsets of T cells. Most of T cells belong to one of two

subsets. T lymphocytes called helper T cells have a molecule CD4 on their surface. Helper T cells help B lymphocytes to produce antibodies and another function of these cells is that they help phagocytic cells (such as macrophages and dendritic cells) to destroy ingested pathogens. The presence of CD4 molecules determinates that CD4 T cells bind epitopes that are part of MHC II. The other type of T lymphocytes is T cells called cytotoxic. The main function of these cells is to kill attacking cells of the body that could be infected for example by viruses. The cytotoxic T cells have on their surface co-receptor called CD8. These lymphocytes bind epitopes that are part of MHC I. The MHC I is expressed by almost all the cells of the body, on the other hand only APCs such as dendritic cells, macrophages and B cells can express MHC II (Hořejší and Bartůňková 2005, Elgert 2009, Porth 2010)

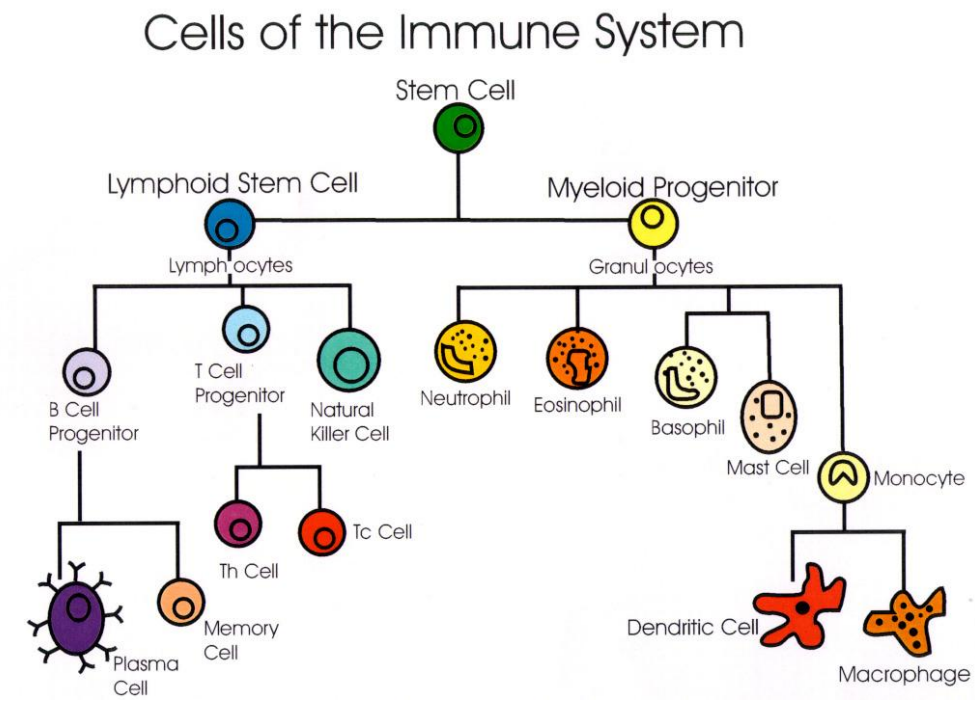

Figure 4:Differentiation of various types of leucocytes from stem cells

Source: file:///C:/Documents%20and%20Settings/Pc/Plocha/pranzatelli-immune.htm

2.3 The role of CD4 T cells and CD8 T cells in diabetes

Type-1 diabetes is caused by T-cells mediated destruction of insulin producing beta cells in pancreas. Presence of both class MHC I restricted CD8(+) T cells and class MHC II restricted CD4(+) T cells participates in pathogenesis of autoimmune diabetes. A previous study has shown that CD4+ T cells can induce type-1 diabetes in NOD-scid mouse model if they were derived from a diabetic donor, but to induce type-1 diabetes in NOD-scid mice with the transfer of CD4+ T cells obtained from pre-diabetic mice the presence of CD8+ T cells is necessary (Christianson et al. 1993). In another study the role of CD4+ and CD8+ T cells in the pathogenesis of NOD mouse (a mouse strain that spontaneously develops diabetes) was tested on athymic NOD nude mice that are completely free of T cells and pathological changes. It was found that $CD4^+$ T cells play a key role in the development of insulitis and that $CD8^+$ T cells migrate into the islets and are subsequently, with the aid of $CD4^+$ T cells, differentiated into killer cells which act against β cells (Yagi et al. 1992). By Phillips et al. (2009) have shown that the development of type-1 diabetes requires presence of both CD8+ and CD4+ T cells. They have proven that in the absence of CD8+ T cells diabetes was not induced which can support the idea that CD8+ T cells may be the final effector cells (Phillips et al. 2009).

It has been shown that in the pancreatic infiltrate and in the pancreas draining lymph nodes where the initial presentation of islet antigen by dendritic cells (DCs) to islet antigen-specific T cells occurs, many different types of cells such as natural killer cells, NKT cells and DC subsets are present and could contribute to the beta-cell destruction (Turley et al. 2003). CD8+ and CD4+ T cell-mediated beta-cell destruction is thought to be caused by production of cytokines such as interferon gamma that induce expression of the death receptor FAS (also known as CD95) and chemokine production by β-cells. Beta-cell apoptosis could be mediated through the activation of FAS by FAS ligand-expressing activated T cells. The inflammation is enhanced by chemokine production by beta-cells which further leads to recruitment of mononuclear cells to the site (Eizirik et al. 2009). In addition, IFNγ can activate macrophages and induce increased pro-inflammatory cytokine production, including interleukin-1β and TNF. Extensive studies in rodent models have demonstrated a very important role of islet beta cell-expressed proteins (autoantigens) such as insulin, GAD65/67, ICA 105 (IA-2), Carboxypeptides H,

Peripherin, etc, which are the targets of the autoimmune process in type 1 diabetes (Castano et al. 1990, Chatenoud and Bach 1993, Christie et al. 1994).

2.4 The role of Treg cells in autoimmune diabetes

The Treg cells are characterized by immunosuppressive activity. The naturally activated subset of CD4+ T cells (Tregs) is the best defined T cell population with immunosuppressive property. These Treg cells contribute to self tolerance and protect from a variety of autoimmune disease. Treg cells express the interleukin (IL)-2Rα chain CD25 and Foxp3 (forkhead fox p3) transcription factor, which controls the development and function of Tregs (Fontenot et al. 2003). CD4+Foxp3+ regulatory T cells (Treg) are known to regulate a variety of immune responses, such as the progression of autoimmune diabetes (T1D) (Zheng and Rudensky 2007, Sakaguchi et al. 2008). In previous studies the punctual and specifical ablation of Treg cells was investigated in the BDC2.5/NOD mouse model (described later) of type 1 diabetes. Within 3 days after this ablation, diabetes was developed and Natural killer cells and Interferon gamma were activated within 7 hours of Treg cells ablation. Interferon gamma had an impact on the gene-expression program of the local CD4+T effector cell population and supported the aggressive attack of these effector T cells on Langerhans islets. This shows that Treg cells play a key role in protection from type-1 diabetes. Autoimmune diabetes may occur as a result of lack of Treg cell compartment due to a mutation in the Foxp3 gene (Feuerer et al. 2009).

2.5 Cholera toxin and mutants

Cholera toxin has been shown to be a strong immunogen and mucosal and parenteral adjuvan which elicites mucosal and systematic B and T cell responses against unrelated co-administrated antigens in experimental models (Williams et al. 1999, Sanchez and Holmgren 2008). Cholera toxin (CT) consists of two parts. The subunit A with ADP-ribosylating property which is associated to a non-covalently linked pentametric ring of B subunit, responsible for cell binding (Rappuoli et al. 1999). The main receptor for B subunit is the GM1-ganglioside receptor (Holmgren 1973) which is

present on all nucleated cells and the binding to this receptor can cause the accumulation of the cholera toxin in CNS. The enzymatically active subunit A is composed of 2 chains A1 and A2 (Rappuoli et al. 1999).

CTA1-DD is a protein construct which is composed of the A1- subunit of cholera toxin and two D subunits of *Staphylococcus aureus* protein A. CTA1-DD lacks the B subunit of cholera toxin and cannot bind to GM1-ganglioside receptors. This gene fusion protein has kept the adjuvant effect, but unlike cholera toxin, this construct is nontoxic. After i.n. administration, CTA1-DD has been shown to have an adjuvant activity, which is comparable to the adjuvant effect of cholera toxin (Hasselberg et al. 2010, Hasselberg et al. 2009).

CTA1R7K-DD is a mutant of CTA1-DD. The A1 subunit is enzymatically inactive by a point mutation where amino acid arginine is replaced by lysine. The single point mutation results in the loss of ADP-ribosylating property. CTA1R7K-DD protein constructs induce tolerance and promotes peptide-specific tolerance in TCR transgenic CD4+ T cells (Hasselberg et al. 2010, Hasselberg et al. 2009).

In a previous study the protective effect of fusion protein CTA1R7K-COL-DD against collagen-induced arthritis (CIA) following i.n. administration was investigated. The mechanism behind the tolerance to CIA appears to be mediated by peptide-specific regulatory T cells induced by mucosal exposure of the peptide containing CTA1R7K-COL-DD vector. This study was the first where it was found that ADP-ribosylation unequivocally can control the outcome of tolerance (Hasselberg et al. 2009).

