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Treating diabetes using novel biocompatible biomaterials for islet cell transplantation creating an immune privileged site

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Abstract

Diabetes mellitus is a metabolic disease affecting 422 million people worldwide, which puts a massive financial burden on health systems and patients. Type 2 diabetes is characterized by progressing insulin resistance, due to unhealthy nutrition, lacking exercise and other environmental factors. Type 1 diabetes patients are dependent on insulin shots to compensate their lack of insulin, caused by the destruction of insulin producing beta cells by autoantibodies. ELASTISLET is an innovative attempt of transplanting functional pancreatic islets for establishing a long lasting and autonomous insulin therapy in type I diabetes patients. These islet cells are immunoisolated by a semi-artificial capsule composed of Elastin-like recombinamers (ELRs), which should be inert and semipermeable to exchange nutrients, oxygen, glucose and insulin. Nevertheless, immune cells and antibodies have to be isolated from the immune privileged site. Furthermore, the formation of a fibrotic capsule, around the implant, should be avoided. Our aim is to test whether this coating material is triggering an immune, allergic or foreign body response upon intraperitoneal and subcutaneous implantation in BALB/c mice *in vivo*. Differential cell count, cytokine measurement and skin histology suggest that the ELRs do not trigger inflammation or cytokine production. However, the serum IgG1 titer was elevated compared to the negative control. Moreover, a second implantation of the material did not cause an elicitation of IgG1 antibodies. These results support that the ELRs might be sufficient for islet cell transplantation. However, it remains to be determined, whether a long-term and self-contained insulin therapy can be established by transplanting these polymers as a coat for islet cells.

Zusammenfassung deutsch

Diabetes mellitus ist eine metabolische Krankheit, die 422 Millionen Menschen weltweit betrifft. Dies bedeutet eine große finanzielle Belastung für weltweite Finanz- und Gesundheitssysteme. Typ 2 Diabetes PatientInnen leiden an einer Insulin Resistenz, welche oft auf ungesunde Ernährung, fehlende Bewegung und andere Umwelteinflüsse zurückzuführen ist. Typ 1 Diabetes PatientInnen sind von Insulin Injektionen abhängig, um fehlende Insulinproduktion zu kompensieren, welche durch die Zerstörung von pankreatischen Beta Zellen durch Autoantikörper verursacht wird. ELASTISLET ist ein innovativer Versuch pankreatische Beta Zellen zu transplantieren, um eine permanente und autonome Insulin Therapie, für Typ I Diabetiker, zu schaffen. Diese Inselzellen sind durch eine semi-artifizielle Hülle immunisoliert, welche aus Elastin-ähnlichen Rekombinanten (ELRs) besteht, die inert und permeabel für den Austausch von Nährstoffen, Sauerstoff, Glukose und Insulin ist. Dennoch soll diese Hülle Immunzellen und Antikörper von dieser immun privilegierten Nische ausschließen. Weiterst soll die Bildung einer fibrotischen Kapsel um das Implantat verhindert werden. Unser Ziel ist es zu testen, ob dieses Umhüllungsmaterial Immunreaktionen, Allergien oder Fremdkörperreaktionen, nach der *in vivo* Implantation in BALB/c Mäusen, hervorruft. Die differentiale Zellzählung, Zytokin Messung und Histologie zeigen, dass die ELRs keine Entzündungen oder Zytokin Produktion verursachen. Dennoch war der IgG1 Titer des Blutserums erhöht. Eine zweite Implantation des Materials hat keine verstärkte Antikörperproduktion verursacht. Diese Ergebnisse unterstützen unsere These, dass die ELRs für die Transplantation von Inselzellen geeignet sind. Es bleibt noch herauszufinden, ob dieses Material eine permanente und autonome Insulin Therapie durch ummantelte Inselzellen ermöglichen kann.

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1 Introduction

1.1 Diabetes Mellitus

Diabetes mellitus is a heterogeneous metabolic disorder, which affected about 422 million people worldwide in 2014[1], and is predicted to affect around 642 million people in 2040[2]. Due to unhealthy life styles the disease prevalence is constantly on the rise, especially in western countries. The manifestation of diabetes is chronic hyperglycemia, caused by defective insulin secretion or insulin impact. There are two main forms of diabetes mellitus:

1.1.1 Type 1 diabetes

An autoimmune disease, which causes the destruction of insulin producing pancreatic beta cells by autoantibodies. This form of diabetes is age independent, but often becomes manifested in children and young adults, where insulin production starts to gradually diminish. Type 1 diabetes patients have a genetic predisposition for the disease, but environmental influences, like nutrition and stress may contribute to its' progression[3]. Typical symptoms are frequent urination, constantly feeling hungry, thirsty and tired, weight loss, retarded growth, dry and itchy skin, impaired eyesight, slow wound healing, numbness in feet or toes and life threatening ketoacidosis[4]. Type 1 diabetes is divided into two subtypes:

Subtype 1a: Also called 'immune mediated diabetes', an autoimmune disease with chronic inflammation of the pancreas, causing destruction of insulin producing beta cells. The disease is manifested very slowly, and can already show symptoms before the acute phase begins (pre-type-1-diabetes). Clinical pathologies of this disease are production of autoantibodies against the insulin producing beta cells, inflammation of pancreatic islets and other autoimmune pathologies. Cause of the autodestruction of islet cells can have many causes, like immune stimulation, vitamin D deficiency, short lactation, viral infections, hygienic conditions etc. but also a genetic predisposition can contribute to the

progression of the disease[5]. Genes that are known to contribute to the development of type-1-diabetes are Human-Lymphocyte-Antigens (HLA) genes. This gene family express proteins that play crucial roles in the body's immune responses, for instance the major histocompatibility complex (MHC). Specifically, certain haplotypes of the genes *HLA-DQA1*, *HLA-DQB1*, and *HLA-DRB1* are linked to type 1 diabetes[6]. Nevertheless, not all people with these gene variations develop immune mediated diabetes, which is a character, which shows the complexity of the disease. In about 10% of diabetic children, one parent is affected by type 1 diabetes. If both parents are affected, their offspring has a 10-20% risk of developing the disease. Diagnosis is usually done by autoantibody measurement. Type 1 diabetes patients develop one or several of following autoantibodies: insulin antibodies (IAA), glutamic acid decarboxylase antibodies (GADA), insulinoma-associated autoantigen 2 antibodies (IA2A) and zinc transporter 8 antibodies (ZnT8A)[7]. A patient's treatment is very complex, and can vary from person to person. It does not only include insulin therapy, but also regulating factors that affect insulin requirement, like nutrition, exercise and stress.

Subtype 1b: A less frequent subtype, which is also termed 'idiopathic type 1 diabetes'. This form of diabetes mellitus causes the depletion of pancreatic beta cells, but the mechanisms, unlike subtype 1a, are not immunogenic. Although the causes of the depletion are not known, it is believed to have a genetic background, due to the heaped occurrence in certain ethnic groups[8]. Patients experience periodical ketoacidosis and insulin deficiency, which makes a therapy more delicate. To distinguish subtype 1b from subtype 1a, the HLA status, and presence of autoantibodies is investigated. Due to the scattering characteristics of this disease, it is hard to diagnose and treat patients.

1.1.2 Type 2 diabetes

Caused by insulin resistance, combined with a deficiency of beta cells to produce insulin. Type 2 diabetes has a strong evidence of being inherited under non-mendelian rules. It is shown that the possibility of inheriting diabetes to the offspring is about 40%, if one parent is affected, and 80%, if both are[5].

Nevertheless, in most cases, an unhealthy life style causes the manifestation of this disease. Nutrition containing a high amount of fat and sugar, combined with a lack of physical exercise, is often the cause of type 2 diabetes[9]. A constantly high blood glucose level causes overexposure of insulin to the body's cells, which can result in insulin resistance. Due to the contribution of life style, the disease often occurs in elderly people, or people with low social status (see epidemiology of diabetes mellitus). Type-2-diabetes is in most cases diagnosed, when 55-60% of all insulin producing pancreatic cells are still functional. Typical manifestations of the disease are reactive hyperinsulinemia, where an excess amount of insulin is circulating the blood. The body tries to compensate the insulin resistance by a longer secretion of the hormone, which can be effective for some years. In the end, resistance to insulin cannot be compensated anymore, and the high glucose level of the patients' blood will cause hyperglycemia.

1.1.3 Gestational diabetes

Glucose intolerance can occur during pregnancy for the first time and is often reversible, although it can last until after pregnancy in some cases[10]. Causes are changing hormone balance and suboptimal nutrition of pregnant women. Especially overweight woman have a 15-30% risk of developing the disorder[11]. In most of the patients, a healthy diet and physical activity can antagonize the high glucose levels. In only 15% of patients additional insulin has to be applied[10]. Consequences of gestational diabetes are high blood pressure, higher risk of infection, and the development of type 2 diabetes mellitus. Furthermore, not only the mother is affected by the disorder. Nutrients can be passed through the placenta and cause a high blood glucose levels in the fetus. Consequences can be premature birth, due to the baby's greater size and weight. Often a caesarian section is recommended for delivery. Furthermore, the child's risk for obesity and diabetes mellitus are higher[11].

1.2 Glucose metabolism

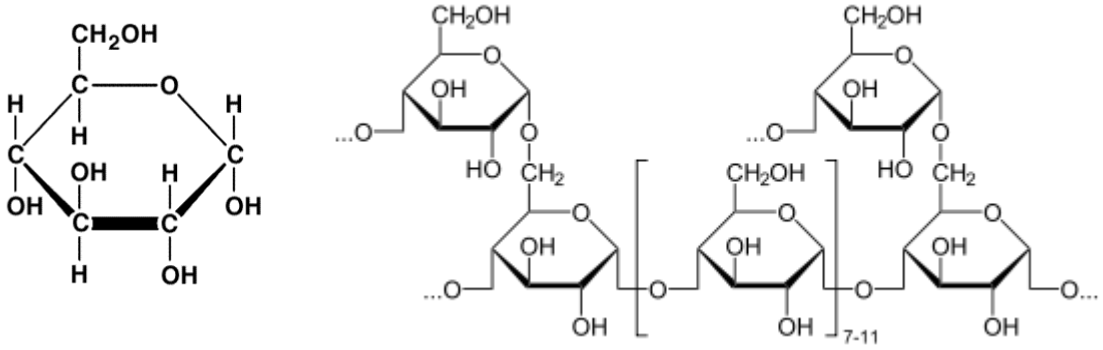


Figure 1.1. Chemical structure of D-glucose[12] (left) and glycogen[13] (right), displayed in the Haworth stereochemical projection. The C1 atom of the hexose contains an aldehyde group, which forms a hemiacetal with the C5 atom of the glucose monosaccharide. In nature glucose always appears in D-conformation. Glycogen is a polysaccharide made up of glucose molecules that are linked via α 1-4 glyosidic bonds with the adjacent molecules, and branched via α 1-6 glyosidic bonds with other glycogen chains.

The monosaccharide glucose is a break down product of carbohydrates, which we consume with our food and drinks. Starch is the major component of carbohydrates. The breakdown occurs in the intestine, which also yields fructose and galactose but in a smaller amount than glucose. Glucose is an essential energy provider, and needs to be stored for times of starvation. Storage occurs in the hepatocytes of the liver, in the form of glycogen. If the glucose level in the blood circulation is higher than required - usually after the intake of food - the hormone insulin gets secreted, to cause the transfer of glucose to liver, where it will be stored as glycogen. The transformation of glucose to glycogen is mediated by the protein glycogenin, and catalyzed by the enzyme glycogen synthase[14]. If the blood glucose level is too low, glycogen will again be transformed into glucose, and released into the blood, mediated by the hormone glucagon. The conversion of glucagon into glucose is mediated by a metabolic pathway, called glycogenolysis[14].

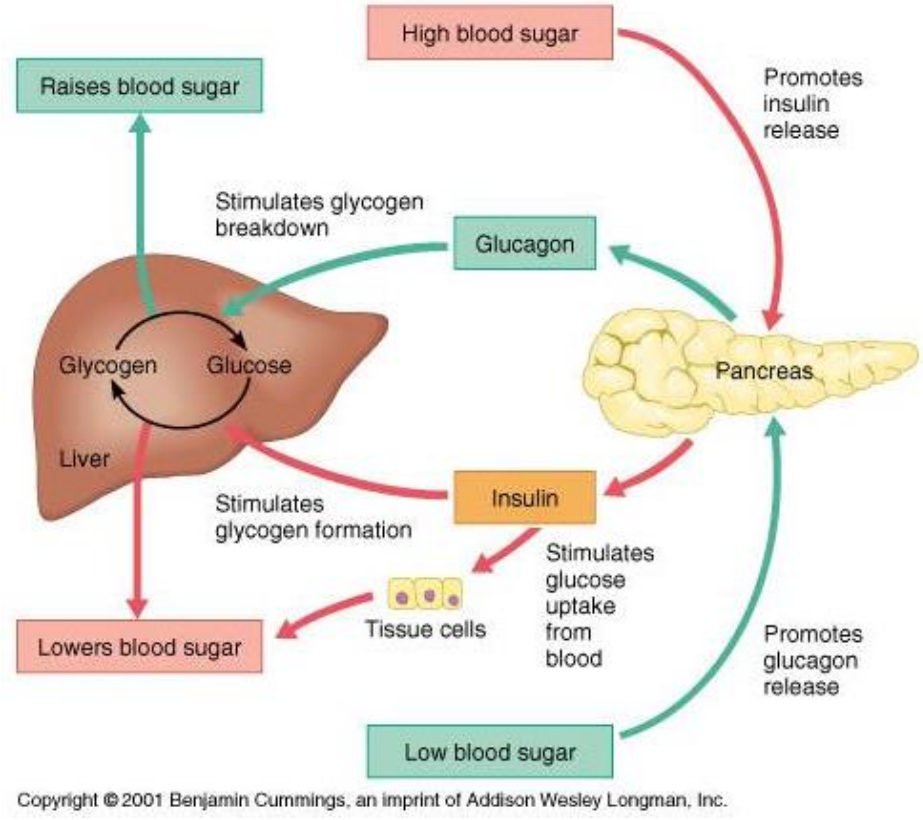


Figure 1.2. Schematic overview of blood glucose regulation[15]. In times of low blood glucose, the pancreatic alpha cells produce glucagon, to mediate the transformation of glucose to glycogen, to be released into the blood stream. As a response to high blood glucose concentrations, insulin will be synthesized by pancreatic beta cells, to convert glucose into glycogen, and store it in the liver.

Glucose is an energy source for the cells of the human body. Energy is yielded in biochemical processes like glycolysis or the pentose phosphate pathway[14]. Glycolysis in an anaerobe metabolic pathway, which breaks down glucose to lactate, with the gain of 2 mols of ATP. The pentose phosphate pathway generates ribose-5-phosphate, while gaining 1 mol of NAPDH.

A healthy individual should have a blood glucose level between 3,3 and 6,1 mmol/l[16]. If the concentration is higher, the person suffers from hyperglycemia, if it is lower, from hypoglycemia. Diabetes patients are in the constant risk of hyperglycemia after meals, due to the body's inability to lower the glucose in the blood, and store it in the liver and cells. However, in times of starvation, the inability to increase the blood glucose level could cause hypoglycemia. A patient is diagnosed with diabetes mellitus, if the blood glucose level is higher than 6,1

mmol/l[5]. A test method, to determine the glucose levels, is the oral glucose tolerance test, where 75g of glucose, dissolved in water, are orally applied to the patient. At 0, 60 and 120 minutes blood samples are taken, to determine the glucose concentration[5].

1.2.1 Insulin

Insulin is a metabolism regulating hormone, secreted by pancreatic beta cells. These beta cells are located in islets of Langerhans, in the pancreas, which are made up of several different cell types. An average human pancreas contains about 1.000.000 islets[17], and one ilset contains between 3.000 and 4.000 cells[18]. Apart from its' endocrine function, the pancreas secretes digestive enzymes into the bowel.

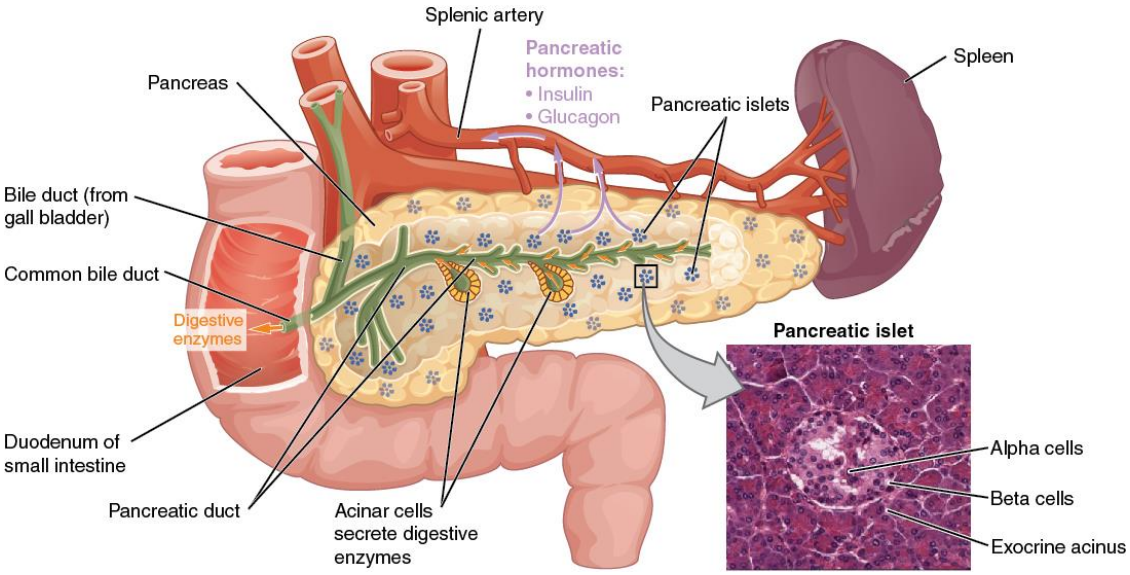


Figure 1.3. Anatomy of the human pancreas[19]. Digestive enzymes are produced in the acinar cells of the exocrine tissue, and secreted through the pancreatic duct into the small intestine. The hormones, produced in the islets of Langerhans, are secreted into the splenic artery, to enter the blood circulation.

Apart from beta cells, the islets of Langerhans contain alpha cells, which produce glucagon, the antagonist of insulin. This hormone stimulates the liver to convert glycogen into glucose, to raise the blood glucose level in times of starvation.

Alpha cells make up around 15-20% [20] of the total cells in the islets. Furthermore, there are delta cells, the producers of somatostatin, which is a hormone regulating other endocrine pathways. 3-10% [20] of total cells in the islets are delta cells. Other endocrine and also exocrine regulating cells in the islets are gamma cells, which make up 3-5% [20] of total cells in an islet. The smallest cell population, of only 1% [20], are epsilon cells, which produce ghrelin. This protein is necessary to stimulate hunger.

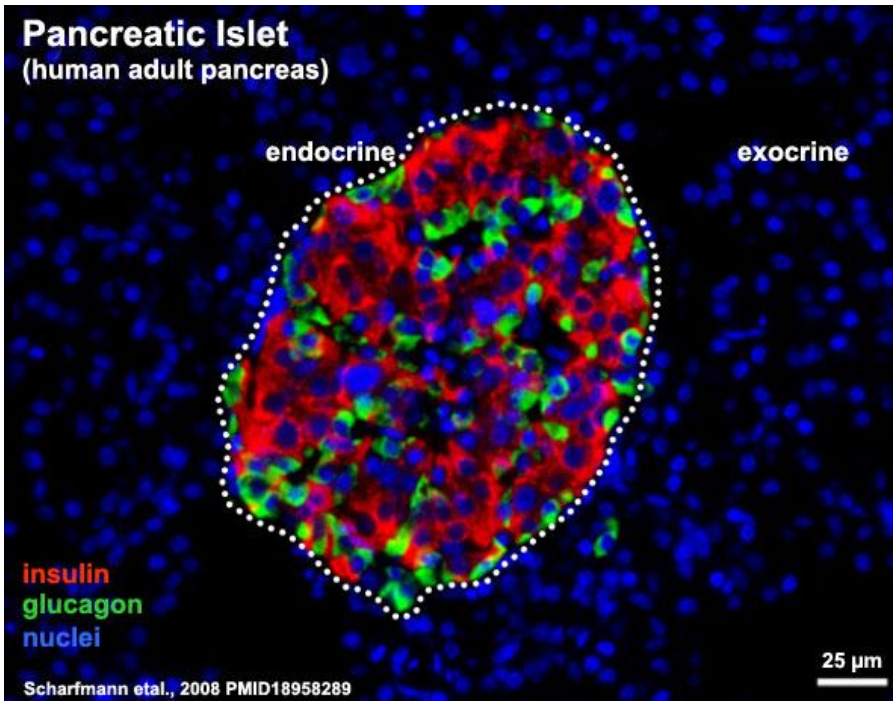


Figure 1.4. Pancreatic hormones stained with fluorescent markers[21]. Insulin, located in beta cells, is stained red. Glucagon, in alpha cells, appear green. Nuclei of all cells are stained blue, to show the total amount of cells in the islet and surrounding tissue.

1.2.2 Insulin Synthesis

The gene coding for insulin in humans, the ISN gene, is located on chromosome 11, and consists of 3 exons[22]. Transcription and alternative splicing of this gene generates four mRNAs, and will be translated into a 110 amino acid containing pre-protein. In the endoplasmatic reticulum a signal peptide of the pre-proinsulin is removed and then packaged into vesicles in the Golgi apparatus, where it receives its correct conformation. Finally, proteases cleave the pre-protein into the C peptide, which dissociates form the complex, and the B peptide, which

forms disulfide bonds to the A peptide, to form the mature Insulin hormone, ready to be secreted.

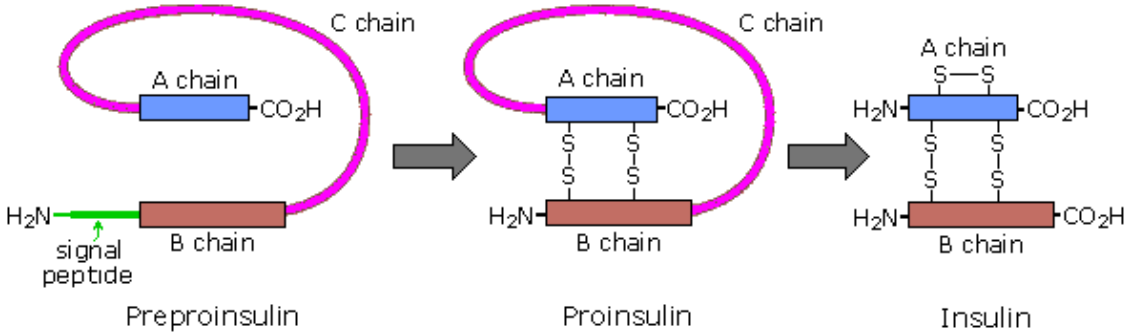


Figure 1.5. Cleavage of preproinsulin into mature insulin molecule[23]. At first the N-terminal signal peptide gets cleaved off in the endoplasmatic reticulum to become proinsolin. Furthermore, the C peptide gets cleaved and dissociates from the protein complex. Finally the A peptide and B peptide of the proinsulin form disulfide bonds to become the mature insulin molecule

1.2.3 Insulin activity

Secretion of the mature insulin hormone is dependent on the glucose level of the blood. If the glucose level is too high, the pancreas is stimulated to produce and secrete insulin. Glucose will be taken up by cells in need for it, which is carried out by glucose transporters in their membranes. At first the glucose molecules get phosphorylated by hexokinases, to be channeled into different metabolic pathways. Translocation of the transporters, into the membrane, is mediated by insulin. There are 14 different isoforms of the hydrophobic transmembrane transporters. The most common in mammalian cells is GLUT1, which is most abundant in the central nervous system[14]. If all cells in need for glucose are saturated, the remaining molecules will be taken up through the GLUT2 transporter of hepatocytes in the liver[14]. Inside the cells, the glucose molecules get phosphorylated by the enzyme glucokinase, which gets expressed, if the intracellular glucose concentration is high. Insulin is responsible for the initiation of glucokinase production. Once expressed, the enzyme will be translocated from the nucleus to the cytoplasm, to phosphorylate its substrate. The generated glucose-6-phosphate can be used for several metabolic pathways, like glycolysis, gluconeogenesis, pentose phosphate pathway, production of saccharides, or it's

storage in the liver as glycogen. If blood glucose levels are low, insulin levels drop and glucagon gets expressed by the pancreatic alpha cells, leading to the conversion of glycogen into glucose, to be finally released from the liver.

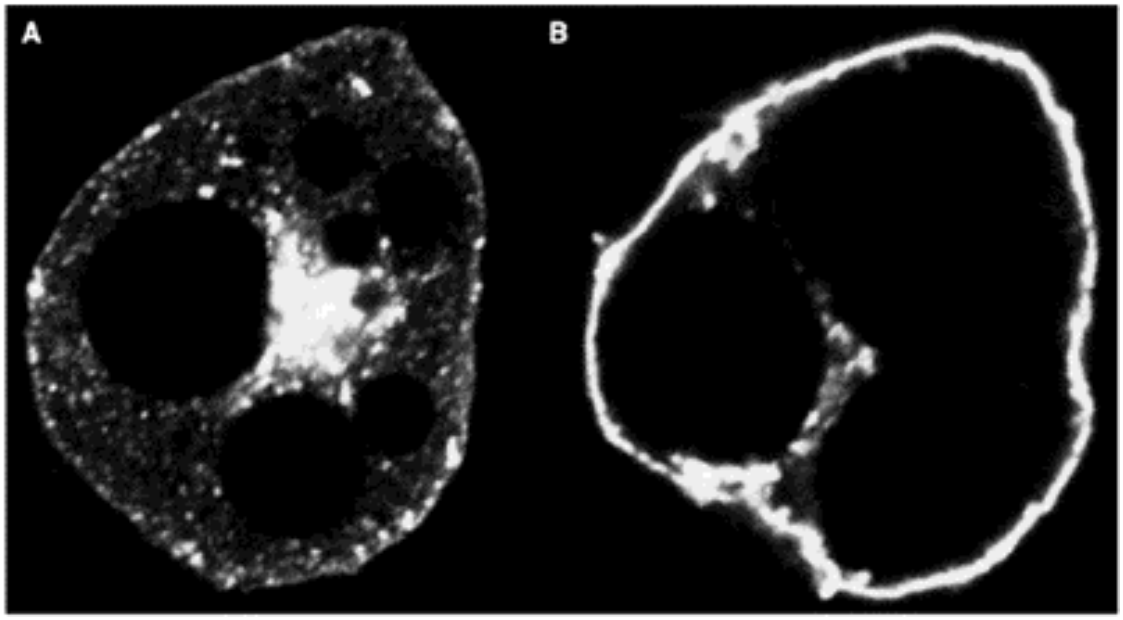


Figure 1.6. Images of an adipocyte with fluorescent labeled GLUT4[24]. (A) Without the stimulation of insulin, GLUT4 transporters are located in vesicles in the cytoplasm. (B) Once insulin binds to receptors on the vesicles, the transmembrane transporters get translocated into the cell membrane, to take up glucose.

1.3 Epidemiology of Diabetes Mellitus

In 2014, around 422 million people were suffering from diabetes worldwide, mostly type 2[9]. In contrast, only 108 million people were affected by the disease in 1980, which means a rise of 234% in only 23 years. According to the World Health Organization (WHO), the prevalence of diabetes in adults lies at 8.5%, and it is a major cause of blindness, kidney failure, heart attacks, strokes and lower limb amputations. In 2012, the disease caused around 1.5 million deaths worldwide, 43% were younger than 70 years. Unfortunately not all cases are being diagnosed - it is estimated that around 73,8 million patients worldwide have not been diagnosed yet[25]. Only 5-10% of all of diabetes patients suffer from type 1, and the prevalence stays relatively stable[7]. On the contrary, type 2 diabetes has a rising prevalence. Additionally, the disorder does not affect all countries, ethnicities, and ages in the same rate. Type 1 diabetes can be manifested at

every age, but statistically the highest incidence is between 10 and 15 years[9]. Worldwide it affects around 15 million more men, than women[9]. Furthermore, the prevalence of developing type 1 or type 2 diabetes is lower in rural, than in urban areas. Strikingly, developing countries, which are adopting to western life style have the highest risk for diabetes, like China, India or Brazil. Nevertheless, in developed countries, like the UK, certain ethnicities are more susceptible, for example South Asian and African-Caribbean, compared to the Caucasian population. In the US, especially the African American and Hispanic population is at a fivefold times higher risk. In Germany the prevalence for diabetes is 7,2%, but for people with a low social status it is 10,9% [25]. This can be correlated with higher obesity rates in these ethnic groups, which is a result of unhealthy life style. Missing or moderate exercising and foods containing a lot of fat, sugar and red meat, are often associated with the development of type 2 diabetes.

Worldwide, the increase of patients with diabetes lies between 2.8 and 3.0% every year, 3.9% in Europe. The International Diabetes Federation predicts that by 2040, around 642 million adults will be affected by diabetes mellitus[26].

	2015	2040
Total world population	7.3 billion	9.0 billion
Adult population (20-79 years)	4.72 billion	6.16 billion
Child population (0-14 years)	1.92 billion	-
Diabetes (20-79 years)		
Global prevalence	8.8% (7.2-11.4%)	10.4% (8.5-13.5%)
Number of people with diabetes	415 million (340-536 million)	642 million (521-829 million)
Number of deaths due to diabetes	5.0 million	-
Health expenditure due to diabetes (20-79 years)		
Total health expenditure, R=2* 2015 USD	673 billion	802 billion
Hyperglycaemia in pregnancy (20-49 years)		
Proportion of live births affected	16.2%	-
Number of live births affected	20.9 million	-
Impaired glucose tolerance (20-79 years)		
Global prevalence	6.7% (4.5-12.1%)	7.8% (5.2-13.9%)
Number of people with impaired glucose tolerance	318 million (212.2-571.6 million)	481 million (317.1-855.7 million)
Type 1 diabetes (0-14 years)		
Number of children with type 1 diabetes	542,000	-
Number of newly diagnosed cases each year	86,000	-

Figure 1.7. Statistics on diabetes worldwide, in 2015 and estimated in 2040[26].

1.4 Prevention of Diabetes Mellitus

Unfortunately, the autoimmunity to pancreatic islet cells of type 1 diabetic patients cannot be prevented. Nevertheless, an early diagnosis can improve a patient's live quality. Children of diabetic parents have a higher risk of developing the disease themselves that is why they should be observed and examined in their childhood and early adolescence.

Type 2 diabetes can be prevented by maintaining a healthy life style. Well-balanced nutrition, combined with regular exercise is the key of avoiding insulin resistance. Smoking and high blood pressure can also increase the risk of developing type 2 diabetes[27]. Additionally, due to reduced motility of elderly people, the development of type 2 diabetes later in life is more prevalent. Furthermore, a lower social class is a risk factor for the development of insulin resistance, because fast food is often cheaper and more available. A solution to this problem could be by educating people about the risks, and to encourage them to lead a healthier life style.

1.5 Manifestation of Diabetes Mellitus

Diabetes has a huge disease pattern, which can substantially affect a patients' life quality. Typical symptoms before the diagnosis of the disease are thirst and frequent urination resulting from dehydration, weight loss, nausea, impaired sight, skin irritations, loss of appetite, muscle cramps, retarded growth in children and adolescents, psychological problems, fatigue and attention deficits[5]. Diabetes patients are constantly at risk of hyperglycemia, which could lead to falling into a ketoacidotic coma. In 5% of children and 1% of adult patients, this condition is the primary manifestation of the disease[5]. Diabetic ketoacidosis is characterized by hyperglycemia, hyperosmolarity and ketonemia[28]. Hyperglycemia, a life threatening high blood glucose level, is as a result of a lacking, or impaired insulin function. As a consequence, the body tries to lower the circulating glucose, by withdrawing it in the urine. This can lead to hyperosmolarity, a more concentrated blood due to dehydration[29]. Ketonemia is the accumulation of ketone bodies in the blood, which are break down products of fatty acids. In high doses these

ketone bodies can be toxic[30]. 2 – 10% of diabetic ketoacidosis cases have a fatal outcome, although in areas with poor health supply it can be up to 50%[31]. Apart from acute pathologies, diabetes can trigger a wide variety of secondary diseases, affecting the nervous system, blood vessels, heart, kidneys, eyes, sweat glands, etc. As a severe outcome, the 'diabetic foot syndrome' can lead to foot amputations, due to infected ulcers that do not heal regularly.

1.6 Therapy of Diabetes Mellitus

The therapy of Diabetes Mellitus is a great financial burden for patients and global health care systems. Due to the rising disease prevalence, therapy costs are constantly on the rise. Norway has the highest expenses worldwide: 11.851 US$ a year. In the US the expenses lie around 320 billion US$, in China around 51 US$ annually[25]. However, these numbers are not a result of the higher patient numbers in those countries, but of the good quality of patient care. Third world countries can often not pay treatments and new technologies, which help treat diabetes. Therefore the death rates of diabetic patients are higher, and the patients receiving treatment are lower[27]. Sometimes the disease does not get diagnosed at all, due to the patients' missing access to health institutions.

In theory, there are several therapy methods for type 1 and 2 diabetes. Some of them are still experimental or in need of improvement. The most conventional treatment is insulin therapy. Pancreas transplantation are less frequent, and islet cell transplantation is still experimental, but extensively investigated.

1.6.1 Insulin therapy

Due to lacking alternatives, insulin therapy is still the most conventional treatment of type 1 diabetes mellitus. Insulin shots are usually of human origin or human analogues. Highly purified hormones of animal origin are rarely used anymore[5]. Since the hormone gets broken down by digestive enzymes, it cannot be taken orally and has to be injected into fat tissue, to be resorbed and distributed by the blood stream. There are four conventional insulin types, differentiating in duration and peak of its' activity and how quickly they begin to work[32]:

- Rapid-acting insulin – reaches blood stream about 15 minutes after injection and can last for 2 to 4 hours. The peak of its effectiveness is approximately 60 minutes after application.
- Regular/ short acting insulin – begins to work after 30 minutes, and can last for 3 to 6 hours. It peaks after 2 to 3 hours after injection.
- Intermediate-acting insulin – starts to work after 2 to 4 hours, and its effect can last up to 12-18 hours, although the peak is 2-4 hours after application.
- Long-acting insulin – requires a few hours to reach the blood stream, but can last up to 24 hours after application of the dose.