2.6 Chromogranin A as an autoantigen and diabetogenic BDC2.5 T cells

The BDC2.5 clone is prepared from cells from the spleen and lymph nodes of diabetic NOD mice (Haskins et al. 1989, Haskins 2005). BDC2.5 CD4 cells are specific against Chromogranin A, present in the pancreas (Judkowski et al. 2001, Yoshida et al. 2002, Stadinski et al. 2010). The BDC2.5 CD4 cells can readily be identified using antibodies recognizing their T-cell receptor. It has been shown, that the BDC2.5 T cell line is highly pathogenic after the adoptive transfer into young NOD mice and that they accelerate the development of type-1 diabetes (Haskins and McDuffie 1990). In addition, the incorporation of the genes encoding BDC-specific TCRs into T cell–deficient

NOD.*scid* mice (Haskins and McDuffie 1990, Peterson et al. 1995, Peterson and Haskins 1996) rapidly induces type-1 diabetes (Burton et al. 2008). There are several studies where the transgenic BDC2.5 cells have been used, providing insights to the type 1 pathogenesis.

Induction of diabetes depends on age of recipient mouse. BDC2.5 CD4 cells can not induce diabetes in NOD mice older than 3 weeks of age or in adult NOD/*scid* mice. Also, the BDC2.5 TCR transgenic NOD mice themselves do not all develop diabetes, only in 10-20% of these mice the disease occurs (Gonzalez et al. 1997). The reason why not all the BDC2.5 TCR transgenic NOD mice develop diabetes could be the presence of regulatory T cells in these animals and the fact that the regulatory T cells can prevent beta islet cells from being destroyed (Gonzalez et al. 2001)

NOD-scid mouse is a mouse strain with multiple defects in adaptive as well as nonadaptive immunologic function (such as lack of CD4 and CD8) (Shultz et al., 1995). A previous study shows that after the transfer of transgenic BDC2.5 CD4 cells into NOD-scid mice the onset of type-1 diabetes is very quick (Burton et al. 2008, Peterson and Haskins 1996). BDC2.5/NOD-scid mouse is a simplified and robust model for autoimmune type-1 diabetes. In the present study the mouse model where transgenic BDC2.5 CD4 cells are transferred to NOD-scid mice to induce diabetes was used.

3 OBJECTIVES

In this project we investigate whether the CTA1R7K-ChrA-DD fusion protein provides therapeutic protection against type-1 diabetes following i.n. administration. In CTA1R7K-ChrA-DD the peptide part is modified to be specific for the BDC2.5 CD4 cells. The peptide part is denoted ChrA in the protein construct, and the same peptide sequence will be called PS3 when used only as a free peptide.

4 MATERIALS AND METHODS

4.1 Mice and induction of type-1 diabetes

For this project NOD-scid mouse model (in the age of 6-8 weeks; both sexes) and BDC2.5 transgenic NOD mice (in the age of 6-12 weeks; both sexes) were used. All these mice were kept under pathogen-free conditions at Department of Experimental Biomedicine (University of Göteborg). Mice were killed by cervical dislocation.

Diabetes was induced by the transfer of BDC2.5 CD4 cells isolated from BDC2.5 transgenic NOD mice (donor mice) into NOD-scid mice. The spleen was taken from donor mouse and put in PBS. Then the spleen was mashed and cells were centrifuged and dissolved in PBS. Splenocytes were counted with trypan blue (1/10 dilution in PBS). Before the transfer of splenocytes into NOD-scid mice, a flow cytometry was used to prove the percentage of BDC2.5 CD4 T cells. Unpurified splenocytes containing 50 000 or 500 000 CD4 cells were transferred i.p. into NOD-scid mice.

4.2 Antigen and immunomodulators

The PS3 peptide (SRLGLWVRME) was obtained from JPT peptide technologies (Berlin, Germany) and the fusion protein was expressed and purified by MIVAC development (University of Gothenburg) and provided by Professor Nils Lycke (fig. 5). The novel plasmids of the pSY-nCTA1-DD vector that contained the PS3 sequence witch was inserted between the CTA1 enzyme and DD-targeting moiety were constructed. The point mutation where amino acid Arginine is replaced by Lysine was made to yield a fusion protein called pSY-nCTA1R7K-ChrA-DD. The construction of the fusion protein is describe in the study by Hasselberg (Hasselberg et al. 2009).

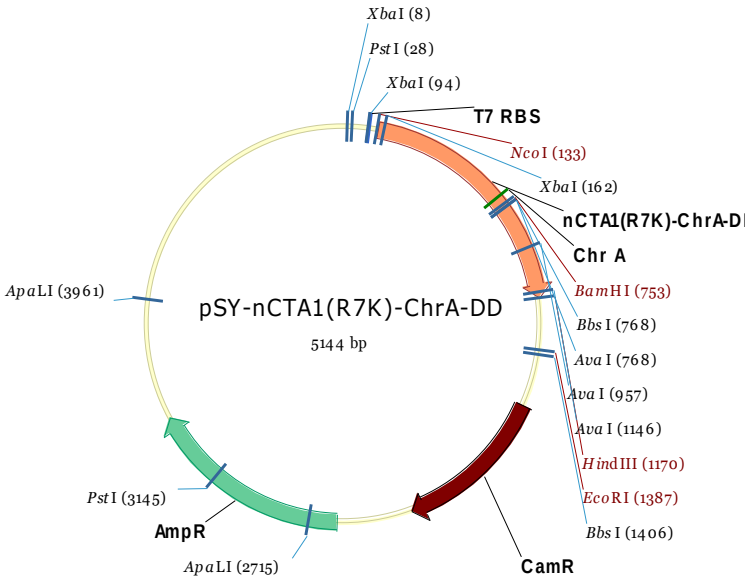

Figure 5: Depiction of the pSY-nCTA1R7K-ChrA-DD plasmid, where the CTA1R7K-ChrA-DD encoding DNA was cloned into the plasmid by MIVAC development

4.3 Induction of tolerance (treatment with CTA1R7K-ChrA-DD construct)

The CTA1R7K-ChrA-DD protein was administered intranasally at 5µg/dose in 20µl PBS, control mice received only PBS. Two treatment regimens were performed; either treatment only once at the day of transfer of BDC2.5 cells, or the treatment of four different days (-2), 0, 4, 6 (0 being the day of transfer of BDC2.5 cells) (fig. 6). Following days the level of glucose in the urine was monitored using urine dipstick analysis.

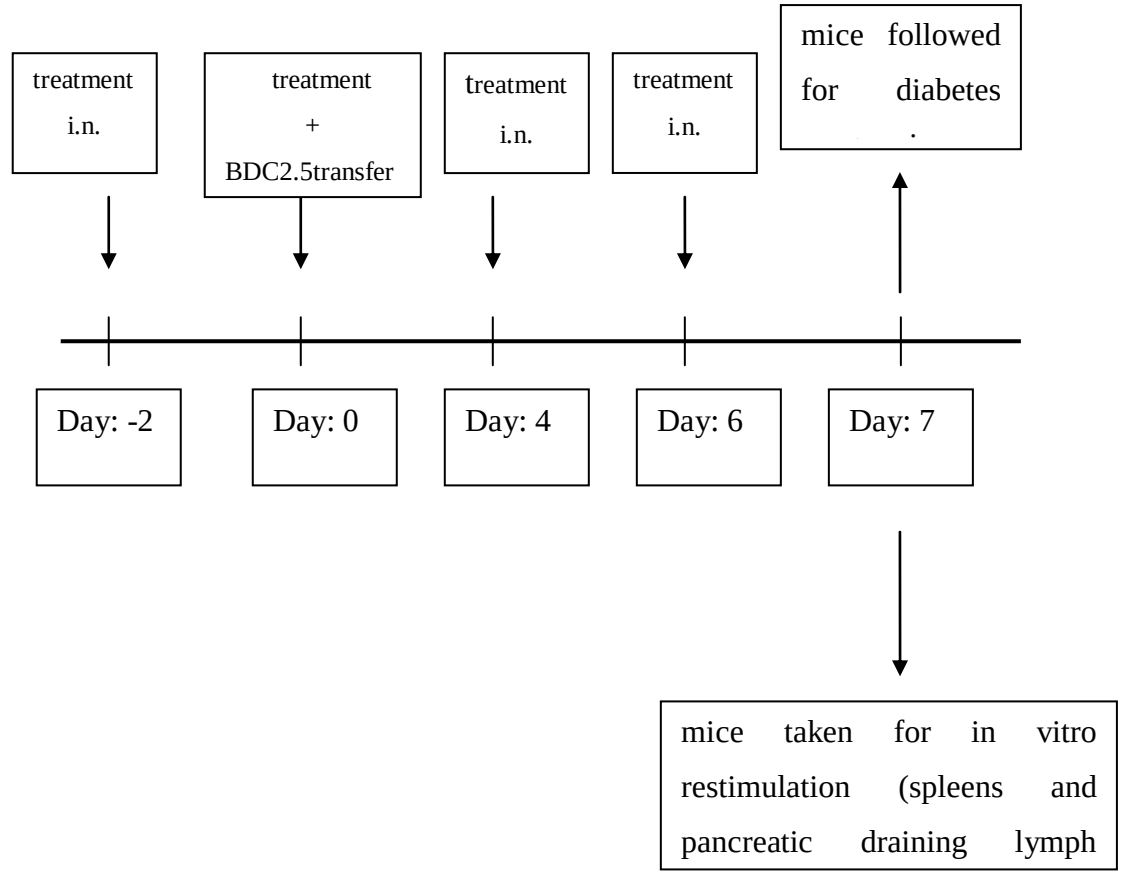

Figure 6: Treatment regiment

Treatment regiment. The therapeutic treatment with 5µg CTA1R7K-ChrA-DD protein was initiated 2 days before transfer of splenocytes, containing 50 000 or 500 000 BDC2.5 CD4 cells, and repeated the same day as the transfer and then twice after transfer (days 4 and 6). At day 7 the part of mice was taken for *in vitro* restimulation and other mice followed for disease progression.