The required dose of insulin is individual in every patient. If some pancreatic beta cells are still able to secrete insulin, they require lower doses of the hormone. Monitoring and protocolling blood glucose levels 2-4 times a day is of great importance to improve treatment. Furthermore, the insulin dose depends on the carbohydrates a meal contains, and the physical activity a person's routine includes[3]. Most patients in the U.S. require a dose of 100 U (100 units / ml), but in severe cases the patients might even need up to 500 U[32]. Conventional type 1 diabetes insulin therapy includes two mixed insulin injections daily. They are composed of rapid or short acting, combined with intermediate acting insulin, which is applied in the morning and evening before meals[3]. Occasionally the late dose is split between a dose before evening meals and a dose later at night, to prevent night time hypoglycemia. Other strategies are the application of rapid or regular acting insulin before every food intake, or injection of intermediate or long acting insulin once or twice a day. Last but not least, an intensive therapy of rapid acting insulin can be administered. Therefore the insulin dose is calculated according the measured blood glucose level and the amount of carbohydrate, which is about to be eaten. This method requires constant blood glucose measurement, and the exact carbohydrate amount of a meal. A side effect of insulin therapy can be weight gain, since more glucose is taken up by cells. This problem can be controlled with an individual diet and regular exercise. Reducing the dose of insulin is often not an option, because it may result in hypoglycemia.

For type 2 diabetes patients, early diagnosis can have a big influence on the diseases' malignity. The sooner an adjustment of diet and physical activity occurs, the slower the disease will progress. Sometimes glucose lowering agents can be effective as a treatment. These drugs can increase insulin secretion, or decrease carbohydrate digestion, to prevent hypoglycemia. In counties with poor medical supply, or people who cannot afford health insurance, type 2 diabetes cannot be treated optimally at an early stage, which could have an effect on the severity of the disease. Furthermore, it might get diagnosed at a late stage or not at all, which can have fatal effects.

A more independent way of applying insulin is via insulin pumps. These devices contain sensors, which can determine the patients' blood glucose level. If the glucose concentration in the blood is too low, a pump will apply the right dose of short or rapid acting insulin through an infusion in the skin. The infusion site has to be changed every three days, due to the risk of infection[33]. Furthermore, the device is capable of recording the patients' blood glucose level, to optimize the disease treatment. Additionally, the pump can be taken off for a short period of time. This technology is especially useful for patients who are incapable, or troubling with the routine of observing their blood glucose levels, and injecting insulin themselves. Insulin pumps are used by type 1 patients only[33].

Diabetes mellitus has a great impact on a patients' life quality. The need of injecting insulin, which requires frequent control of the blood glucose levels and self-applied hormone doses, combined with life style adjustments, can restrict a patient in many daily activities. Another approach, apart from insulin therapy, is to transplant pancreatic islets, or even the whole organ into a patient. This would take the responsibility of applying insulin, which can ultimately increase a persons' life quality.

1.6.2 Pancreas transplantation

Pancreas transplantation is a common surgery, done in type 1 diabetes patients with pancreatic failure. In 90% of the cases the pancreas gets transplanted together with both kidneys, which are failing in many patients with advanced

diabetes. However, in most of the cases, the kidneys get transplanted first and the pancreas later on. Unfortunately, the waiting list for such surgeries are very long - approximately 3 years in the U.S.[34]. In fact, in the last decade, the number of patients on the transplantation list have been rising by 150%[35]. However, the increase in the performed surgeries was only 21%[35], which is a result of the lacking organ donors. Most organs are taken from brain dead patients, but not all of them are sufficient for transplantation. Many donors are either too old, or had an unhealthy life style, and are therefore unfit for donation. Only 6-8% of the donors in the U.S. were older than 45 years[36]. The one-year survival of pancreas grafts is 79%, and 85% for kidney-pancreas grafts[36]. Despite these relatively successful surgeries, many patients have to consider other treatments.

1.6.3 Islet cell transplantation

A long term treatment for type 1 diabetic patients would be the transplantation of functional pancreatic islet cells. A better life quality, compared to conventional insulin therapy, could be guaranteed, due to the independence to insulin shots. Optimally, the glucose levels should be autoregulated without the constant risk of hyperglycemia. There are two methods of pancreatic islet cell transplantations: allo- and auto-transplantation.

Allo-transplantation is the transfer of a donors' cells into a patient with type 1 diabetes. Therefore the donors' pancreas is used to isolate islets of Langerhans. By adding collagenase to the organ, the connective tissue gets digested and separated from the islet cells[37]. After transferring the islets in an aqueous solution, they get injected into the patients' portal liver vein through an infusion[38]. The cells are transferred into the blood stream, and should directly sense and respond the host's glucose level.

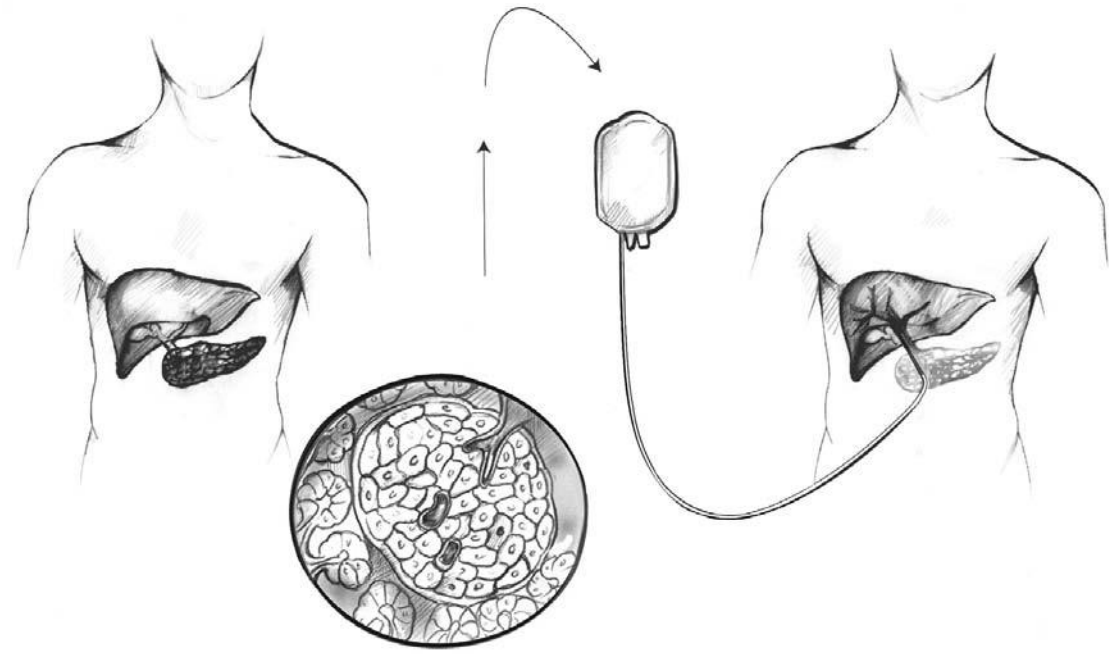


Figure 1.8. Scheme of islet cell allo-transplantation from a deceased donor into a type 1 diabetes patient. The cells, added to a liquid pahse, enter the blood stream through the portal liver vein, using an infusion[38].

However, this method bears three main obstacles, which limit successful and long lasting insulin production. Pancreatic beta cells will be provided by a donor, which means that these cells will be seen as antigens by the recipient's immune system. Further details of the foreign body response will be given in chapter XY. Ultimately, the transplanted cells could be rejected by the patient. A common pharmaceutical tool in organ transplantation, is to administer immunosuppressive drugs. A study by Nanji et al.[39] shows that immunosuppressive drugs might lead to insulin resistance, by deteriorating beta cell functions. The second big challenge, which islet cell transplantations bring along, is the decrease in insulin secretion over time. After 5 years, the insulin production often reduces down to 10% of the initial amount. Keymeulen et al.[40] suggest this is due to suboptimal cell number and quality. Their studies suggests that a patient should receive about 0.5-5.0 x 10^6 beta cells per kilogram of body weight. A third limitation, like in kidney-pancreas transplantations, is the lack of suitable donors.

Auto-transplantation is for type 2 diabetes patients, who still have a partial functional beta cells. In this method, the patients' own pancreatic islet cells are used for transplantation. The benefit of this therapy is that the cells will express the patients' own MHC complex, and will not be seen as foreign bodies. Despite that, it is not a permanent treatment, because cells lose their ability to produce insulin over time. Furthermore, the islet cells might not produce the optimal dose of insulin a patient requires, since they cell numbers are often too low.

Due to the limitations, the method of auto- and allo-transplantation of pancreatic islet cells is not a standard procedure. A strategy to avoid the clearance of the cells by the immune system, is to coat the islets with a material, to immunoisolate them from the body. Therefore, different materials have been used in several studies. These materials include natural polymers that occur in the body itself, like endothelial cells[41] and polyphenoles[42]. Furthermore, foreign materials, like Alginate[43], were used. However, these studies have not shown permanent success, because the cells could not provide optimal insulin secretion. Another approach would be, to construct a coating system consisting of synthetic molecules, mimicking natural structures of the body. These bionic materials should have the benefits of being inert and open to modifications, to add certain features to the coating polymers.

1.7 ELASTISLET

ELASTILET is a project funded by the European Commission, and involves 11 partners from 8 countries. Each institution has a different task, and provides many specialists from the disciplines of medicine, immunology, chemistry, cell biology etc.

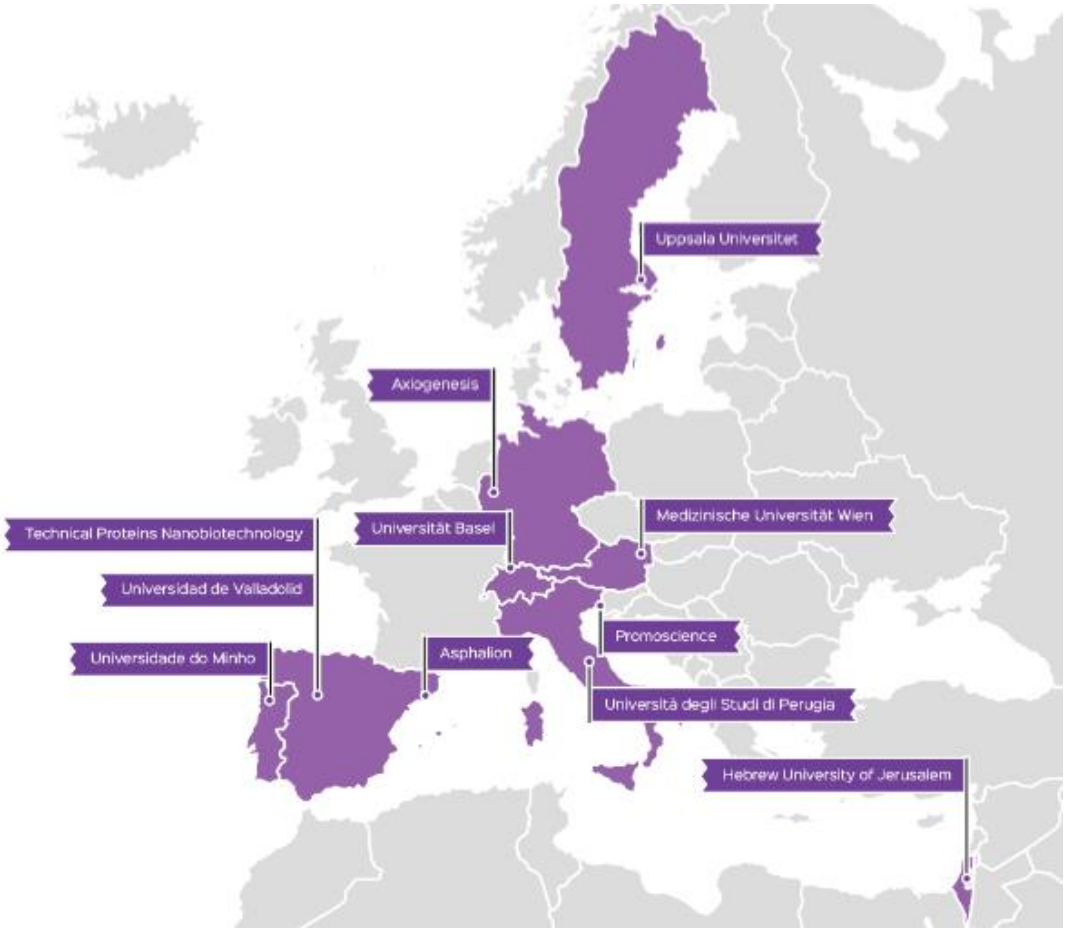


Figure 1.9. Partners participating in the ELISTAILET project[44].

The aim of ELASTISLET is to potentially cure type 1 diabetes mellitus, by transplanting encapsulated pancreatic islet cells, for advanced insulin therapy. The goal is to create a tailored biomaterial, which is semi-permeable, immunoisolated and biocompatible. Furthermore, the capsule should promote angiogenesis, to provide access of the transplanted islet cells to the hosts' blood stream. The beta cells should sense the patients' high glucose level and respond to it, by producing the sufficient amount of insulin. The semi-permeability of the capsule should guarantee that immune cells and its mediators, like macrophages and antibodies, are isolated from the immune privileged site. On the other hand, supplies, like nutrients and oxygen, should be able to pass the border. Otherwise, the cells inside the capsule would not be able to survive for a long period of time. Last but not least, glucose and insulin should be able to penetrate the capsule, to balance the blood glucose concentration. Therefore, angiogenesis around the implant should be promoted, to guarantee an optimal

interaction of the host and the islet cells, and the direct release of insulin into the patients' circulation. The formation of a fibrous capsule around the implant should be avoided, because it would isolate the islets from the host tissue and blood stream. Ultimately, the islet cells could die, due to the missing supply with nutrients and oxygen. These requirements towards this immune privileged site are a great challenge for the synthesis of the coating material. Optimal results of the project should provide an immunoisolated implant, which can interact with the donor's blood glucose levels and preserve long lasting insulin production, which is cost efficient and environmentally sound.

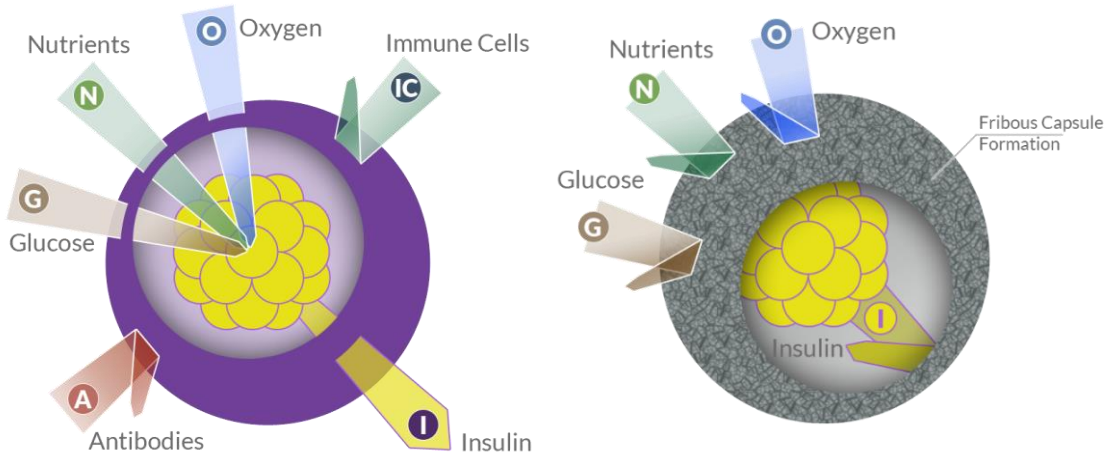


Figure 1.10. Coating strategy of the islet cells[44]. Nutrients, oxygen, glucose and insulin should be able to penetrate the polymers, while immune cells and antibodies should be excluded from the immune privileged site (left). The formation of a fibrous capsule around the implant could cut off the cells from the surrounding tissue and the blood stream, which will ultimately lead to apoptosis (right).

1.8 Elastin-like recombinamers (ELRs)

Elastin like recombinamers have gained on great importance in biotechnological research. Their properties make them versatile tools for several bioengineering techniques, including protein purification, assistance in protein folding, drug delivery, and tissue regeneration. ELRs are semi-artificial materials, which mimic the extracellular matrix protein elastin, making them biocompatible and immune stealth to the host, to avoid an immune or allergic response.

Elastin is a filamentous protein of the extracellular matrix, and one of the most important structural proteins of the human body. It is located in skin, lung,

cartilage and arterial walls, and has the properties of being extremely flexible and elastic. These features provide elasticity to tissues, and its' structure allows the filaments to undergo more than one billion stretching-relaxing events without breaking[43]. The primary sequences of elastin contain mainly the amino acids Alanine, Glycine, Proline and Valine in different combinations (ex. VPGG, VPGVG, APGVG), which are organized in repeated motifs. Apart from its structural properties, elastin has shown to contribute to modulating cell behavior and promoting tissue repair[45].

Elastin-like recombinamers are synthetic filamentous polymers, which mimic the basic structure of elastin. Because they contain certain chemical modifications, they are semi-artificial. ELRs can be synthesized using genetic engineering techniques, to exhibit a specific structure or function, which is not present in the natural polymer. However, the created material should not show too many aberrations towards natural elastin, so it can remain immune stealth. An important feature that has to be considered when constructing such polymers, is the transition temperature (T_t). It is defined by its concentration, amino acid composition, pH, and ionic strength[46]. Below the transition temperature, the ELR is hydrophilic and unfolded, and above, it starts to undergo hydrophobic folding and consequently encapsulate itself form aqueous environments. Genetic engineering allows us to synthesize ELRs, but the process involves many challenges. The primary issue is to construct a stable end product. Looking at the level of gene expression, mutations of the primary sequence should be avoided. This requires a stable expression system. *Eschericha coli* is the most commonly used organism for protein production, and exhibits a high yield of the end product. However, specific amino acids have to be added to the medium, or the aminoacyl-tRNA pool hast to be increased, by creating metabolically engineered strains, to acquire a high yield protein production[46]. Another problem is the accumulation of endotoxic byproducts. To avoid that, a widely used eukaryotic expression system for ELRs is the yeast species *Pichia pastoris*[46]. A slightly lower yield, than the use of E.Coli is achieved, although the accumulation of endotoxins is avoided. Furthermore, the advantage of using a eukaryotic organism is the intracellular machinery, which allows post translational modifications of the yielded proteins.

Plants can be used as expression systems too, for example tabacco seeds, or leaves, showing the benefit of a higher protein yield[47]. Nevertheless, this method can be more cost and time extensive. After the expression of the protein, it needs to be purified. The most commonly used method, which takes advantage of the ELRs biological features, is inverse transition cycling (ITC)[48]. This method separates the polymers from other molecules, by increasing the samples' temperature. This causes the polymers to aggregate, due to its' specific transition temperature. After centrifuging of the sample, a pellet, containing the ELRs, will be resuspended, by lowering the T_t again. To optimize the purification, the whole procedure can be repeated several times[48].

ELASTILET is using two different ELR polymers, for coating the islet cells:

1) VKV: contains the amino acid Lysine, linked to additional azide and cyclo-octyne groups, for chemical crosslinking of the individual ELR molecules. A covalent bond between the free electrons of the azide, and the triple bond of the cyclooctyne group will cause the formation of the ELR hydrogel.

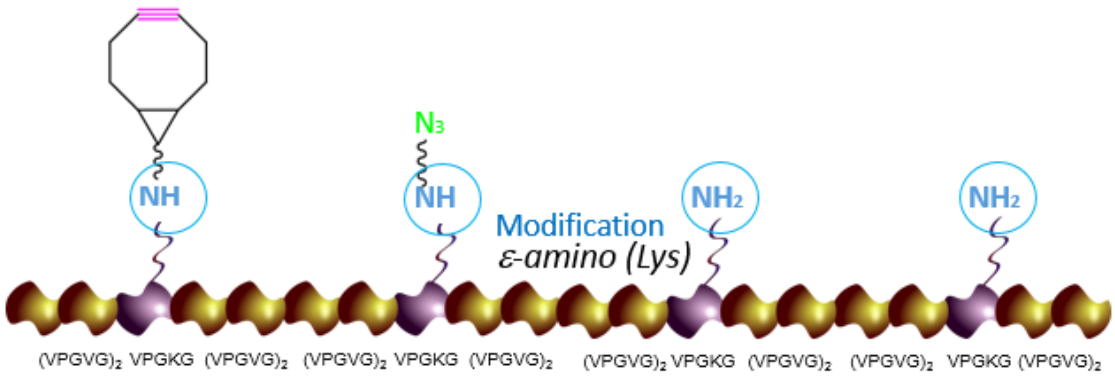


Figure 1.11: Diagram of a VKV polymer. The molecule is a repeat of four VPGVG sequences, followed by one repeat, where the second Valine is substituted with a Lysine. These Lysine's contain either an azide, or a cyclooctyne group, for chemical crosslinking to other ELR polymer.

2) RGD: A variant of the VKV polymer, with additional cell adhesion domains (RGD domains). This polymer will be located on the inside of the sphere

for adhesion to the ilset cells and on the outside for adhesion to the host tissue.

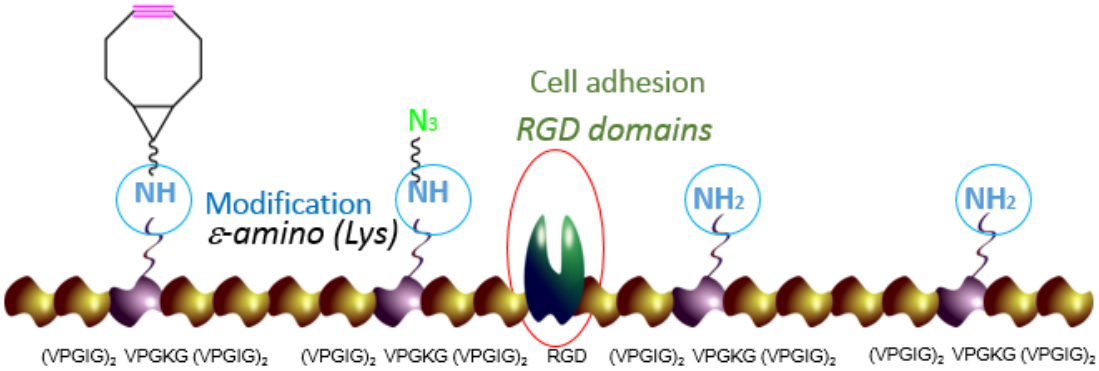


Figure 1.121 Diagram of a RGD polymer. The RGD cell adhesion domain contains the amino acid sequence AVTGRGDSPASS, and is flanked by VKV modules. The VKV's Lysines contain either an azide or a cyclooctyne group, for chemical crosslinking to other ELR molecules.

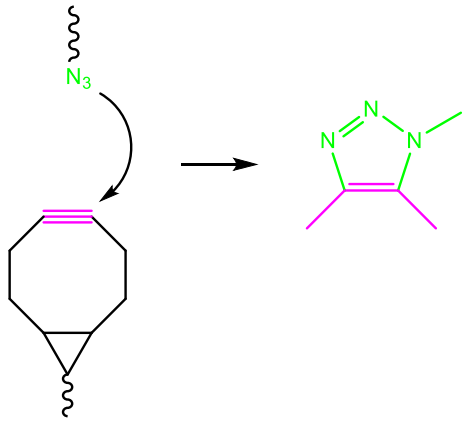


Figure 1.13. Chemical crosslinking of the ELR-polymers for hydrogel formation. The azide and cyclooctene will form a covalent bond between the free electrons of the azide group and the triple bond of the cyclooctyne, once they are added to each other and put on room temperature.

1.9 The innate and adaptive immune system

During its' life time, a human body is exposed to millions of potentially harmful substances, like bacteria, viruses, allergens, toxins, etc. To distinguish these antigens from self- structures, and protect the body from them, a complex, and

tightly regulated immune system is required. Its tasks is, to recognize these structures, while providing tolerance to self- structures. Furthermore, it has to initiate and regulate effector functions, to destroy, or degrade the foreign structures. Finally, an immunologic memory, to facilitate the defense against the next encounter to a specific antigen, should be provided. The primary defense against substances from the outside are barriers, like the skin, mucus, antimicrobial peptides, tight junctions, and chemical substances, to avoid their entrance into the system. If these barriers fail, or are overcome, the immune system is activated, to neutralize and remove them. The human immune system is divided into two sectors: the innate, and the adaptive immune system. They are distinguished by their function, specificity, mediators and speed of action.

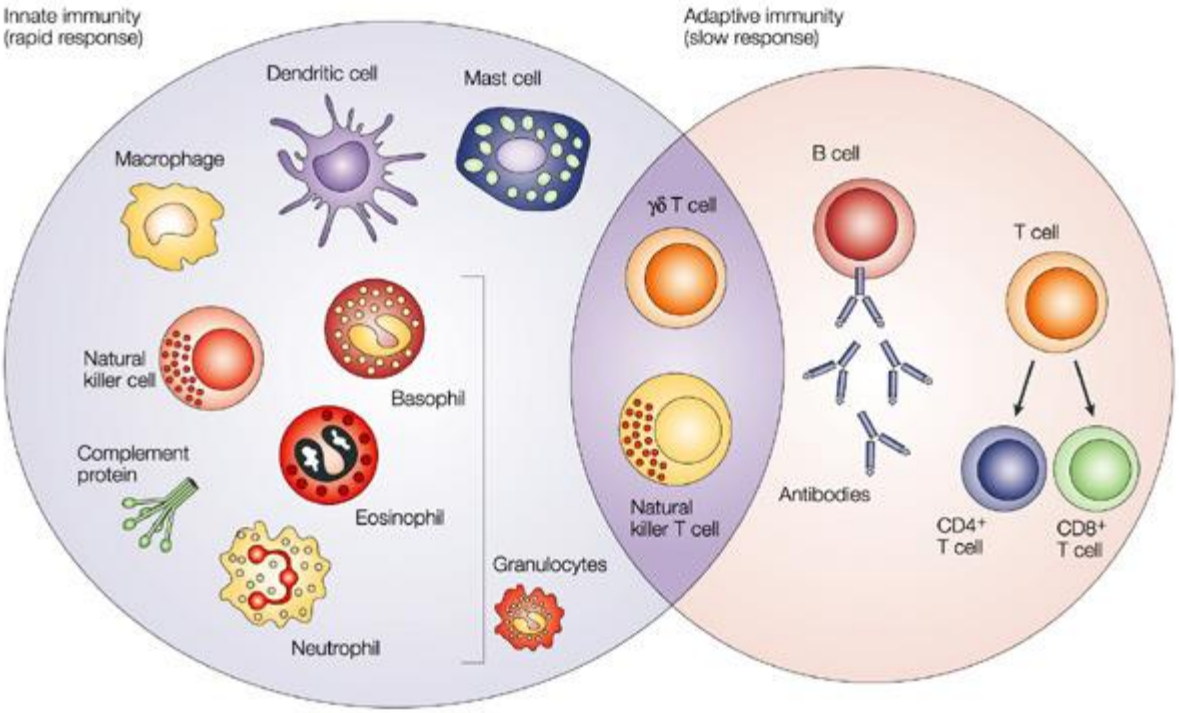


Figure 1.14. Cells of the innate and adaptive immune system[49].

1.9.1 The innate immune system

The innate immune system is the first line of defense against antigens. As the name indicates, it is a pre-existing system, found in different forms, in animals

and plants. The compliment system can form protein membrane attack complexes, to destroy harmful cells. Cells of the innate immune system originate as hematopoietic stem cells in the bone marrow[50]. They migrate to peripheral tissues, the lymphatic system or the blood stream, to differentiate into immune effector cells, ready to be activated. The fate of a cell is determined by transcriptional regulation. Within the innate cell line are four further progenitors: Common myeloid progenitors develop to become mature dendritic cells. Granulocyte macrophage progenitors become neutrophils, eosinophils, basophils, mast cells or macrophages. Furthermore, blood platelets and erythrocytes share the same progenitor. Granulocytes contain toxic proteins and enzymes, to combat bacteria and parasites, and contribute allergic inflammation. Mast cells contain granules, which trigger inflammation, once they are released. Macrophages and dendritic cells are antigen presenting cells (APCs). They can recognize antigen structures with their pattern recognition receptor (PRR) and phagocytize them. To activate other immune cells, they display them on their surface major histocompatibility complexes (MHCs), for antigen presentation. This mechanism links the innate to the adaptive immune system.

1.9.2 The adaptive immune system

Compared to the fast, and unspecific innate immune response, the adaptive immune system gets activated about three to four days after an infection. If innate immune mediators are unable to clear a pathogen, or foreign body, antigen presenting cells, cytokines, or chemokines trigger an adaptive response. Cells of the lymphoid lineage originate from pluripotent hemapoietic stem cells, and differentiate into a common lymphoid progenitor. Further differentiation will cause the development of B- lymphocytes, T- lymphocytes and natural killer (NK) cells. B-cells can differentiate into antibody producing plasma cells. T helper (Th) cells have the ability to activate other immune effectors. Cytotoxic T- cells can directly attack and destroy infected, or mutated cells of the body. Regulatory T-cells (Treg cells) suppress and regulate immune responses.

1.9.3 Antigen presentation

Antigen-presenting cells are cells, which have the ability, to present an antigen on its surface MHC, to present it to naïve T-cells. Dendritic cells, macrophages and B-lymphocytes are APCs. There are two classes of MHC molecules: MHC class I binds molecules from the cytosol, for example from bacteria and viruses, which replicate in this compartment. This protein complex is present on most of the cells in the human body, and its' absence is a strong indication for foreign materials or organisms. Secondly, there are class II MHC molecules, which display peptides that have entered the host cells via vesicles. It is mainly expressed by APCs and lymphocytes. Cells, which express a class I MHC on their surface bind the T-cell receptor (TCR) with a CD8 co-receptor on cytotoxic T-cells. These effector lymphocytes furthermore cause apoptosis of the infected cells, to avoid replication of the organism. Class II MHC bind the TCR with a CD4 co-receptor on Th-cells. There are different subsets of Th-cells: T_H1 lymphocytes are activated in cells, infected with intravesicular pathogens. T-bet and STAT4 are the transcription factors thriving T_H1 differentiation. Furthermore, IL-2 and IL-12 cause naïve T-cells to develop this subtype. IFN-γ is the main cytokine produced by T_H1 cells. T_H2 cells differentiate, if the host is infected with extracellular pathogens. Key transcription factors are GATA3, and STAT6, and differentiation is initiated by the cytokine IL-4. T_H2 cells mainly produce IL-4, IL-5 and IL-13. Dendritic cells are the most conventional APCs, since they express both MHC classes, and can activate CD4+ and CD8+ cells. Macrophages mostly present antigens via the MHC class II pathway, to activate T-helper cells. These are helping to destroy the infected macrophage. B-cells often express MHC class II, to bind and activate Th-cells. They trigger the B-cells to proliferate into antibody-producing plasma cells.

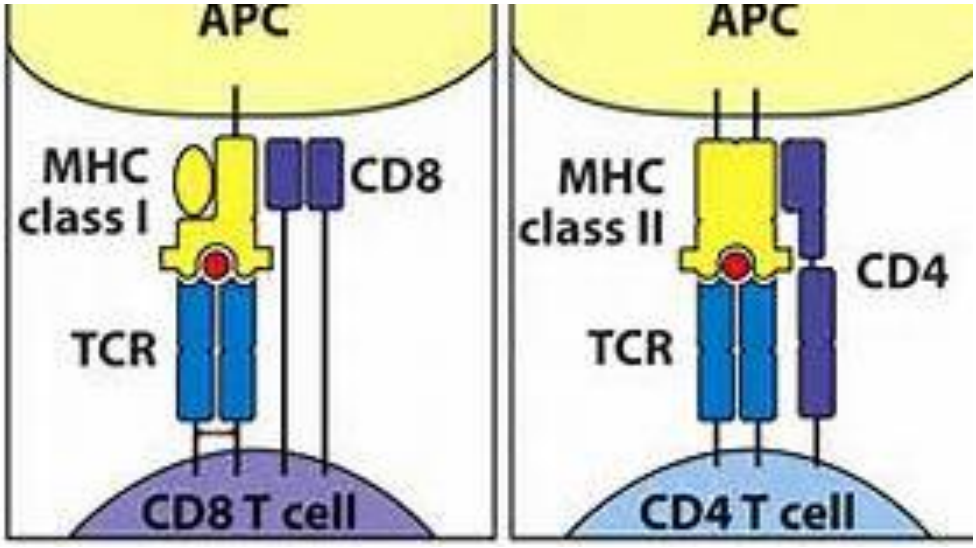


Figure 1.15. Schemes of the class I (left) and class II (right) MHCs[51]. MHC I receptors on an antigen-presenting cell contain the antigen, which directly binds to the TCR of a cytotoxic T-cell. The CD8 co-receptor binds the MHC, but not the antigen itself. MHC II receptors on an antigen-presenting cell contain the antigen, which directly binds to the TCR of a T-helper cell. The CD4 co-receptor binds the MHC, but not the antigen itself. The co-receptors ensure the binding to the right effector T-cells.

1.9.4 B- cell differentiation and antibody production

Like T-cells, B-cells express an antigen binding receptor on their surface. The B-cell receptor (BCR) is a membrane bound immunoglobulin (IgM), which is anchored with its carboxy-terminal Fc region into the plasma membrane. The antibody binding Fab region, at the amino-terminal end is responsible for specific antigen binding. Signal transduction is carried out by the signaling proteins Igα and Igβ[50].

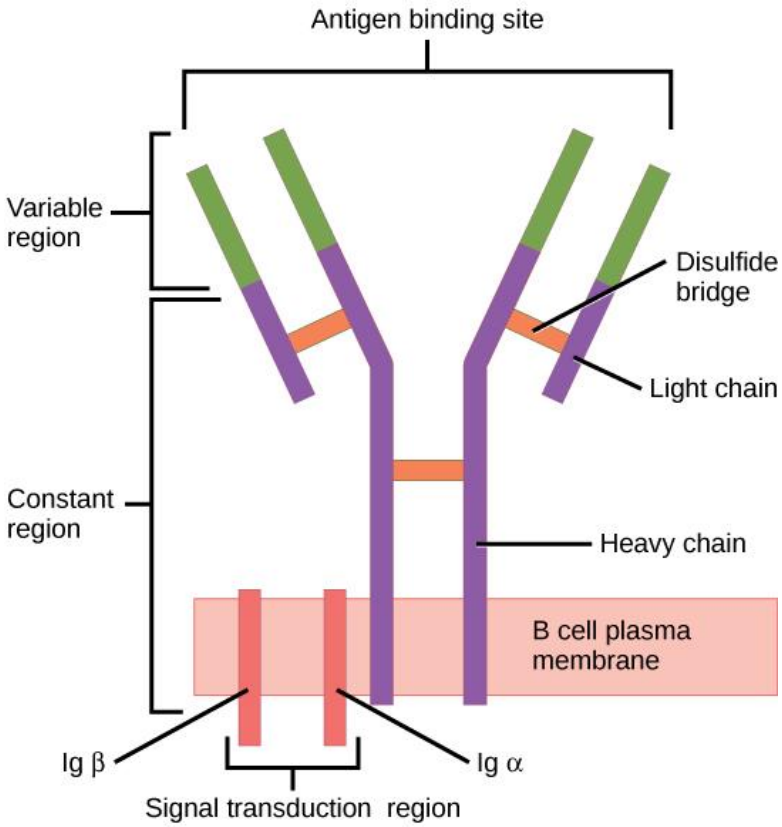


Figure 1.16. Scheme of a B-cell receptor[52]. The c-terminal Fc region of the IgM is anchored into the plasma membrane. The variable domain on the n-terminal Fab region is responsible for binding the specific antigen. Signal transduction, after antigen binding, is carried out by the signal peptides Igα and Igβ.