4.4 In vitro restimulation with PS3 peptide

At day 0 NOD-scid mice were given the transfer of splenocytes, containing 50 000 BDC2.5 CD4 cells. Mice were treated four times i.n. with 5µg CTA1R7K-ChrA-DD (fig. 6). As controls, mice treated with phosphate buffered saline were used. At day 7 they were sacrificed by cervical dislocation. Spleens and pancreas draining lymph nodes were taken from BDC2.5 transferred and treated NOD-scid mice and the cells from these organs were used for *in vitro* restimulation.

Triplicate cultures of splenocytes and lymph node-cells were performed in 96-well microwell plate (167008, Nunc) with 3 μg/ml PS3 peptide, with 1μg/ml PS3 peptide, with 1μg/ml Con A and only with medium (RPMI complete) containing RPMI (Invitrogen AB, Sweden), 10% FBS (Sigma AB, Sweden), 1% HEPES (1mM;Invitrogen AB, Sweden), 1% PEST (Invitrogen AB, Sweden), 1% Nap (10mM;Invitrogen AB, Sweden), 0.1% 2-mercaptoethanol (0.5mM;Invitrogen AB, Sweden). Splenocytes were added in the amount of 400 000 cells/well and lymph node-cells in amount of 63 700 cells/well. The plates were incubated for 48/72 hours at 37˚C in humidified 5% CO_2. Cytokine production and cell proliferation were measured after 48/72 hours of incubation.

4.5 Measuring of cell proliferation

^{3}H thymidine incorporation was used to determine the proliferation rate of splenocytes and lymph node cells after *in vitro* stimulation with PS3 peptide. ^{3}H thymidine (Amersham Bioscience) stock was diluted in RPMI complete (dilution factor: 1/40). For all proliferation assays, cultures were incubated for 48 hours or 72 hours at 37 ˚C in a 5% CO_2, pulsed and harvested 6-24 hours later for determination of ^{3}H tymidine incorporation using liquid scintillation and luminescence counter 1450 MicroBeta Trilux. Results were expressed as counts per minute (cpm).

The principle of this test is based on the fact that proliferating cells double their DNA before dividing. In the replication process the radioactive thymidine is inserted in the new synthesized DNA. The faster the cells divide the more cells become radioactively labeled and the more radioactive counts per minute can be measured. Thus growing cells give a high signal in this test method. Resting or dead cells do not replicate and so they were less or not radioactive.

4.6 Measuring of IFN-gamma production

Interferon-gamma production was quantified using ELISA test. The supernatant for ELISA was taken after 72 hours of incubation. This test was performed according to manufacturer`s protocol**.**

ELISA plate (Greiner, 655061, 96F microwell plate without lid, VWR) were coated with 50 µl capture antibodies (R&D Systems Europe, Abingdon, UK)) diluted in PBS (final concentration: 4µg/ml) and than incubated overnight at room temperature. After coating wells were blocked with 0.5% BSA-PBS (buffer) and after this, the plate was incubated for 1 hour at 37˚C. 2 fold-dilution of standard (Duoset ELISA) diluted in buffer (final concentration: 2ng/ml) in duplicate and supernatant in triplicate were added to the plates, followed by incubation overnight at 4˚C. Conjugate (DY485) diluted in 0.5% BSA-PBS (final concentration: 400ng/ml) where then added to each well for 3 hours at room temperature. Then fallowed addition of avidin-HRP diluted in buffer and incubation for 45 minutes. It continued with preparation of substrate solution (TMB stock solution diluted to 0.1mg/ml in 0.05M phosphate-citrate buffer and H_2O_2- 2 µl per 10 ml of solution). The reaction was stopped by adding 25 µl of 1M H_2SO_4. The enzymatic reaction was analyzed using a spectrophotometer and the plate was read at 450 nm. Data are expressed as concentration cytokines (ng/ml), as extrapolated from standard curve with recombinant cytokines.

In this study cytokine sandwich ELISA was used. This test can specifically detect and quantitate the concentration of cytokines. This method makes use of highly purified capture antibodies (anti-cytokines antibodies) which are coated (noncovalently adsorbed) onto plastic microwell plates. The unbound antibodies are washed away and the immobilized antibodies serve to specifically capture soluble cytokine proteins present in samples which were added to the wells. After rinsing to remove unbound material, the captured cytokines proteins are detected by biotin-conjugated anti-cytokine antibodies (detection antibodies) followed by an enzyme-labeled avidin-HRP.

Very popular enzymes are these which convert a colorless substrate to a colored product. In this study TMB solution as a substrate and HRP as an enzyme were used. The enzyme (HRP) acts as a catalyst to oxidize substrate (TMB solution) in the presence of hydrogen peroxide to produce a blue color. Reaction was stopped with H_2SO_4 to cause complex to turn yellow.

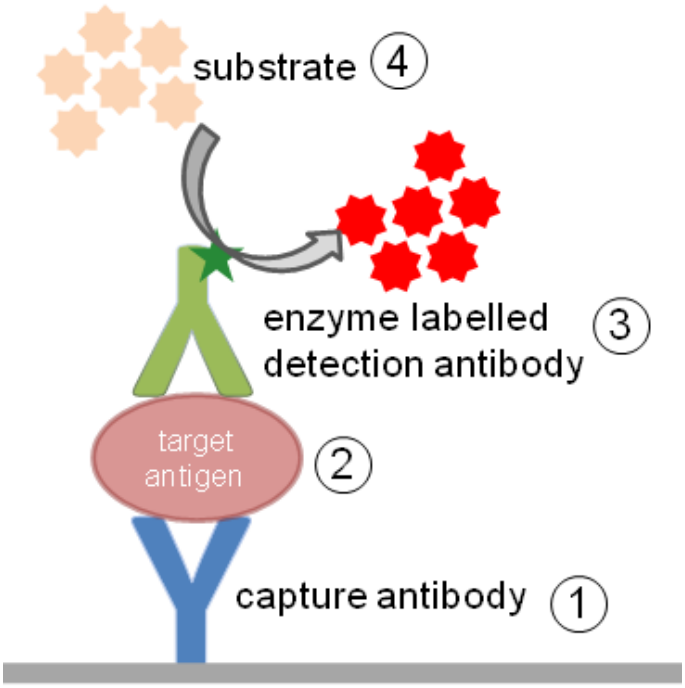

Figure 7: Sandwich ELISA method

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Sandwich ELISA method. The first step is to coat the plate with capture antibodies. In the second step the antigens are added. In the third step the detection antibodies labelled with enzyme are added. Finally (the forth step), a substrate is added to the plate.

5 RESULTS

5.1 Activation of BDC2.5 CD4 cells by in vitro restimulation with the PS3 peptide

At first we wanted to test if BDC2.5 cells can be restimulated *in vitro* with the PS3 peptide. Splenocytes were taken from a BDC2.5 transgenic mouse and cultured in the presence of various concentrations of the PS3 peptide or ConA. Con A is a CD4 cell mitogen and was used as a positive control. This experiment was performed twice (with two spleens from different mice), with similar results. One of the two experiments is presented (fig. 8, 9, 10 and fig. 11).

Proliferation was measured by H^3-thymidine incorporation. We found that the PS3 peptide induced a proliferative response in BDC2.5 cells (fig. 8), which was similar in magnitude to the proliferation gained with ConA (fig. 9). Further, the IFN-gamma production in the same cell cultures was measured by ELISA. It was found that the proliferative responses were accompanied by increased production of IFN-gamma, both after peptide and after ConA restimulation (fig. 10 and 11). Thereby, we concluded that we could use the PS3 peptide for *in vitro* restimulation of BDC2.5 cells in following experiments.

A

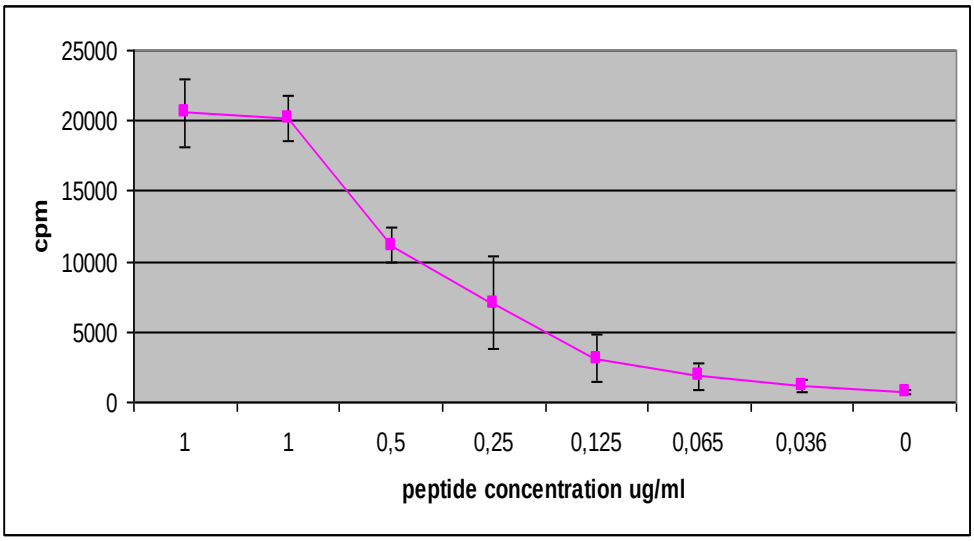

Figure 8: Proliferative ability of the PS3 peptide (A) in comparison with Con A (B) measured by 3H thymidine incorporation - (A)

B

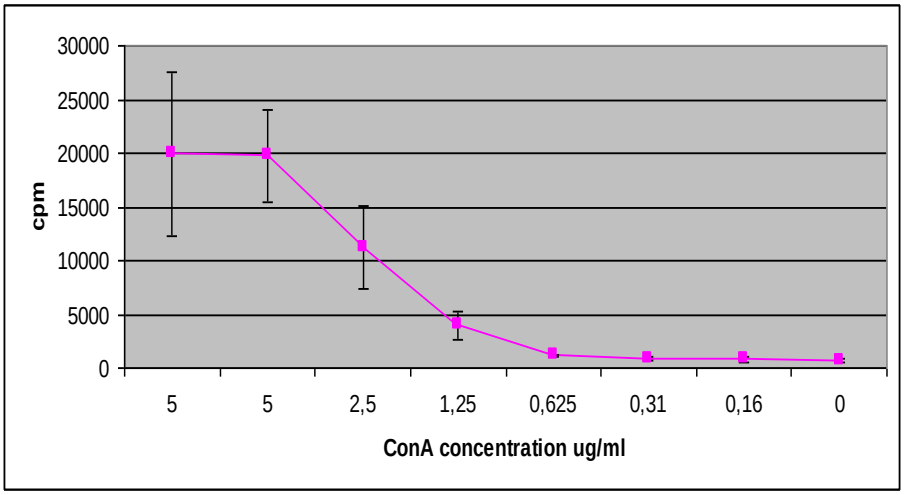

Figure 9: Proliferative ability of the PS3 peptide (A) in comparison with Con A (B) measured by 3H thymidine incorporation - (B)

A

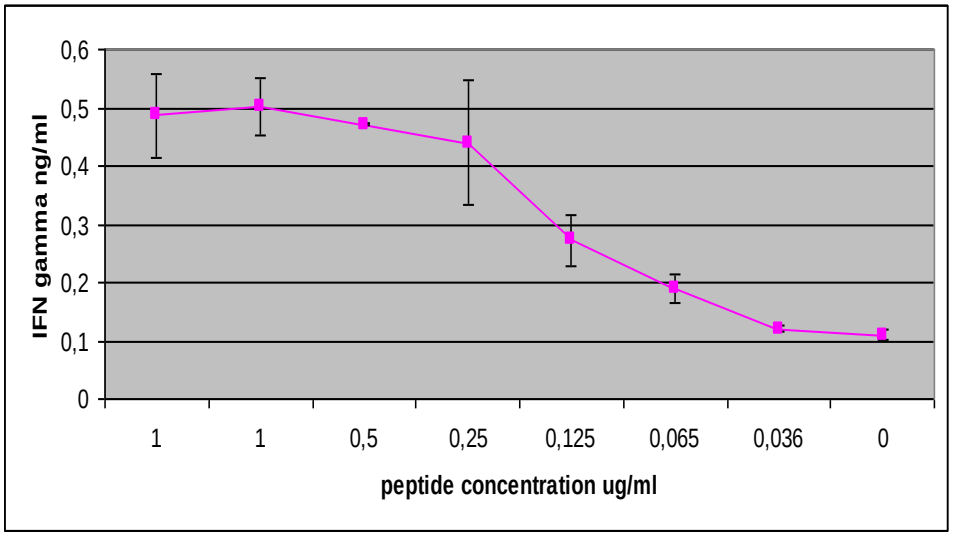

Figure 10: INF-gamma production was measured by ELISA. The graphs show the concentration of INF-g in supernant (in ng/ml) after in vitro restimulation for 3 days with PS3 peptide (A) or with Con A (B) - (A)

B

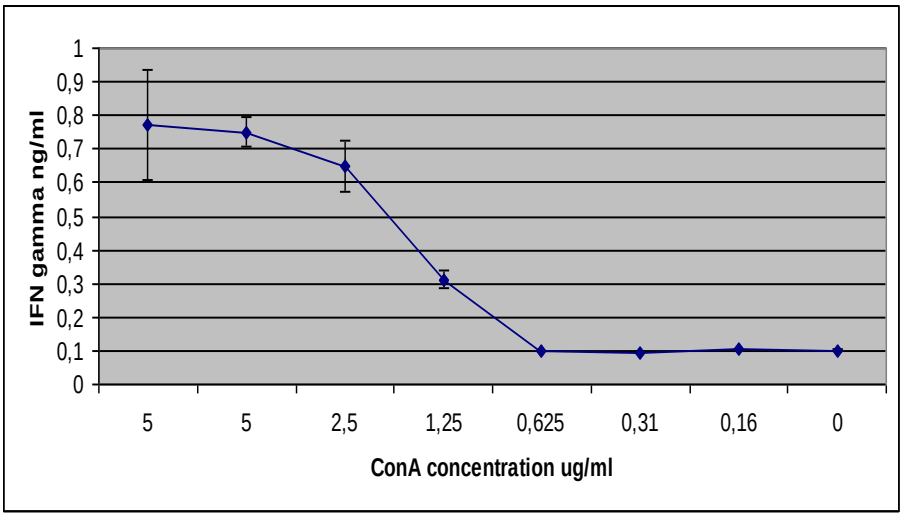

Figure 11: INF-gamma production was measured by ELISA. The graphs show the concentration of INF-g in supernant (in ng/ml) after in vitro restimulation for 3 days with PS3 peptide (A) or with Con A (B) - (B)

5.2 Prevention of type-1 diabetes by one intranasal CTA1R7K-ChrA-DD treatment

In this experiment, we investigated if CTA1R7K-ChrA-DD administered intranasally could inhibit type-1 diabetes.

We used the mouse model where transgenic BDC2.5 cells were transferred into NOD.scid mice. Previously in our group, 500 000 purified CD4 cells from a transgenic BDC2.5 mouse has been used to induce diabetes in NOD.scid mice (N. Kadri, personal communication). We choose to transfer unpurified splenocytes from a BDC2.5 transgenic mouse, containing either 500 000 or 50 000 CD4 cells. On the same day, the mice were given 5 µg CTA1R7K-ChrA-DD or PBS (control mice) intranasally. The glucose levels in the urine were measured during the following days. In both the case when the mice received 500 000 or 50 000 BDC2.5 cells the mice developed diabetes, starting from the ninth day after cell transfer. Treatment with CTA1R7K-ChrA-DD had no protective effect on disease development, since 100% of the treated mice became diabetic (fig. 12).

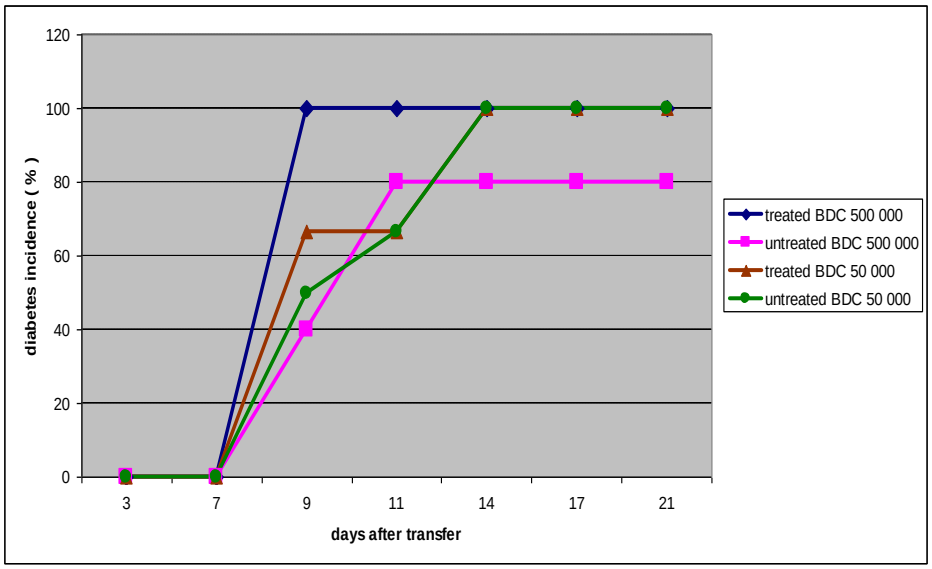

Figure 12: The protective effect of intranasal treatment with CTA1R7K-ChrA-DD protein

The protective effect of intranasal treatment with CTA1R7K-ChrA-DD protein. NOD-scid mice were transferred with 500 000/50 000 BDC2.5 cells. The mice were treated with PBS (untreated) or were treated intranasally with 5 µg of CTA1R7K-ChrA-DD in the same day as BDC2.5 transfer. The level of glucose in the urine was measured for 21 days.

5.3 Repeated treatments with CTA1R7K-ChrA-DD to inhibit type-1 diabetes

In the previous experiment we found that the NOD.scid mice transferred with BDC2.5 splenocytes and treated only once with CTA1R7K-ChrA-DD became diabetic, so we changed the protocol. We wanted to see if an increase in the amount of treatments could prevent diabetes, so this time we treated the mice four times, both before (-2) the day of (0) and after transfer (days 4 and 6). We chose to only transfer splenocytes containing 50 000 CD4 cells.