Once a naïve B-cell encounters an antigen, it engulfs and degrades it, to bind and present peptides on its surface MHC. This MHC-bound peptide can furthermore be recognized, and bound by an effector Th1 cell, specific for the same antigen. This occurs at the border of the T- and B-cell zones in primary lymphoid follicles. The Th1 cell is now activated to produce and secrete cytokines. This causes the B-cell to develop into an antigen-producing plasma cell. Apart from the interaction of the MHC II complex, with the TCR and CD4, another co-receptor plays an important role in the B-cell - Th1 cell interaction. CD 40, on the B-cell, binds the CD40 ligand (CD40L) on the Th1 cell, which enhances the expression of more co-stimulatory molecules, and cell cycle activity in the B-lymphocyte.

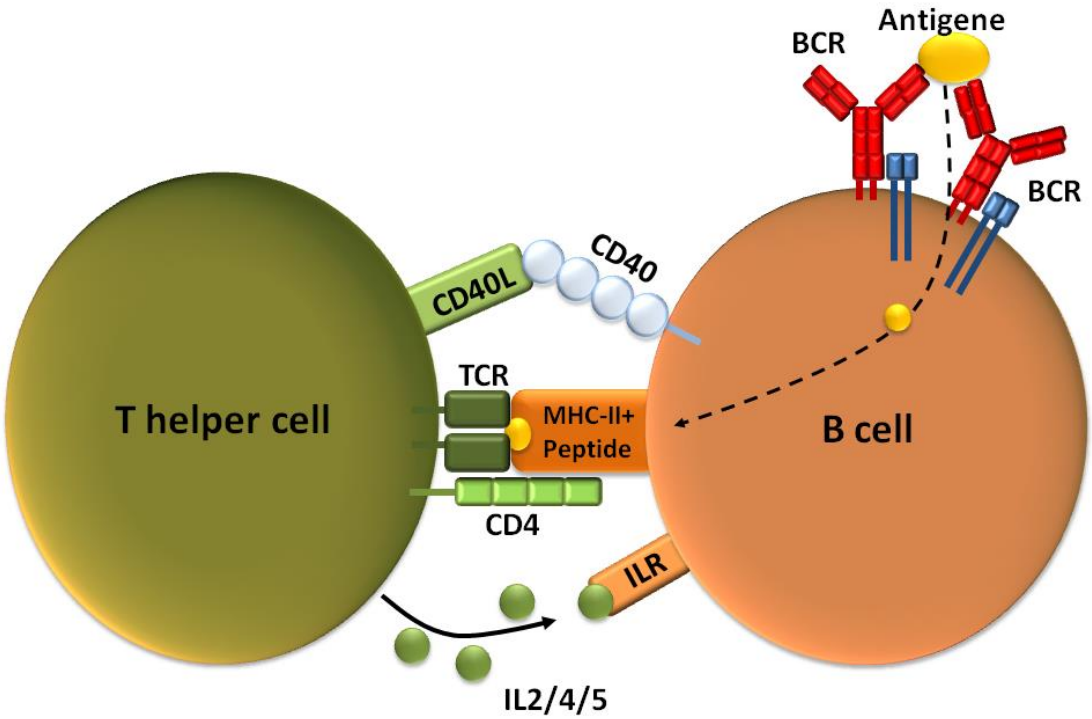


Figure 1.17. Interaction of a Th1 and an antigen-presenting B-cell[53]. The B-cell contains the antigen, bound with its' MHC II, which gets recognized by the TCR. The CD4 co-receptor binds the MHC-peptide complex. The B-cells CD40 co-receptor interacts with the CD40 ligand on the Th1 cell. After binding, and signal transduction, the Th1 cell secretes cytokines, which bind the plasma membrane bound Interleukin receptor, on the B-cell. This event will cause signal transduction, and differentiation of the mature B-cell, into an antibody producing plasma cell.

Antibody production is initiated by Th1 secreted cytokines of the interleukin family (IL-2, IL-4, IL-5, IL-6), by binding the B-cells interleukin receptor (ILR). IL-4 and CD40L binding initiate clonal expansion and antibody production of the cells. IL-5 and IL-6 play important roles in later phases of plasmablast and plasma cell development. Once B-cells are activated, they form germinal centers, inside lymphoid follicles, where they expand and proliferate. At first plasma cells secrete mainly IgM, but they undergo DNA rearrangements, and start to produce other immunoglobulin isotypes. This process is called class switching. The different immunoglobulins are determined by their structure. Cytokines control, which isotype will be expressed by a plasma cell. IL-4 induces IgG1 and IgE production.

IFN-γ induces the secretion of IgG3 and IgG2a. TGF-β thrives class switching towards IgG2b and IgA[50]. Consequently, all isotypes have different functions, and are expressed in different tissues. IgA is a secretory Ig, which gets mostly produced in the mucosa. Although, IgD is known to be expressed at B-cell surfaces, its function is still not entirely clear. IgE is expressed at basophil, and mast cell surfaces, and is involved in defense against parasites. IgE also causes type I hypersensitivity reactions. IgG, the most abundant antibody, is mainly present in the blood serum. Humans express four IgG subtypes: IgG1, IgG2, IgG3 and IgG4. Mice have five different IgG subtypes: IgG1, IgG2a, IgG2b, IgG2c and IgG3. Nevertheless, in both species, IgG1 is the most abundant subtype[54].

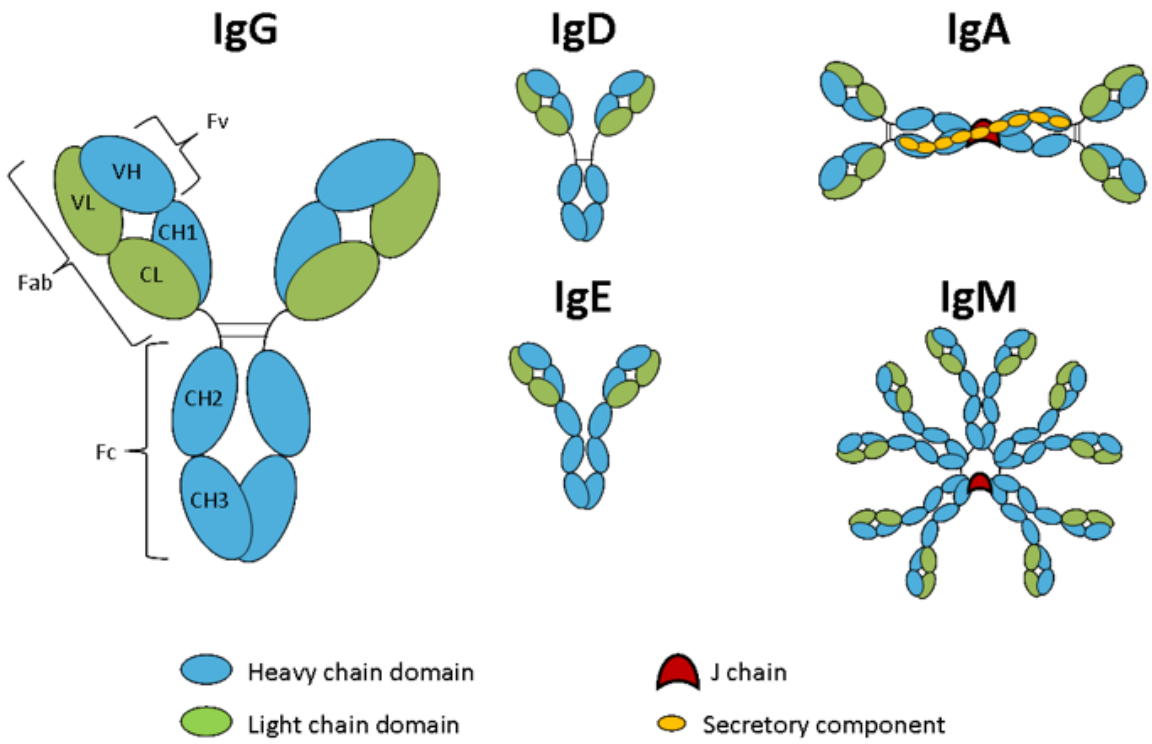


Figure 1.18. The structures of the immunoglobulins isotypes[54]. An antibody contains heavy and light chains. They are linked via hinge domains, which give them a Y-structure. The Fc domain is constant, while the Fab domain is variable and presents the binding sites for the specific antibodies. IgG, IgD and IgE are single Igs. They vary in their heavy chain domains, which are called alpha, delta, epsilon, gamma or mu. They furthermore have different hinge structures. IgA is a dimer, linked via a J chain, while IgM is a pentamere.

1.9.5 Immunologic memory

Apart from becoming effector lymphocytes, both, B- and T-cells, can develop towards memory cells. They remain dormant, until the host encounters another challenge of the specific antigen. The second response is much faster, and more effective. This mechanism is used in vaccines, where the patient gets injected with a harmless amount of an antigen, to create an immunologic memory. Nevertheless, these memory cells can sometimes be harmful, by causing hypersensitivity reactions and allergies. Here, a usually harmless antigen causes a massive immune response, which can in some cases be fatal. The reason why some people develop these reactions is still unclear.

1.9.5.1 Memory B-cells

While antibody producing plasma cells leave germinal centers and get chemoattracted by chemokines towards the site of infection, some B-cells remain in the lymph follicles and become memory cells. They do not produce and secrete antibodies, until they are activated by its specific antigen, or an APC. Until that second encounter they get homed into tissues, or circulate the blood stream.

1.9.5.2 Memory T-cells

Naïve T-cells, which get presented to an antigen, can either become an effector, or a memory T-cell. Like memory B-cells, they circulate the system, or get homed into peripheral tissues. Once their TCR binds its antigen, they get activated, and produce cytokines, to stimulate clonal expansion of effector B- or T-cells. Memory T-cells are known to play important roles in many diseases, like cancer, or allergic asthma.

1.10 Foreign body response to biomaterials

After implantation of a foreign material, the body responds with a series of immunologic reactions. During the first days, protein adsorption, the formation of a provisional matrix, and an acute inflammatory response of the innate immune

system occur. On account of the tissue injury, blood clotting and swelling take place, at the surgery site. Furthermore, an influx of body fluids occur, which delivers important mediators of the immune system. Moreover, the process of wound and tissue healing is initiated. After several days, chronic inflammation, involving an adaptive immune response, will be initiated.

1.10.1 Protein adsorption

Due to the interaction of the biomaterial with the body's tissue and blood, protein adsorption to the foreign body's surface occurs right after implantation. A provisional fibrin protein matrix is formed, for the process of blood clotting, including the influx of blood platelets, to cause blood coagulation. Furthermore, the complement system is activated, which consists of blood circulating proteins that target antigens and can cause opsonization, chemotaxis, inflammation, cell lysis, and apoptosis[55].

1.10.2 Acute inflammation

Acute inflammation is conducted by the innate immune system. The response is fast and unspecific towards the antigen. At first vasodilation, the widening of blood vessel, occurs, which can cause a decrease in blood pressure[56]. In addition, the vessels' permeability gets increased, which causes vascular fluids to enter the surrounding tissues. Furthermore, neutrophilic granulocytes get chemoattracted by pro-inflammatory cytokines and chemokines, to migrate to the inflammatory site. Approximately three days after the initiation of inflammation, monocytes and macrophages start to infiltrate the tissues. Acute inflammation takes place in the first days after implantation, and if the foreign body cannot be removed, chronic inflammation is initiated.

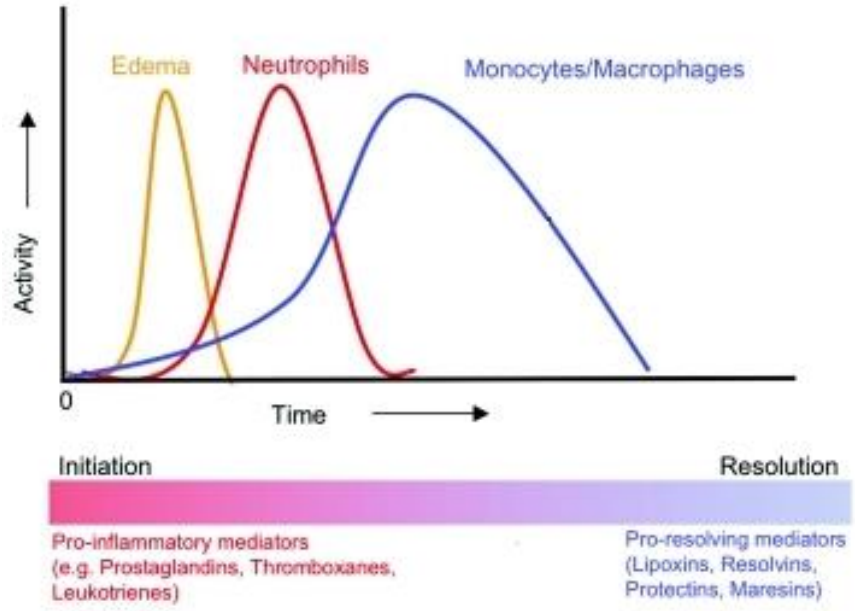


Figure 1.19: Acute inflammation starts with the formation of edemas, followed by neutrophil infiltration. Usually two to three days after the injury, the neutrophil response is abrogated, and monocyte/macrophage infiltration is initiated at the site of inflammation[57].

1.10.3 Chronic inflammation

If the foreign body cannot be cleared by acute inflammatory mediators, chronic inflammation will be initiated. This process causes the recruitment of monocytes, macrophages, and lymphocytes to the foreign material's surface, and anoikis, the regulated cell death of detached cells, will take place. This will consequently lead to angiogenesis, the formation of blood vessels, and fibrosis, the proliferation of connective tissue, around the implant.

1.10.4 Macrophage activation

One of the most important immune cells in chronic inflammation, after implantation of foreign materials, are macrophages. The macrophage cell line originates in the bone marrow, where pluripotent hematopoietic stem cells differentiate, to become common myeloid progenitors[50]. Further the cytokine granulocyte/monocyte colony stimulating factor (GM-CSF) drives their development towards myeloblasts. Finally, they differentiate towards monocytes, which circulate the blood stream. Once they enter the tissue, they become tissue

macrophages. These phagocytes can furthermore be activated by T_H1-cells, which bind to CD40, with is CD40 ligand. In response, the TH1-lymphocytes will secrete IFN-γ, which binds to its receptor on the macrophage surface. Other non-immune activators, like endotoxins, fibronectin, and chemical mediators also have the ability to activate these phagocytes. Activated macrophages can secrete a wide variety of cytokines and chemokines, which generate cross-talk to other immune cells. Table 1 displays the cytokine profile of macrophages.

Secreted cytokines	**Activating cytokines**	**Inhibiting cytokines**
IL-1α	IL-1α	IL-4
IL-1β	IL-1 β	IL-10
IL-6	IL-3	IL-13
IL-8	GM-CSF	TGF-β
IL-10	IFN-γ	
IL-12		
IL-18		
IL-23		
IL-27		
OSM		
TNF-α		
G-CSF		
GM-CSF		

Table 1.1. Macrophages secrete a wide variety of cytokines, for cross-talk with other cells. Furthermore they can be activated and inhibited by other immune cells[50].

Other cells that can differentiate from common myeloid progenitors are dendritic cells, neutrophils, eosinophils, basophils, mast cells, erythrocytes, and blood platelets. The development is determined by gene expression and stimulation with cytokines and growth factors.

1.10.5 Foreign body giant cells

Macrophages get chemoattracted to the site of implantation of foreign materials by chemokines, like CCL2, CCL3 and CCL13, CXCL4, but also by cytokines, like IL-1, and histamine[58]. Once they reach their destination, the macrophages adhere to the materials' surface. Furthermore, the integrin receptors, on the cell surface, will interact with the materials' surface and initiate downstream signaling. These pathways ultimately cause remodeling of the cytoskeleton, formation of podosomes, and spreading of the cells on the implants' surface. If the foreign materials is larger than 5µm, macrophages cannot ingest it. To overcome this problem, they will fuse with surrounding macrophages, to form a foreign body giant cell. These cells are characterized by multiple nuclei in one big cell. IL-13 and IL-4 are known to regulate the process of fusion. These cytokines upregulate the expression of mannose receptors on the cell's surfaces, which will furthermore cause endocytosis, phagocytosis, and fusion of the cells[58]. Finally, the whole implant will be covered in macrophage layers, trying to ingest the material. However, the immunologic responses towards a foreign body can alter, depending on its' size, shape, surface, and tissue location.

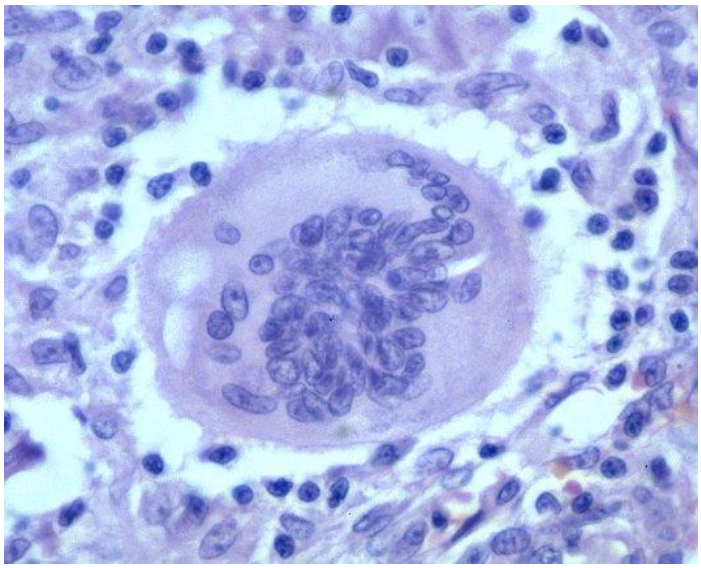


Fig 1.20. Foreign body giant cell stained with hematoxylin and eosin[59]. One giant cell contains multiple nuclei (blue) as a result of macrophage fusion.

But how do foreign body giant cells degrade its target? Their phagolysosomes contain a low pH, where reactive oxygen species are generated, and hydrolases and degrading enzymes reside. These can be released to interact with the implant's surface, and eventually cause its degradation. Whether it will be degraded or not depends of the materials surface. Activated macrophages secrete a wide variety of cytokines (see table 1), which will attract inflammatory cells towards the immune privileged zone.

1.10.6 Extracellular matrix remodeling

1.10.7 Fibrosis

Activated macrophages do not only cause the degradation of a foreign body, but also of the surrounding tissue. Reactive oxygen species, hydrolases, and degrading enzymes also attack the body's' own cells. Nevertheless, this activates regenerative processes, like the construction of components of the extracellular matrix and angiogenesis[60]. TGF-β is a cytokine known to promote fibrosis in various organs[50]. It activates fibroblast to become myofibroblasts, which secrete procollagen, a precursor of the mature collagen fiber. Procollagen consists of three α-strands, organized in a helical fiber, connected via hydrogen bonds. The α-strands are polypeptide chains, built up of the three amino acids glycine, proline, and hydroxyproline. Procollagen gets cleaved by peptidases and can furthermore form mature collagen fibrils. By forming covalent bonds, those fibrils can form complex collagen networks. Collagen represents the most important matrix component of the extracellular matrix. It is the most abundant protein in humans and found in skin, bone, cartilage, and ligaments[61]. If the implantation of a foreign material causes a huge inflammatory response, with the recruitment of macrophages, a fibrous capsule might be formed around the implant. This is an undesired effect, because the implant will be separated and cannot interact or exchange molecules with the surrounding tissue.

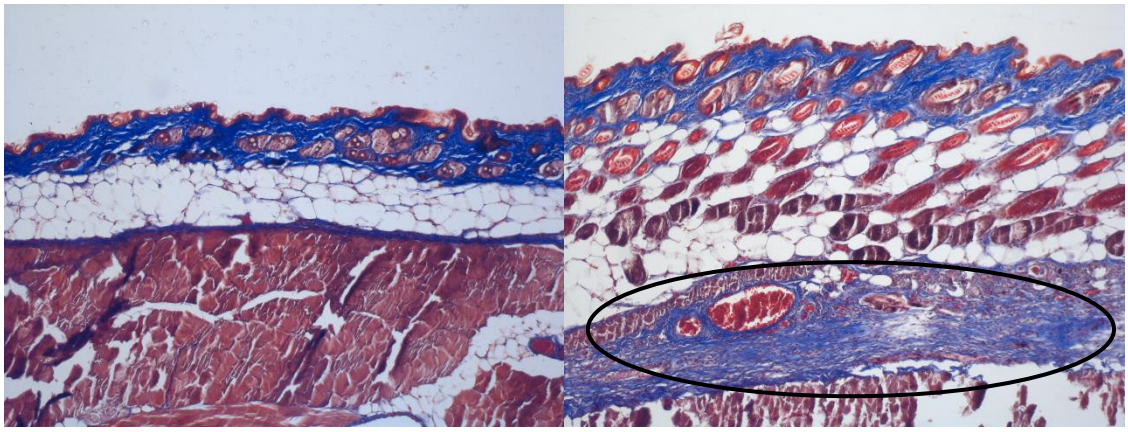


Figure 1.21. Histology sections of normal (left) and implanted (right) skin, stained with hematoxylin, eosin, and annilin blue. The marked area indicates fibrosis, as a reaction to tissue injury and the foreign body response.

1.10.8 Angiogenesis

Angiogenesis is the formation of new blood vessels. It plays a crucial role in the process of wound healing[62]. After implantation of coated islet cells, the implant should have access to the blood stream, to assure a good supply with oxygen, nutrients, and glucose. Furthermore, the produced insulin should be released into the blood, to ensure its fast response to rising blood glucose levels. Endothelial cells develop from angioblasts, after stimulation with growth factors and morphogens, most importantly fibroblast growth-factor-2 (FGF-2), epidermal growth factor (EGF), and vascular endothelial growth factor A (VEGF-A)[63]. In general, there are two types of angiogenesis that can take place in all tissues and organs: sprouting and intussusceptive angiogenesis[64]. Sprouting angiogenesis is the *de novo* formation of blood vessels. Due to the low oxygen concentration in non-vascularized tissues, new vessels are required nearby. Parenchymal cells sense the low oxygen levels and secrete VEGF-A, to create a growth factor gradient. Angioblasts get attracted to the tissue, and initiate the formation of blood vessels. Intussusceptive angiogenesis is the division of existing vessels, and is therefore also called splitting angiogenesis. This process is faster and needs less resources, than sprouting angiogenesis. It often occurs in tissues with high blood flow, to reduce the pressure in the existing vessels. Regulation of intussusceptive angiogenesis is not completely understood yet, but VEGF-A and many other growth factors are involved in this process.

2 Aim

Aim of our experiments is to determine, whether the ELRs trigger an immune, allergic, or foreign body response, upon implantation in female BALB/c mice. Therefore, we use a high throughput intraperitoneal, and a subchronic *in vivo* mouse model. These experiments should provide data on the response of the immune system towards the native coating material. Ultimately, we expect information on whether the material is safe for coating islets, to treat type 1 diabetes.

3 Materials and Methods

3.1 Materials

ELR-biogels for i.p. and s.c. implantation

- RGD-N_3
- VKV-cyclo

Controls

- Vitoss® (β-Tricalcium Phosphate and type 1 collagen foam)
- Alginate (1.8% Alginate, 1.2% $CaCl_2$)
- egg white from chicken eggs
- RGD-VKV-OVA (VKV-cyclo dissolved in 1x PBS (200mg/ml) + RGD-N_3 dissolved in 1x PBS (200mg/ml) containing 100mg/ml chicken egg ovalbumin (OVA)

Surgery equipment

- Anesthesia for surgery: 10 ml Ketanest (25 mg/ml ketamine-hydrochloride, Pfitzer ®), 0.75 ml Rompun (2% xylazin.hydrochloride, Bayer ®), and 14,25 ml dH2O
- 1 ml syringes + needles
- Ear punch, hair dyes
- Shaver
- Autoclaved scissors and forceps
- 70% Ethanol
- Polyglactin suture (Vicryl Plus, Ethicon® Lot Nr. KAP424)
- Polyester suture (Ethibond Excel, Ethcon®, Lot Nr. GM8GTLP0)
- Warming plate
- Eye ointment (VitA-POS®)

Materials for i.p. assessment

- Ice
- 15 ml falcons
- Scissors and forceps
- 1 ml pipette + tips
- 1x PBS (Gibco®, Cat# 14190)
- 10 ml syringes + needles

Materials for s.c. assessment

- Anesthesia to kill: 10 ml Ketanest (25 mg/ml ketamine-hydrochloride, Pfitzer ®), 1.5 ml Rompun (2% xylazin.hydrochloride, Bayer ®), and 13.5 ml dH2O
- Scissors and forceps
- 1 ml syringe + needle
- 1.5 ml Eppendorf® tubes
- 7,5% Paraformaldehyde
- Histology cassettes

Materials for total cell count

- Cell counting chamber
- Tryphan blue solution
- 1x PBS (Gibco®, Cat# 14190)
- Olympus ® light microscope

Materials for differential cell count

- 1x PBS (Gibco®, Cat# 14190)
- Shandon® Cytospin 2 centrifuge
- Cytospin tubes
- Microscope Slides (Thermo Scientific®, 76x26 mm, frosted ends)
- KWIK DIFF ® cell staining kit

Materials for Cytokine ELISA

- IL-1β: ready-SET-Go! Kit from eBioscience ® (Lot Nr. E09327-1637)

- IL-2: ELISA MAX kit from Biolegend ® (Lot Nr. B189154)
- IL-4: ELISA MAX kit from Biolegend ® (Lot Nr. B176237)
- IL-5: ELISA MAX kit from Biolegend ® (Lot Nr. B217084)
- Multiscan FC microplate reader (Thermo Scientific®)

Materials for skin histology

- 7,5% Paraformaldehyde
- Paraffin
- Microtome (Heidelberg® 400)
- Microtome blades (Feather®, s35)
- Microscope Slides (Thermo Scientific®, 76x26 mm, frosted ends)
- Water bath (Kunz® instruments)
- VE- water
- Brushes

Materials for H&E staining

- Xylene (Fluka®, Chemika, Ref: 95690)
- Ethanol Absolute (AustrAlco Österr. Alkolholhandels GmbH, 2104 spillern. 5L bottle)
- Hematoxylin (Sigma Aldrich® Hematoxylin solution, Gill No. 3, Ref: GHS 332- 1L, Lot # 106K4352. Sigma Aldrich Chemie GmbH)
- Eosin Y: (Eosin Y solution alcoholic, Sigma Aldrich®, Cat no. HT110132-1L)
- Fuming HCl (HCl, 37% Merck, Ref: K32970717 409, 1.00317. 1000)
- Mounting media: Eukitt (O. Kindler GmbH and Co., Germany, Chargen No. C106)
- 1% HCl (Sigma Aldrich ®) in dH2O
- Staining racks
- Staining containers
- Tap water
- VE- water
- 1ml syringe

- Cover slips (Thermo Scientific®, 24x60 mm)

Materials for Masson's Trichrome Staining

- Masson's trichrome staining kit (Sigma Aldrich ®, HT 15)
- Bouin's solution (Sigma Aldrich ®, HT10132-1L, Lot # SLBJ3855V)
- Weigert's iron Hematoxylin set (Sigma Aldrich ®, HT1070-1SET, Lot # SLBL5829V)
- 99% Acetic Acid (Sigma Aldrich ®)
- Staining racks
- Staining containers
- Tap water
- VE- water
- 1ml syringe
- Cover slips (Thermo Scientific®, 24x60 mm)

Materials for antibody ELISA

- 96 well plate (Nunc® maxisorp 96 well plate, flat bottom)
- 1x PBS (Gibco®, Cat# 14190)
- Tween 20 (Bio-Rad®, Cat # 170-6531, Lot# 164973A)
- Bovine Serum Albumin (Sigma-Aldrich®, Cat # A-2153, lot. 70H0184)
- Goat anti mouse IgG1 biotin (cat.# 1070-08 from Southern biotechnology associates Inc. USA®)
- Goat anti mouse IgG1 biotin (cat.# 553419 from BD Pharmingen®)
- Streptavidin - HRP (SA-HRP, Southern Biotech®, 7100-05, D126-786H)
- TMB substrate (3.3´,5.5´-tetrametylbenzidine) (BD OptEIATM®, TMB substrate Reagent Set, BD Biosciences, Cat.No.555214)
- ELISA washing buffer (1x PBS, 0.1% Tween 20)
- Blocking buffer (2% BSA, 1x PBS)
- Carrier buffer (1% BSA, 1x PBS)
- Stop solution (0.18 M H_2SO_4)
- OpsysMR DYNEX® plate reader

Mice

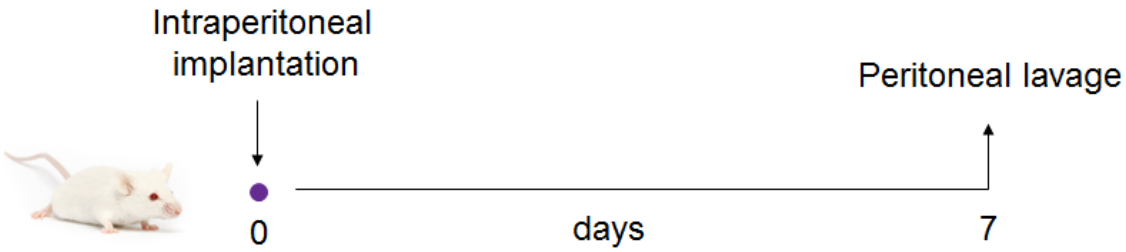
For all experiments female BALB/c mice were used, purchased from Charles Rivers, 6-8 weeks old, housed at the University of Vienna, and fed with normal mouse chew from SSNIFF.

3.2 Methods

3.2.1 Experimental setup

The aim of our experiments was to determine, whether the RGD-VKV biogel induces inflammation, an allergic or foreign body response in BALB/c mice upon implantation. Therefore we use two different *in vivo* mouse models, to implant the gel and examine the immune response. A high-throughput intraperitoneal model, to observe the cellular response towards the gel, and a subcutaneous model, to observe chronic inflammation and antibody responses. To compare these results, we need fitting controls. sham control mimics the surgical procedure, without adding an implant to the peritoneal cavity, or under the skin. Furthermore, we use Alginate, a polysaccharide extracted from brown algae, which is inert, and serves as our negative control. Alginate is a natural polymer, found in the cell walls of brown algae. It has not only shown to immunoisolate its content, if it is used as a coating material, but also induces a minimal immune response[65]. However, a negative control is required too. Since the testing of the polymers is just at the beginning, we needed to test several materials, to find a good negative control. It is required to be of protein nature, have a gel-like aggregation state, and induce a strong inflammation, allergic or foreign body response. We tested three different materials: Vitoss®, a sponge-like scaffold containing β-tricalcium phosphate and type 1 collagen foam, RGD-VKV-OVA, where we add chicken egg ovalbumin to our ELR gels, and cooked egg white from conventional chicken eggs. Only the best of those controls will be used in further experiments.

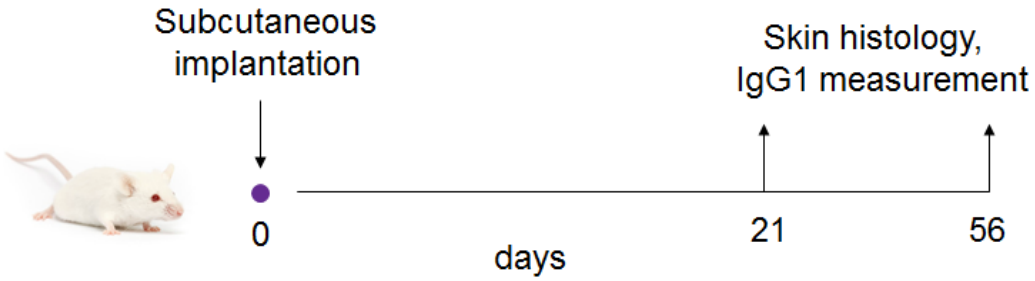
1) **High-throughput intraperitoneal implantation of ELR-biogels**



One day before surgery, we prepared the materials for implantation under sterile conditions. Surgery tools were autoclaved. We implanted the biogel and controls into the peritoneal cavity of the mice, to obtain a quick response of the immune system. The peritoneal cavity is a space in the abdomen, which contains most coelomic organs. It is confined by the peritoneum, a membrane made of epithelial and mesothelial cells. We use it as implantation site, because it guarantees a fast interaction of the body with the materials, due to its' good access to the blood stream and lymphoid vessels. Seven days after the surgery, we euthanized the mice to perform a peritoneal lavage. We analyzed the lavage fluid to determine the immune cell composition, which might change due to the implants, compared to sham control. Moreover, we used the peritoneal lavage fluid to determine cytokine concentrations. We measured following cytokines, using ELISA kits:

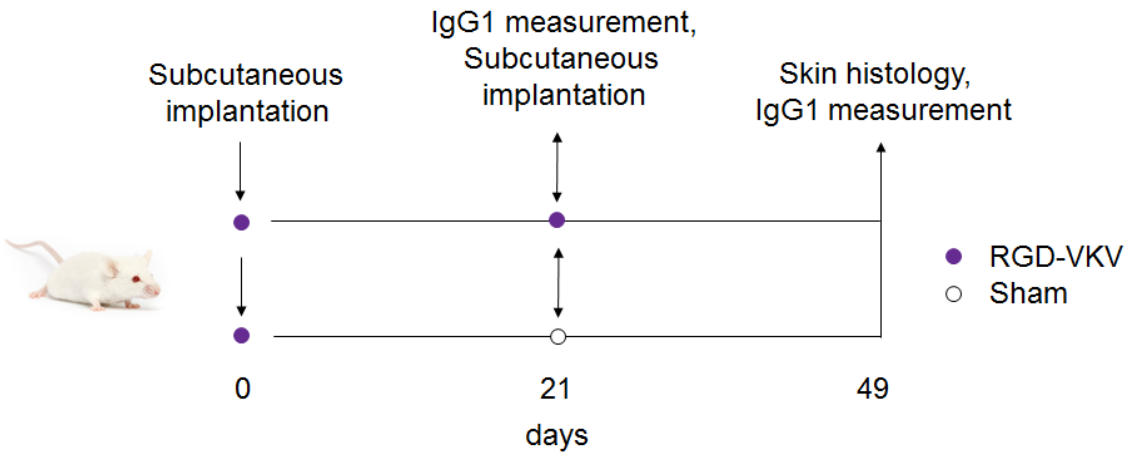
- IL-1β, a marker for macrophage activation and proliferation.
- IL-2, a cell proliferation marker.
- IL-4, a cytokine promoting the fusion of macrophages, to form foreign body giant cells. It also induces antibody class-switching towards IgG1 and IgE, and fibroblast activation to produce collagen.
- IL-5, produced by eosinophils, an indicator for an allergic reaction.