The experiment was repeated twice. In the first experiment, only 25% of the CTA1R7K-ChrA-DD treated mice became diabetic. On the other hand, in the control group (PBS), 100% became diabetic (fig. 13). Thus, the CTA1R7K-ChrA-DD protein induced effective suppression of type-1 diabetes in intranasally treated mice. In contrast, when the experiment was done again using the same protocol to confirm the effectiveness

of the CTA1R7K-ChrA-DD-treatment, the intranasal therapeutic treatment with CTA1R7K-ChrA-DD did not result in protection against diabetes. All the mice (treated and untreated) became diabetic (fig. 14).

A

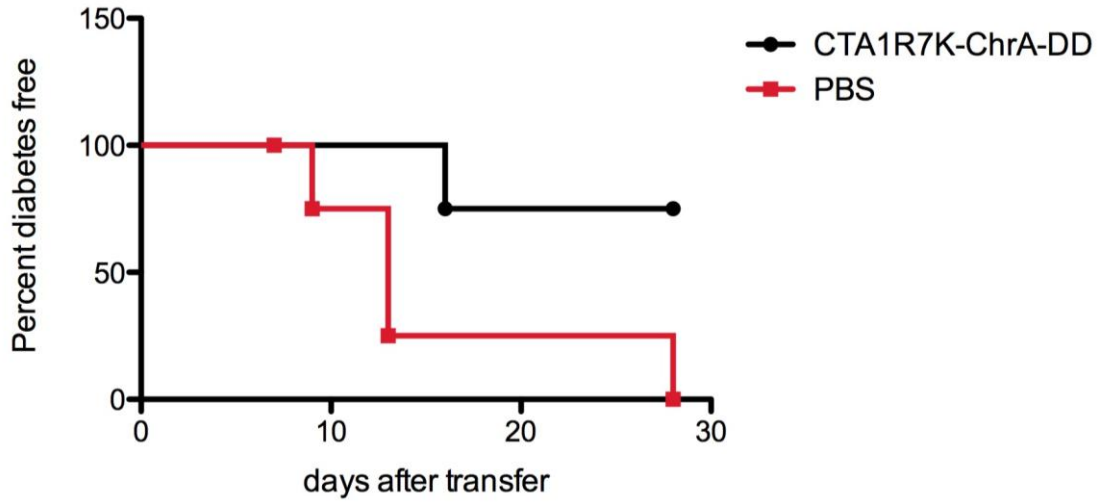

Figure 13: CTA1R7K-ChrA-DD treatment protects against type-1 diabetes- first experiment (A)

B

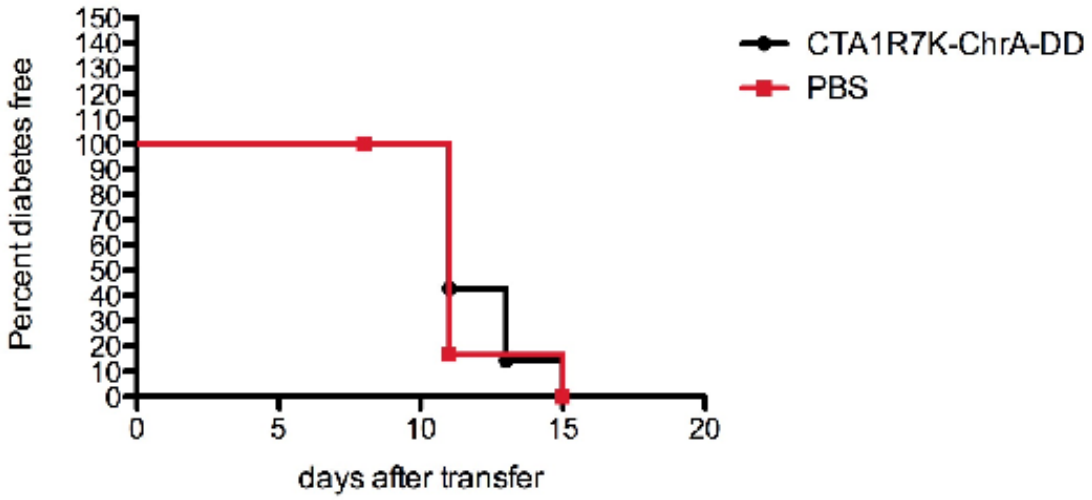

Figure 14:CTA1R7K-ChrA-DD treatment protects against type-1 diabetes-second experiment (B)

CTA1R7K-ChrA-DD treatment protects against type-1 diabetes- first experiment (A) and second experiment (B). NOD-scid mice were immunized with 50 000 BDC2.5 cells. The mice were treated with PBS (untreated) or were treated intranasally with 5 μg of CTA1R7K-ChrA-DD on days (-2), 0, 4, 6 after BDC2.5 transfer. Mice were monitored for the development of diabetes. For the first experiment 4 mice in each group (treated/ untreated) were used. In the second experiment 7 mice were treated with the CTA1R7K-ChrA-DD peptide and 6 mice served as controls (received PBS).

5.4 Cell proliferation and cytokine production (IFN gamma)

Finally, we studied the possible impact of CTA1R7K-ChrA-DD on T cell proliferation and cytokine production (IFN gamma).

As we mentioned above the experiment was done twice using the same protocol. We transferred splenocytes (obtained from a BDC2.5 transgenic mouse) containing 50 000 CD4 cells to NOD-scid mice on day 0. The mice were treated four times (two times before transfer on days (-2), 0 and twice after transfer on days 4 and 6) with CTA1R7K-ChrA-DD administered intranasally or the mice received phosphate buffered saline alone (control mice). After seven days, splenocytes and lymphocytes from pancreas draining lymph nodes were isolated and counted and then reactivated *in vitro* with the PS3 peptide. In both experiments, three mice from either the CTA1R7K-ChrA-DD group or the PBS control group were used. In both experiments we noted that the number of cells was very low (especially in pancreas draining lymphnodes) In the second experiment, the number of cells in the spleen was higher in the CTA1R7K-ChrA-DD treated mice compared to the PBS treated mice, otherwise there was no difference in cell number (fig. 15 and 16). Since we had very few lymph node cells we decided to pool untreated and treated, respectively, cells from several animals for in vitro restimulation. The cells were stimulated *in vitro* in the presence of recall PS3 peptide in various concentrations or with ConA or the cells were cultured alone in medium (RPMI). Proliferation by ^{3}H thymidine incorporation and cytokine production by ELISA were assessed.

The proliferative response was detected 48 hours (only in the second experiment) and 72 hours (in both experiments) after in vitro restimulation. In the first experiment we found that the proliferative response to PS3 differed between CTA1R7K-ChrA-DD-

treated mice and untreated mice (treated with PBS). For splenocytes, we found that those from mice treated with CTA1R7K-ChrA-DD protein demonstrated slightly higher proliferation in response to the PS3 peptide, as compared with cells from PBS treated mice (fig. 17). In contrast, interestingly, cells from pancreas draining lymph nodes showed a reduced proliferative response to the PS3 peptide in CTA1R7K-ChrA-DD-treated mice as compared with untreated mice (control mice). In the repeated experiment the proliferation was measured two times (after 48 and 72 hours of culturing) (fig. 18 and fig. 19). Also in this experiment, the proliferation was higher for splenocytes from CTA1R7K-ChrA-DD treated mice when measured at 48 hours. However, when measured at 72 hours, there is no longer any difference between splenocytes from treated and untreated mice. Moreover, when looking at the proliferation of cells from the pancreas draining lymph nodes, the picture is different between the first and the second experiment (fig. 20 and fig. 21). Importantly, the cpm is considerably lower in the second experiment, less than thousand for the PS3 stimulated cells, as compared with around 7000 or around 15000 cpm for the peptide stimulated cells in the first experiment, even though the same amount of lymphocytes (60 000 cells per well) was used in both experiments. This indicates a difference between the two experiments. However, the trend is the same in the second experiment as in the first, both indicating that the lymphocytes from CTA1R7K-ChrA-DD treated mice proliferate to a lesser extent in response to the PS3 peptide. We could not determine the statistical significance of this difference, since we only had one well for each condition of restimulation.

The IFN-gamma secretion into the culture supernatant was assessed by ELISA after 72 hours of restimulation with the PS3 peptide (at 3μg/ml or 1μg/ml) or with ConA or cells alone. In both experiments we observed that the secretion of IFN-gamma was higher from splenocytes from CTA1R7K-ChrA-DD-treated mice compared with PBS treated mice (fig. 23 and fig. 24). The concentration of IFN gamma was reduced when the splenocytes were cultured with ConA or only cells alone in medium (RPMI). In the repeated experiment, in addition, we measured the IFN-gamma concentration in cells derived from pancreas draining lymph nodes. In untreated mice the IFN-gamma concentration was a bit enhanced in comparison with CTA1R7K-ChrA-DD-treated mice (fig. 25).

A

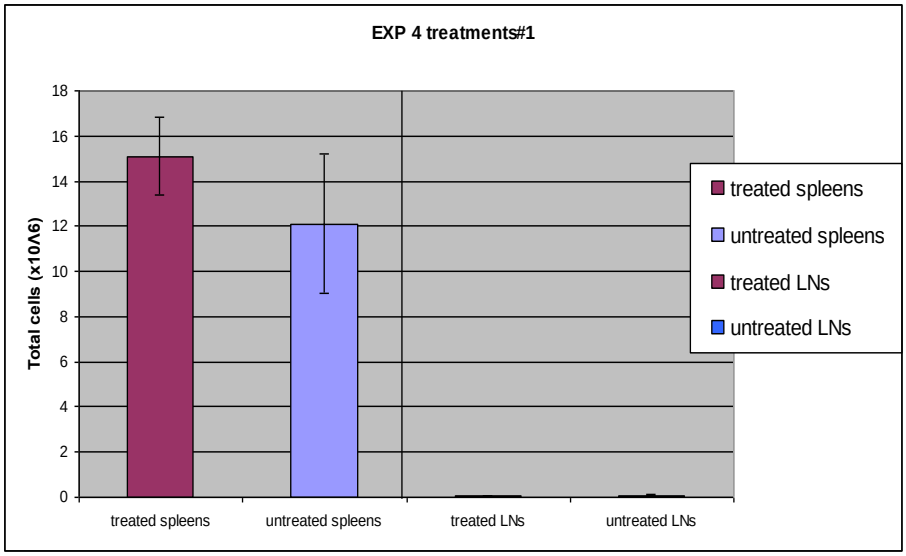

Figure 15: Cell counting - first experiment

B

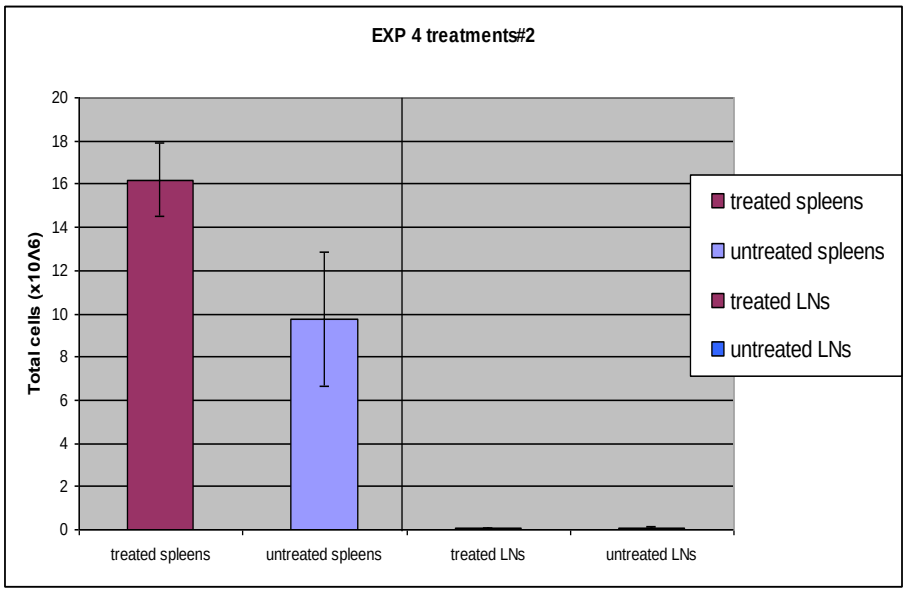

Figure 16: Cell counting – second experiment

A

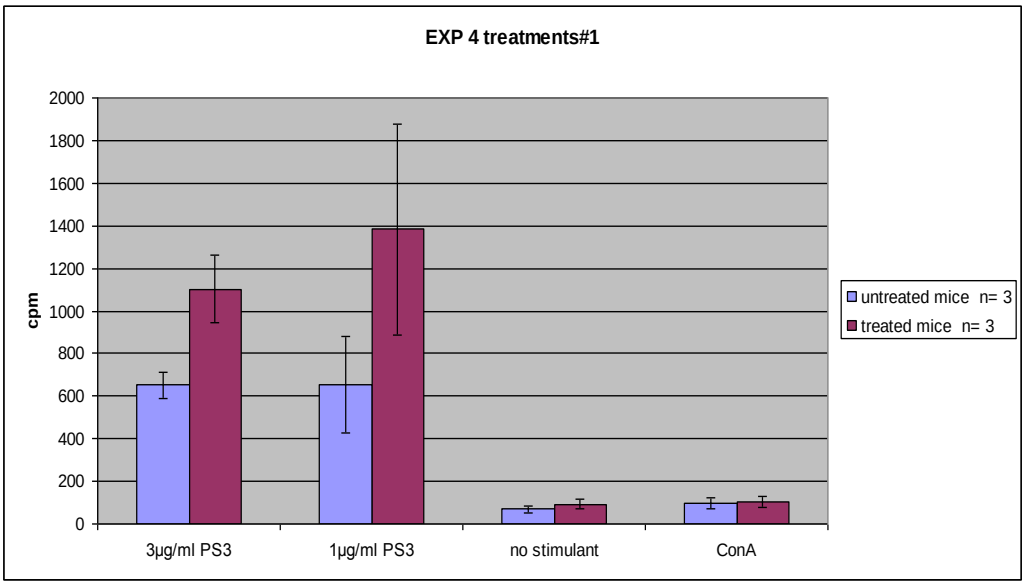

Figure 17: Proliferation of T cells (obtained from spleens) from mice treated with CTA1R7K-ChrA-DD or PBS (untreated) – first experiment (A)

B

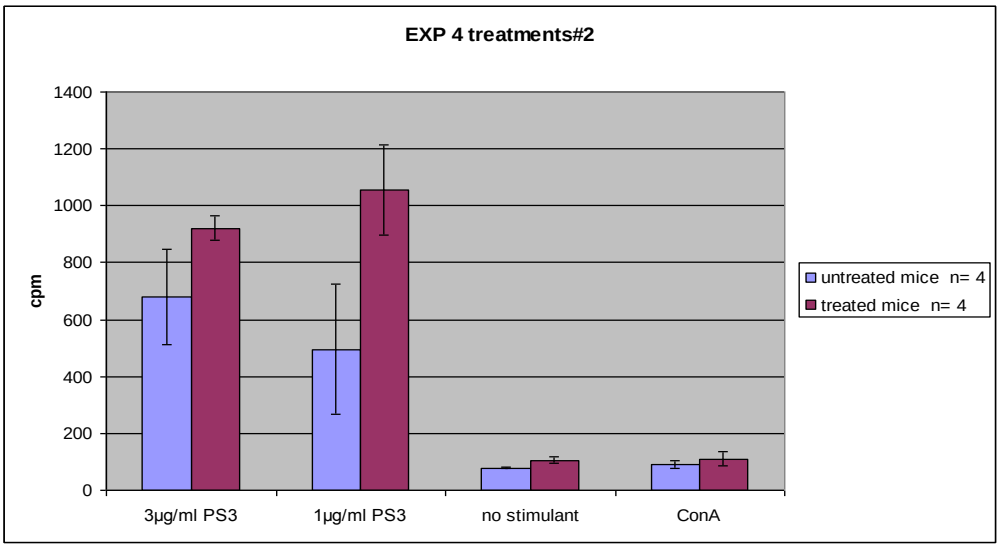

Figure 18: Proliferation of T cells (obtained from spleens) from mice treated with CTA1R7K-ChrA-DD or PBS (untreated) – second experiment (B)

Proliferation of T cells (obtained from spleens) from mice treated with CTA1R7K-ChrA-DD or PBS (untreated) – first experiment (A) and second experiment (B). NOD-mice received 50 000 BDC2.5 CD4 cells. They were treated intranasally with 5µg of CTA1R7K-ChrA-DD or with PBS alone on days (-2), 0, 4, 6 after BDC2.5 transfer.

The spleens were taken 7 days after immunization with BDC2.5 cells. Proliferation was assessed after 48 hours of culturing by 3H thymidine incorporation.

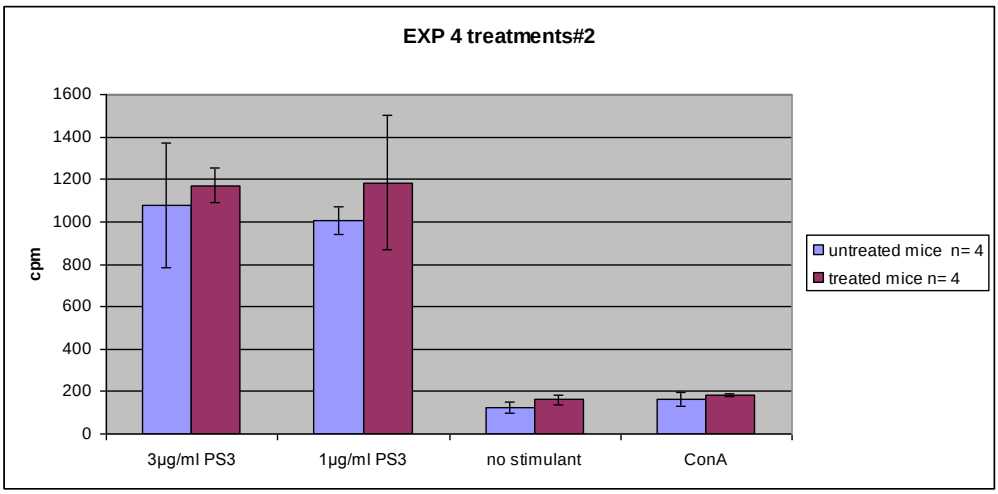

Figure 19: Proliferation of T cells (obtained from spleens) from mice treated with CTA1R7K-ChrA-DD or PBS (untreated) – second experiment

Proliferation of T cells (obtained from spleens) from mice treated with CTA1R7K-ChrA-DD or PBS (untreated) – second experiment. NOD-mice received 50 000 BDC2.5 cells. They were treated intranasally with 5µg of CTA1R7K-ChrA-DD or with PBS alone on days (-2), 0, 4, 6 after BDC2.5 transfer. The spleens were taken 7 days after immunization with BDC2.5 cells. Proliferation was assessed after 72 hours of culturing by ^{3}H thymidine incorporation. 4 mice per group were analyzed (treated/untreated).