2) Subcutaneous implantation of ELR-biogels



One day before surgery, the materials for implantation were prepared under sterile conditions. Surgery tools were autoclaved. We implanted the ELRs, and the controls between the skin and the peritoneum of the abdomen, to determine the long term effects of the materials. In contrast to i.p. implantation, the immune response to the implants is slower. The mice were assessed 21 or 56 days after surgery. Furthermore, we did skin histological examinations, by staining skin samples with H&E and Masson's trichrome techniques, to determine, whether inflammation and fibrosis occurred around the implanted materials. At last we measured the IgG1 titer in the blood sera, to determine the humoral immune response towards the implants.

3) Rechallenge of subcutaneously implanted mice



One day before surgery, the materials for implantation were prepared under sterile conditions. Surgery tools were autoclaved. We implanted the ELRs between the skin and the peritoneum on the left side of the abdomen. 28 days after the initial implantation, the same amount of gel was implanted in 6 mice, at the right side of the abdomen. The remaining 6 mice were rechallenged with sham surgery. 49 days after primary implantation, the mice were assessed for IgG1 measurement, to observe if the second implantation induced an elicitation of RGD-specific antibodies. Furthermore, we did a skin histological examination.

3.2.2 Methodology

3.2.2.1 Preparation of ELR-biogel and controls

All gels and controls were prepared under sterile conditions, using a cell culture hood, sterile pipette tips, and sterile PBS.

Basic gels (VKV-cyclo, RGD-N_3) were dissolved in 1x PBS (c=200mg/ml), at 4°C, overnight. Before surgery, the two basic gels were added 1:1 on a sterile paraffin sheet and incubated at room temperature, for chemical crosslinking of the two polymers. Each mouse was implanted with 4 mg RGD-N_3 and 4 mg VKV-cyclo.

Alginate control was prepared in a 96-well plate, using 122 µl endotoxin free sodium alginate solution (2.2 mg per mouse). By adding 34 µl sterile 1.2% $CaCl_2$, gel formation was initiated. The gel was stored at 4°C until further use.

RGD-VKV-OVA was created by dissolving the RGD-N_3 polymer in 1x PBS, containing 100mg/ml OVA. At the day of surgery, the RGD-N_3-OVA was mixed 1:1 with VKV-cyclo gel and incubated at room temperature, for hydrogel formation. Consequently, each mouse was implanted with 2 mg OVA and 8 mg RGD-VKV gel.

The egg white positive control was created, by cooking egg white for 30 minutes, at 100°C, in a water bath. Furthermore, the coagulated egg was dehydrated in 70% ethanol, for 2 days. Before implantation, the material was washed with 1x PBS, and cut into small disks, using a 6mm biopsy punch.

Vitoss® was cut using a sterile 6mm biopsy punch. Before implantation, the controls were washed with 1x PBS.

3.2.2.2 Intraperitoneal implantation

Mice were anesthetized, by injecting 0.15 - 0.2 mL of anesthesia intraperitoneally. Furthermore, we shaved their abdomen, and disinfected the skin with 70% ethanol. We applied a ~5mm incision to the skin on the abdomen, and the peritoneum, with a sterile scissors for microsurgery. To insert the materials into the peritoneal cavity (except to the sham control mice), we used a sterile forceps. Between the different mice, the forceps and scissors were disinfected with 70% ethanol. Moreover, we sutured the incisions in the peritoneum, by using a sterile and antibacterial polyglactin suture. The incision in the skin was closed with a sterile polyester suture. At last, we disinfected the site of surgery with 70% ethanol, and placed the mice on a warming plate (37°C) for recovery, until they regained consciousness. For preventing their eyes to dry out, we applied eye ointment onto them. We observed the mice until 3 hours after surgery, to guarantee their wellbeing.

Assessment

7 days after surgery, the mice were assessed. After euthanizing them by cerebral dislocation, we performed a peritoneal lavage. Therefore, we injected 3 mL 1x PBS into the peritoneum of each mouse. Furthermore, we applied an incision to the skin, and created a pocked between the skin and the peritoneum. Furthermore, we applied a small hole to the peritoneum, for the PBS to exit the peritoneal cavity. The liquid was collected with a 1 ml pipette in a 15 ml falcon, and stored on ice until cell counting.

Total and differential cell count

For performing a cell count, we used the chilled peritoneal lavage samples. They were centrifuged at 1.300 rpm for 5 minutes. Afterwards, 1 ml of the supernatant was transferred into a sterile 1.5 mL Eppendorf tube, and stored at -20°C for cytokine ELISA. To the remaining samples we added 1 ml 1x PBS, and the pellet was resuspended by vortexing. For the total cell count, we diluted the samples with 1x PBS, and mixed them 1:1 with a Tryphan Blue solution. We pipetted 20 µl of this solution into the counting chamber. For counting, an OLYMPUS ® light microscope, with a 10x magnification objective lens was used. At last, we diluted the remaining samples, depending on the total cell count and added 150 mL to cytospin tubes. The cytospin was run at 8,000 rpm, for 5 minutes, to transfer the cells onto glass slides. Finally, we stained the slides, using a KWIK DIFF cell staining kit. For the differential cell count, we used an OLYMPUS® light microscope, with an oil lens of 40x magnification.

Cytokine ELISA

For cytokine ELISA, we used an ELISA MAX kit from BioLegend®, for IL-2, IL-4 and IL-5, and an ELISA ready-SET-Go! kit from eBioscience®, for IL-1β, according to the given protocols. We measured the plates at OD450 nm, using a microplate reader.

3.2.2.3 Subcutaneous implantation

Mice were anesthetized, by injecting 0.15 – 0.2 mL of anesthesia intraperitoneally. Furthermore, we shaved the mice at the site of surgery, and disinfected them with 70% ethanol. For marking the different groups, we applied ear punches. A ~5mm incision was applied to the skin, and a pocked formed, with a blunt forceps, between the skin and the peritoneum. Furthermore, we added the implants (except for sham control) to the pocket, and sutured the skin, using a polyglactin suture. Finally, we disinfected the wound, using 70% Ethanol, and placed the mice on a warming plate (37°C), for recovery. For preventing their eyes to dry out, we applied eye ointment onto them. Until regaining consciousness, the mice were observed, and then placed back into the cages.

Assessment

21 or 56 days after surgery, the mice were injected with 0.4 ml of anesthesia to kill. We drawed the blood via cardiac puncture, using a 1 ml syringe. The blood was collected in 1.5 ml Eppendorf tubes, and incubated at RT for >1 hour. Next, we centrifuged the samples at 13.800 rpm, for 10 minutes, and pipetted the blood sera into a fresh 1.5 ml Eppendorf tube. They were stored at -20°C, until further use. At the day of antibody ELISA, the samples were slowly thawed at 4°C. For skin histologic sections, we cut out a 1*1 cm square of skin, at the site of implantation. We placed the samples in a histology cassettes, before fixing them in 7.5% paraformaldehyde. Furthermore, we fixed the skin samples in paraffin. For embedding them into paraffin, they were cut into smaller fractions. Afterwards we froze the embedded samples at -20°C, for >24 hours. With a microtome, we created 0.4 mm cuts of the frozen paraffin blocks, and transferred them into a warm water bath. At last, we transferred them on glass slides, and let them dry for the histological staining.

H&E staining

The oven was preheated at 65°C. We incubated the paraffin slides in racks for 45 minutes. Afterwards, we placed the racks in xylene for 20 minutes, to remove

the molten paraffin. Furthermore, the tissue samples were rehydrated in an ethanol gradient (100%, 70%, 50%), for each 1 minute. Then we rinsed the slides with dH2O, and placed them in hematoxylin for 5 minutes. Next, the samples were rinsed with tap water, to remove the excess color. Furthermore, we dipped the slides in 1% HCl for 5 seconds, and rinsed them in dH2O. Then the slides were dehydrated in 50% and 70% ethanol for 30 seconds, before incubating them for 30 seconds in an Eosin Y solution. Afterwards, we rinsed the slides with tap water. At last we dehydrated the tissue, using an ethanol gradient (50%, 70%, 100%), for each 30 seconds. Finally, the slides were air dried, before we mounted them, and added a cover slip.

Masson's trichrome staining

The oven was preheated at 65°C. We incubated the paraffin slides in racks for 45 minutes. Afterwards, we placed the racks in xylene for 20 minutes, to remove the molten paraffin. Furthermore, the tissue samples were rehydrated in an ethanol gradient (100%, 70%, 50%), for each 1 minute. Afterwards, we rinsed the slides with dH2O, and incubated them in preheated Bouin's solution, at 56°C for 15 minutes. Next, the slides were rinsed with tap water, and placed in Working Weigert's Iron Hematoxylin for 5 minutes. We rinsed the slides again with dH2O, and incubated them in Biebrich Scarlet-Acid Fuchsin, for 5 minutes. Furthermore, the slides were rinsed in dH20, and placed in Working Phosphotungstic/Phosphomolybdic acid solution, for 5 minutes. Afterwards, we incubated the racks in Anilin Blue Solution, for 5 minutes. At last, we put the samples in 1% acetic acid, for 2 minutes. Then the tissue samples were dehydrated in an ethanol gradient (50%, 70%, 100%), for each 30 seconds. Finally, the slides were air dried, before we mounted them, and added a cover slip.

Antibody ELISA

Day 1: 96 well plates were coated, using antigen suspensions (type 1 bovine collagen, RGD and Alginate), with a concentration of 0.1µg/50µl. The plates were

incubated at 4°C o/n. Furthermore, the blocking buffer (2% BSA in 1x PBS) was prepared, and stored at 4°C.

Day 2: At first, the coating suspension was discarded, and the plates washed 3x, using 200µl of washing buffer (1x PBS, 0.1% Tween 20). The wells were dried, by tapping them on clean paper sheets. Next, 100µl of blocking buffer was added, and the plates incubated for 2 hours on RT, on a rotating shaker. Furthermore, the plates were washed 3x with 200µl of washing buffer/well. Finally, the samples were thawed and diluted (1:50 for IgG1 and 1:4 for IgE), and 100µl added in duplicates to row A of the plates. To the remaining rows, 80µl of carrier buffer (1% BSA in 1x PBS) was added. Then 1:5 serial dilutions were done, by always transferring 20µl of the sample dilutions to the next row, using a multichannel pipette. This was done until row G, where 20µl of the diluted samples were finally discarded. Row H only contained carrier buffer, and represents the blank. The plates were incubated at 4°C o/n.

Day 3: Sample dilutions were discarded, and the plates washed 3x using 200µl wash buffer. 100µl of secondary antibody, using a 1:1000 dilution of IgG1 and a 1:250 dilution of IgE in carrier buffer, were added and the plates and incubated at RT for 2 hours. After a further washing step, 100µl of streptavidin – HRP (1:8000 with carrier buffer) were added, and the plates incubated at RT for 1 hour. Furthermore, the plates were washed, and 100µl of freshly prepared TMB substrate added. At last, the plates were incubated for 10 minutes, and finally 100µl of stop solution (0.18M H_2SO_4) added, to stop the enzymatic reaction. Plates were measured at 450nm, using a microplate reader.

3.2.2.4 Figures and statistical analysis

All figures and statistics were performed with the software GraphPad Prism 5. We compared the different groups with one-way analysis of variance (ANOVA) and Tukey post hoc test with a 95% confidence interval. We compared all groups to sham, to find out, whether they showed a significant difference. The results are displayed in our figures using following abbreviations: * $p<0.05$, ** $p<0.005$, *** $p<0.001$, or not significant (ns)

4 Results

4.1 Response to implantation of ELR-biogel in the peritoneal cavity

4.1.1 Physical characteristics

Formation of the dissolved polymers occurs at room temperature, after adding the RGD-N_3 and VKV-cyclo gel in a 1:1 ratio to each other. Before implantation, the gel appeared as a white and viscous gel, with a convex form (see figure 3.1). After the gel was in the peritoneal cavity of test mice for 7 days, it seemed to change is shape, color, and solidity. It appeared white-transparent, irregular, and solid (see figure 3.1). An examination under the light microscope is shown in Figure 3.1. The shape of the gel was irregular, and more transparent than before (see figure 3.1).

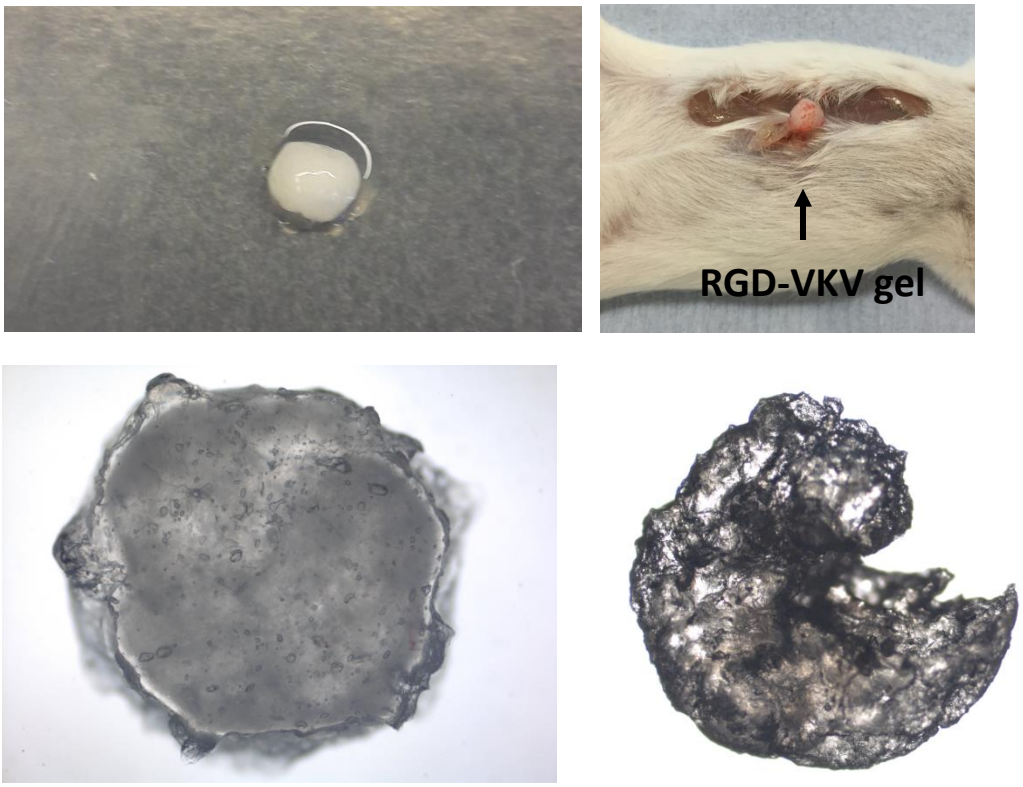


Figure 3.1. RGD-VKV gel before (top left), and 7 days after (top right) intraperitoneal (i.p.) implantation taken with a 10x magnified light microscope. Changes in shape, color, and solidity can be observed. Images of RGD-VKV gels before (bottom left), and after (bottom right) i.p. transplantation taken with a 10x magnified light microscope. Changes in shape and transparency can be observed.

4.1.2 Inflammatory cell populations in peritoneal lavage fluids

The peritoneal lavage fluid of BALB/c mice implanted with egg white induced the highest total cell count compared to sham controls ($p<0.01$), which did not contain an implant, and should therefore not show an inflammatory, or foreign body response (Fig.3.2). Although mice with a Vitoss® implant had a significantly higher cell count compared to sham control ($p<0.05$), egg white induced a higher cellular response than Vitoss® (Fig.3.2). For mice implanted with RGD-VKV-OVA, there was no significant difference to sham and was, therefore, dismissed as a possible positive control for further experiments (Fig.3.2). Neither Alginate nor RGD-VKV implants caused a significant change in total cell counts compared to sham controls ($p>0.05$). Therefore, we concluded that they did not trigger a severe immune response upon i.p. implantation (Fig.3.2).

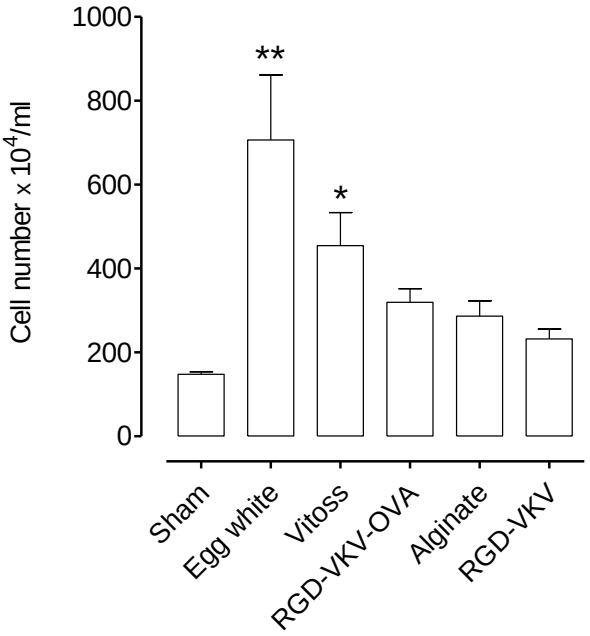


Figure 3.2. Total cell numbers in the peritoneal lavage fluid of BALB/c mice implanted with RGD-VKV biogel and sham, egg white, Vitoss®, Alginate controls, and RGD-VKV-OVA. Data was calculated with mean + SEM; statistics were done using one-way ANOVA; n = 5; * sham vs. Vitoss, $p<0.05$; ** egg white vs. sham, $p<0.005$.

When examining differential cell counts (Fig.3.3, Table 3.1), the largest cell population in all mice were macrophages. egg white and Vitoss® induced a shift

towards eosinophils after implantation (Fig.3.3, Table 3.1), which is an indicator for an allergic response towards the foreign material. Moreover, the absolute cell numbers for egg white revealed the highest number of macrophages (Fig.3.4), eosinophils (Fig.3.5) and neutrophils (Fig.3.6), compared to sham controls. The percentages of cells suggest that Vitoss® triggered the highest number of eosinophils (Table 3.1), however, sham control mice had the highest percentage of macrophages. RGD-VKV-OVA peritoneal lavage fluid contained the highest percentage of neutrophils, although it was not a significant change compared to sham controls (Table 3.1).