A

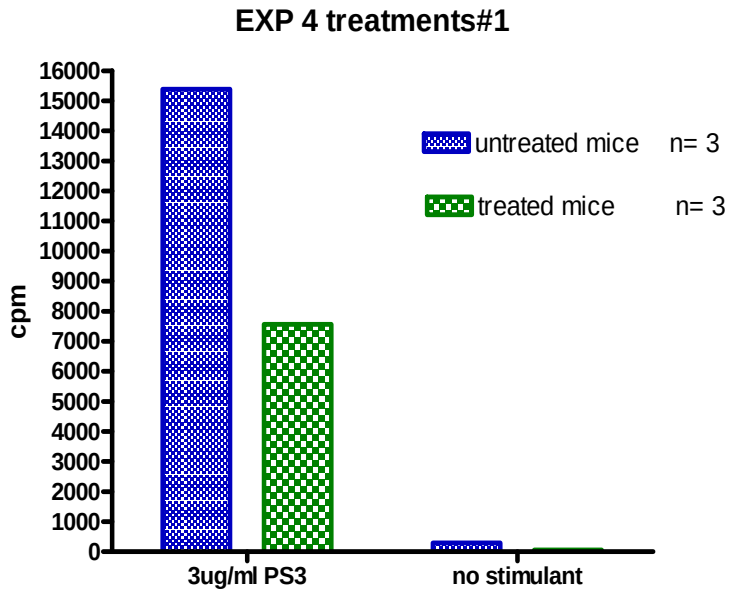

Figure 20: Proliferation of T cells (obtained from pancreas draining lymph nodes) from mice - first experiment

B

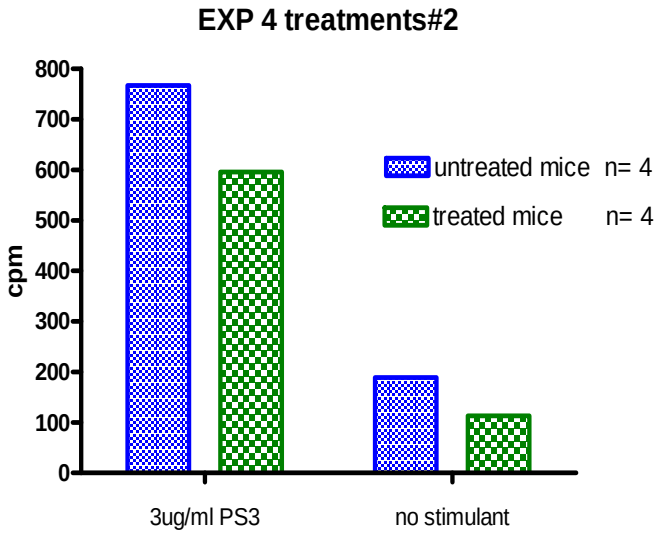

Figure 21: Proliferation of T cells (obtained from pancreas draining lymph nodes) from mice – second experiment

Proliferation of T cells (obtained from pancreas draining lymph nodes) from mice treated with CTA1R7K-ChrA-DD or PBS (untreated) – first experiment (A) and second experiment (B). NOD-mice received 50 000 BDC2.5 cells. They were treated intranasally with 5µg of CTA1R7K-ChrA-DD or with PBS alone on days (-2), 0, 4, 6

after BDC2.5 transfer. The pancreas draining lymph nodes were taken 7 days after immunization with BDC2.5 cells. Proliferation was assessed after 48 hours of culturing by ^{3}H thymidine incorporation.

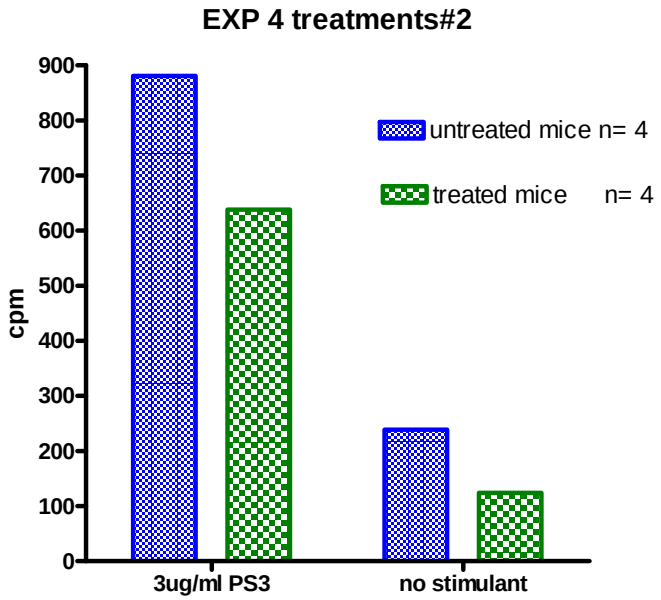

Figure 22: Proliferation of T cells (obtained from pancreas draining lymph nodes) from mice treated with CTA1R7K-ChrA-DD or PBS (untreated) – second experiment

Proliferation of T cells (obtained from pancreas draining lymph nodes) from mice treated with CTA1R7K-ChrA-DD or PBS (untreated) – second experiment. NOD-mice received 50 000 BDC2.5 cells. They were treated intranasally with 5µg of CTA1R7K-ChrA-DD or with PBS alone on days (-2), 0, 4, 6 after BDC2.5 transfer. The pancreas draining lymph nodes were taken 7 days after immunization with BDC2.5 cells. Proliferation was assessed after 72 hours of culturing by ^{3}H thymidine incorporation.

A

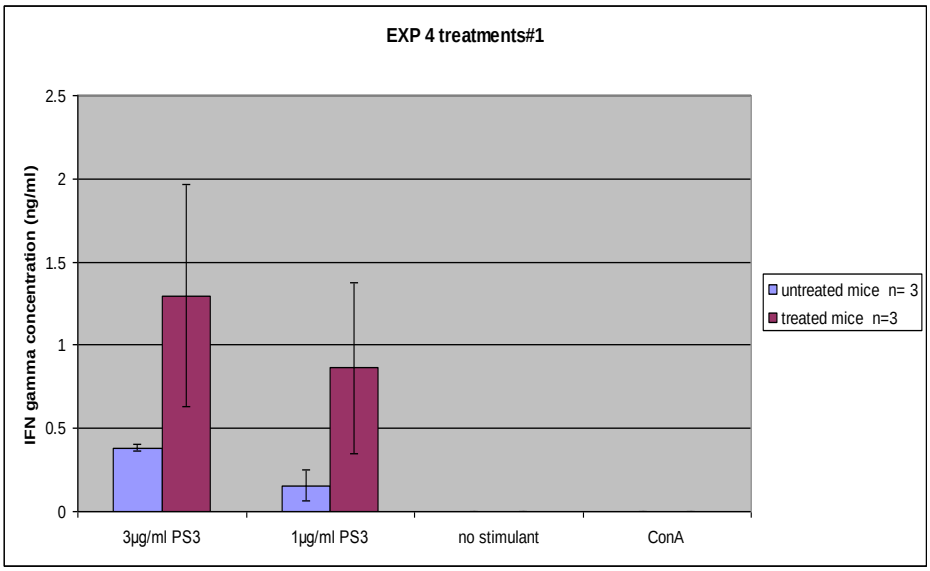

Figure 23: IFN gamma production by splenocytes - fisrt experiment (A)

B

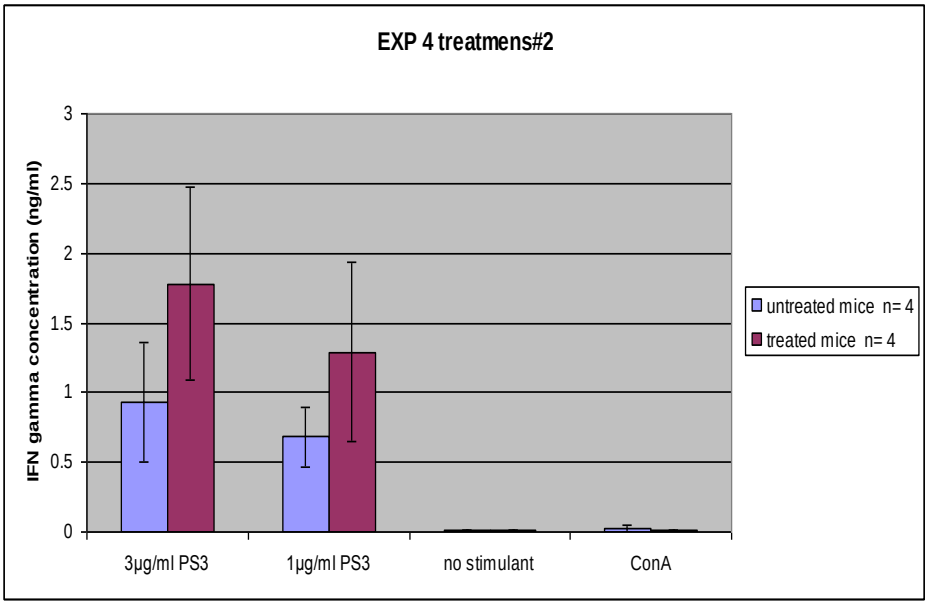

Figure 24: IFN gamma production by splenocytes - second experiment (B)

IFN gamma production by splenocytes - first experiment (A) and second experiment (B). NOD-mice received 50 000 BDC2.5 cells. They were treated intranasally with 5µg of CTA1R7K-ChrA-DD or with PBS alone on days (-2), 0, 4, 6 after BDC2.5 transfer. The spleens were taken 7 days after immunization with BDC2.5 cells Splenocytes were cultured in triplicate in the presence of PS3 peptide (in concentration 3µg/ml or 1µg/ml) or with ConA or only cells in medium. IFN-gamma

production was assessed after 72 h of culturing. The values are given in ng/ml as means +/- SD and represent 3 analyzed mice (first experiment) and 4 analyzed mice (second experiment) in each group (treated/untreated).

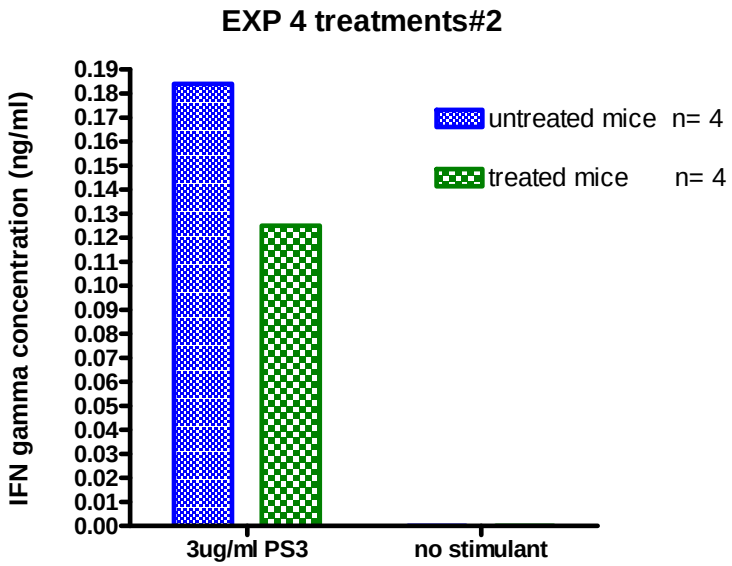

Figure 25: IFN gamma production by pancreas draining lymph nodes – second experiment

IFN gamma production by pancreas draining lymph nodes – second experiment. NOD-mice received 50 000 BDC2.5 cells. They were treated intranasally with 5µg of CTA1R7K-ChrA-DD or with PBS alone on days (-2), 0, 4, 6 after BDC2.5 transfer. The pancreas draining lymph nodes were taken 7 days after immunization with BDC2.5 cells. Cells were cultured in the presence of 3µg/ml PS3. IFN-gamma production was assessed after 72 h of culturing. The values are given in ng/ml.

6 DISCUSSION

In the present study we wanted to investigate if the CTA1R7K-ChrA-DD protein could protect against autoimmune type-1 diabetes in a mouse model where BDC2.5 transgenic cells are transferred to NOD.scid mice. Further, we also wanted to show how the *in vivo* treatment with CTA1R7K-ChrA-DD affected cytokine production (IFN gamma) and cell proliferation upon *in vitro* restimulation.

We used two different treatment protocols where the mice were treated either once, on the same day as BDC2.5 transfer, or four times. In all cases, the CTA1R7K-ChrA-DD construct was administered intranasally. The treatment with CTA1R7K-ChrA-DD only once, the same day as transfer, was not effective, since all mice became diabetic (Figure 10). The reason might be that the dose of CTA1R7K-ChrA-DD construct was too small to yield an efficient inhibition of all transferred BDC2.5 cells. Another reason might be that the treatment was too close in time to the cell transfer, so that the transferred cells had time to travel to the pancreas draining lymph nodes, become activated and continue out to the pancreas and initiate β-cell destruction before the treatment with CTA1R7K-ChrA-DD had the time to inhibit these autoreactive BDC2.5 cells.

Therefore, in the second treatment protocol, we increased the number of treatments, and they were carried out both before and after cell transfer. This treatment protocol was carried out twice. There was a difference in disease protection between these two experiments. The first experiment suggested a protective effect of the CTA1R7K-ChrA-DD protein, since only 25% of the treated mice became diabetic, compared to 100% in the PBS control group. On the other hand, when the experiment was repeated the treatment had no protective effect, all treated mice became diabetic. We can only speculate on the reason for this discrepancy. It might be that the quality of the donor cells differed in between the two experiments. The donor cells in the first experiment could somehow have been less aggressive, as indicated by the fact that diabetes emerged rather slowly in the first experiment, with the last mouse becoming diabetic as late as 28 days after BDC2.5 transfer, and therefore more prone to be affected by the CTA1R7K-ChrA-DD treatment.

The proliferation and IFN gamma production were assessed *in vitro*. Despite the fact that the treatment with CTA1R7K-ChrA-DD peptide had different effects on disease protection in the two experiments, the *in vitro* restimulation gave rather similar results.

Both times, splenocytes from CTA1R7K-ChrA-DD treated mice were more able to proliferate (at least at 48 hours after *in vitro* restimulation) and secrete IFN-gamma upon peptide restimulation compared to the splenocytes from PBS treated mice. This is opposite to what we expected and also in contrast to the study by Hasselberg et al (Hasselberg et al 2010), where treatment rendered the OVA-peptide specific CD4 cells less able to proliferate and produce IFN gamma upon *in vitro* restimulation. We propose that the results of these two experiments differ because different recipient mouse strains and donor cells for transfer were used. The treatment protocols were also different: in this study the mice were treated intranasally at repeated time points whereas only once in the study by Hasselberg et al. Another difference is that in the later study, the mice were immunized with the OVA peptide inserted in the CTA1R7K-OVA-DD construct and later used for *in vitro* restimulation. Such an immunization was not performed in this study. It should also be noted that the CPM values indicating proliferation were only around 1000. This is unusually low, in other experiments 1000 CPM is often the background value. However, since the peptide restimulation gave significantly higher CPM compared to cultures without any stimulant (the negative control), we have decided to consider these signals as true signals.

In contrast to the results with *in vitro* restimulated splenocytes, when investigating the lymphocytes from the pancreas draining lymph nodes, the effect of CTA1R7K-ChrA-DD treatment seemed to have had an inhibitory effect. In the first experiment, the proliferation was clearly reduced in cells from CTA1R7K-ChrA-DD treated mice compared to PBS treated mice. The tendency is the same in the second experiment, even though the proliferation is drastically lower for both treatment groups compared to the first experiment. The second experiment also show a reduction in IFN-gamma production by the lymphocytes from the CTA1R7K-ChrA-DD treated mice.

The effect of CTA1R7K-ChrA-DD on IFN gamma production is interesting in relation to diabetes, since it seems to participate in the development of disease when expressed by autoreactive CD4 cells (Eizirik et al. 2009). It would be locigal if a treatment that could inhibit diabetes also reduced IFN gamma production by autoreactive cells.

7 CONCLUSIONS

To summarize, in this study we aimed at investigating if intranasal treatment with CTA1R7K-ChrA-DD could prevent diabetes and also how this treatment affect the proliferative and cytokine secreting ability of the autoreactive cells. As it was mentioned above, in this project two different treatment protocols were used. The treatment only once was not effective. We could see a protective effect of CTA1R7K-ChrA-DD in one out of two experiments, when the mice were treated with this construct four times. In treated mice it was accompanied by a increased proliferation and IFN gamma production upon *in vitro* restimulation. In contrast, we got opposite results from lymphocytes where the treatment reduced INF gamma production and proliferation.

The present study incites hope for that CTA1R7K-ChrA-DD actually has the ability to induce protection against type 1 diabetes and is a good starting point for further studies. In the future, the transfer and treatment protocol can be modified, for example by lowering the number of transferred BDC2.5 cells, to see if we can find a situation where diabetes is induced but prevented by CTA1R7K-ChrA-DD treatment.

8 ABBREVIATIONS

ADP	-	Adenosine diphosphate
APC	-	Antigen presenting cell
B cells	-	B lymphocytes
BSA	-	Bovine serum albumin
CD4	-	Cluster of differentiation 4
CD8	-	Custer of differentiation 8
ChrA	-	Chromogranin A
CIA	-	Collagen-induced arthritis
CNS	-	Central nervous system
Con A	-	Concanavalin A
cpm	-	Counts per minute
CT	-	Cholera toxin
DCs	-	Dendritic cells
ELISA	-	Enzyme-linked immunosorbent assay
FAS	-	Apoptosis- related factors
FBS	-	Fetal Bovine Serum
FOXP3	-	Forkhead box P3
GAD	-	Glutamic acid decarboxylase
GM1	-	monosialotetrahexosylganglioside
HEPES	-	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	-	Horseradish peroxidase
ICA	-	Islet cells antibodies
IFN γ	-	Interferon gamma
IL	-	Interleukin
MHC	-	Major histocompatible complex
MIVAC	-	The Mucosal Immunobiology and Vaccine Center
Nap	-	Na-pyruvate
NKT	-	Natural Killer T lymphocytes
NOD	-	Non obese diabetic
PBS	-	Phosphate buffered saline

PEST	-	100U/ml penicillin G / 100µg/ml streptomycin sulphate
PS3	-	Peptide Synthesizer
RPMI	-	Roswell Park Memorial Institute
scid	-	Severe combined immunodeficiency
T cells	-	T lymphocytes
TCR	-	T cell receptor
Treg	-	Regulatory T cells
TMB	-	3,3´, 5,5´ - tetramethylbenzidine
TNF	-	Tumour necrosis factor
T1D	-	Type 1 diabetes

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10 LIST OF FIGURES