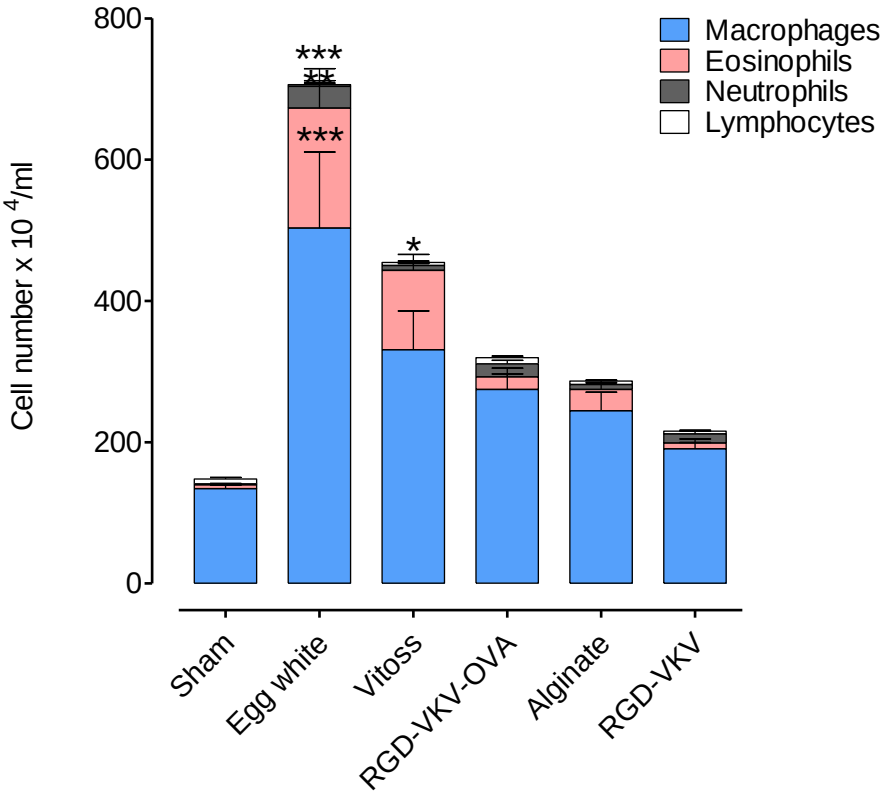


Figure 3.3. Cell numbers of macrophages, eosinophils, neutrophils and lymphocytes counted in the peritoneal lavage fluid of BALB/c mice implanted with RGD-VKV biogel and sham, egg white, Vitoss®, Alginate controls, and RGD-VKV-OVA. Data was calculated in mean ± SEM; *** macrophages and eosinophils: egg white vs. sham, p<0,001; ** neutrophils: egg white vs. sham p<0.01; * eosinophils: Vitoss® vs. sham, p<0.05

Table 3.1. Percentages of immune cell populations calculated from total cell numbers

	Macrophages	Eosinophils	Neutrophils	Lymphocytes
sham	89,96	2,95	1,05	6,22
egg white	74,60	19,48	5,45	0,47
Vitoss®	70,77	23,81	4,07	1,35
RGD-VKV-OVA	85,72	5,40	5,83	3,05
Alginate	73,25	9,37	2,34	2,93
RGD-VKV	87,19	8,30	2,45	2,06

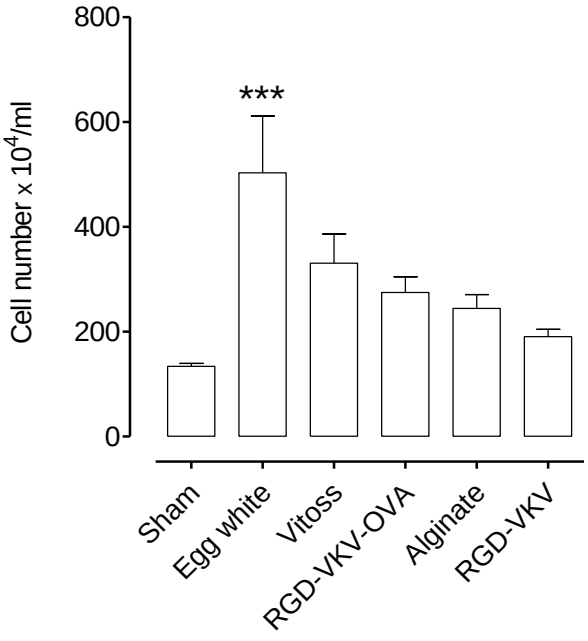


Figure 3.4. Cell numbers of macrophages in the peritoneal lavage fluid of BALB/c mice implanted with RGD-VKV biogel and sham and egg white, Vitoss®, Alginate controls and RGD-VKV-OVA. Data was calculated with mean ± SEM; statistics were done using one-way ANOVA; n = 5; *** egg white vs. sham, $p<0.001$.

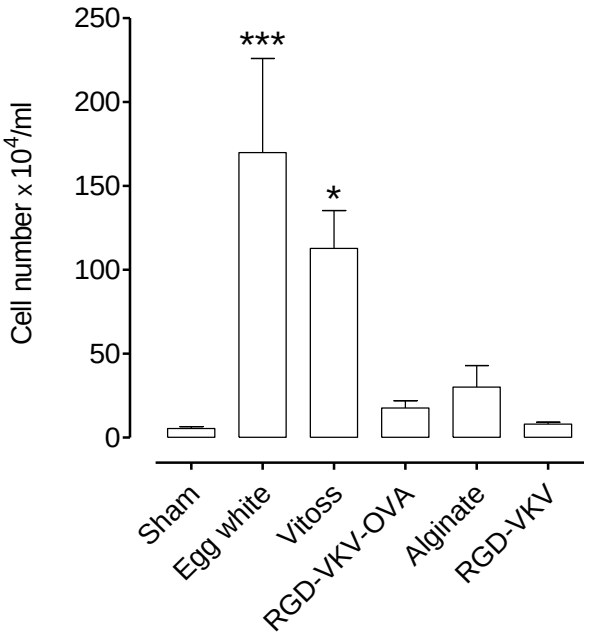


Figure 3.5. Cell numbers of eosinophils in the peritoneal lavage fluid of BALB/c mice, implanted with RGD-VKV biogel and sham, egg white, Vitoss®, and Alginate controls, and RGD-VKV-OVA. Data was calculated with mean ± SEM; statistics were done using one-way ANOVA; n = 5; * Vitoss® vs. sham, $p<0.05$; *** egg white vs. sham, $p<0.001$.

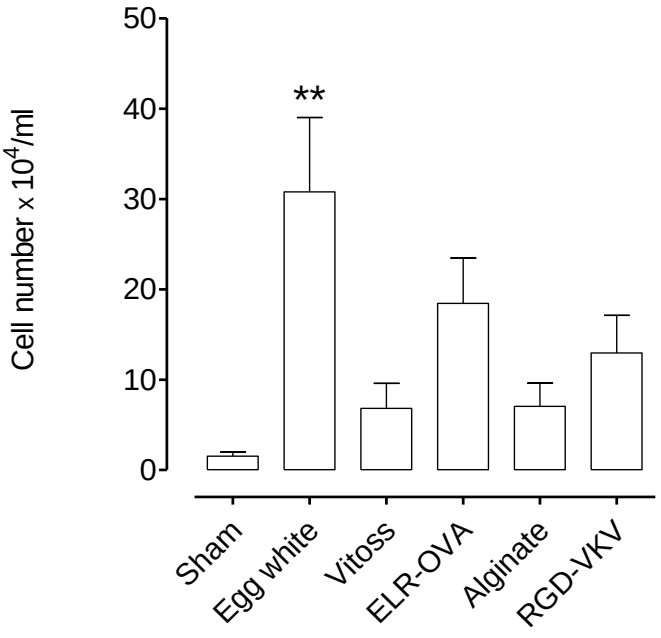


Figure 3.6. Cell numbers of neutrophils in the peritoneal lavage fluid of BALB/c mice implanted with RGD-VKV biogel and sham, egg white, Vitoss®, Alginate controls, and RGD-VKV-OVA. Data was calculated with mean ± SEM; statistics were done using one-way ANOVA; n = 5; * egg white vs. sham. Vitoss ® and Alginate, $p<0.05$.

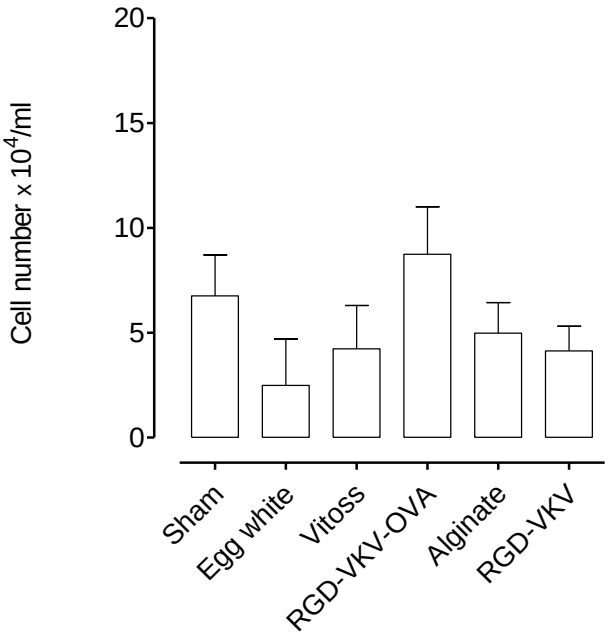


Figure 3.7. Cell numbers of lymphocytes in the peritoneal lavage fluid of BALB/c mice, implanted with RGD-VKV biogel and controls sham, egg white, Vitoss®, Alginate and RGD-VKV-OVA. Data was calculated with mean ± SEM; statistics were done using one-way ANOVA; n = 5; not significant.

4.1.3 Cytokine concentration in peritoneal lavage fluids

After we performed the differential cell count, we used the peritoneal lavage fluid for cytokine measurements. We selected to investigate IL-1β, IL-2, IL-4 and IL-5. IL-1β is a macrophage-related cytokine. Our analysis did not show any significant results for RGD-VKV, egg white, Vitoss, RGD-VKV-OVA or Alginate compared to sham (see figure 3.8). Although the mice implanted with egg white and Vitoss® had increased IL-1β production, the variation between samples was too high and were not statistically significant. IL-2 measurement was only significantly difference for egg white compared to sham controls ($p<0.05$, figure 3.9). Importantly, RGD-VKV gel did not induce an IL-2 response, which means the gel did not simulate cell proliferation upon i.p. implantation. We observed similar results for IL-4 measurements. Only implantation of egg white induced a high IL-4 response in the mice ($p<0.05$, figure 3.10). RGD-VKV-implanted mice did produce more IL-4 compared to sham controls. In this case, we assume that the

fusion of macrophages to form foreign body giant cells is not stimulated. Lastly, we investigated IL-5 concentrations in the peritoneal lavage fluid, to determine whether allergic-type inflammation occurs upon i.p. implantation. Similar to the eosinophil cell count results, egg white induced a significantly higher IL-5 concentration than sham controls ($p<0.001$, figure 3.11). Because both IL-4 and IL-5, are T_H2 cytokines, we conclude that egg white triggers an allergic response. In contrast, we did not detect IL-5 in RGD-VKV peritoneal fluid.

In summary, the cytokine measurements confirm that egg white induces a higher immune response than Vitoss® and is therefore a more suitable positive control.

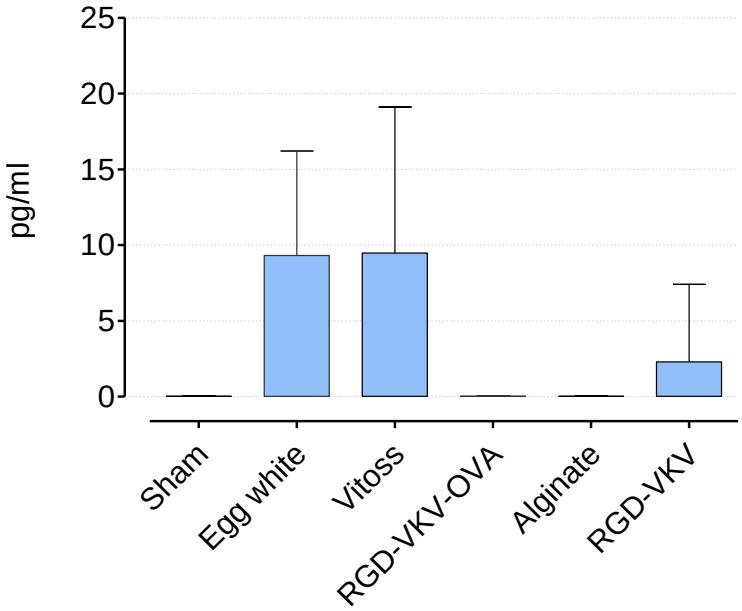


Figure 3.8. IL-1ß concentration in peritoneal lavage fluid of RGD-VKV and sham, egg white, Vitoss®, Alginate controls, and RGD-VKV-OVA; Data was calculated with mean ± SEM; statistics were done using one-way ANOVA; ns

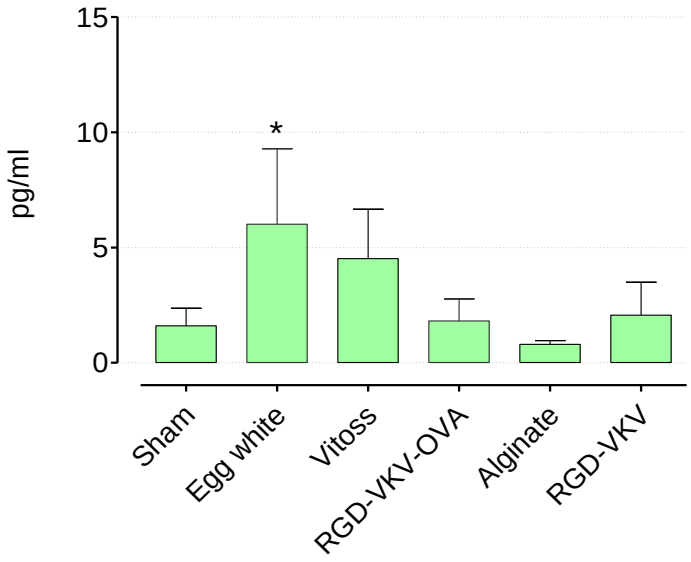


Figure 3.9. IL-2 concentration in peritoneal lavage fluid of RGD-VKV and sham, egg white, Vitoss®, Alginate controls, and RGD-VKV-OVA; Data was calculated with mean ± SEM; statistics were done using one-way ANOVA; n = 5. * egg white vs. sham, p<0.05.

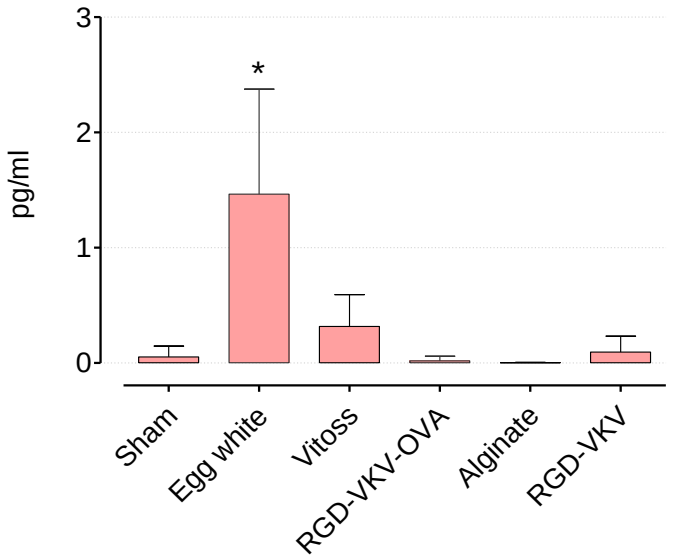


Figure 3.10. concentration in peritoneal lavage fluid of RGD-VKV and sham, egg white, Vitoss®, Alginate controls, and RGD-VKV-OVA; Data was calculated with mean ± SEM; statistics were done using one-way ANOVA; n = 5. * egg white vs. sham, p<0.05

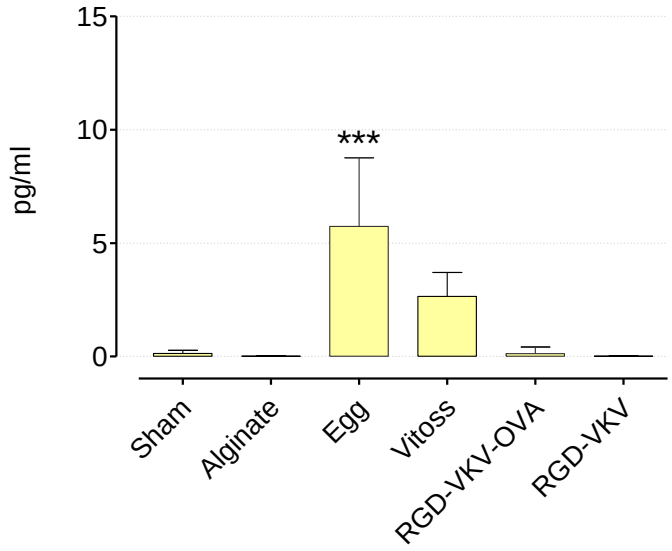


Figure 3.11. IL-5 concentration in peritoneal lavage fluid of RGD-VKV and sham, egg white, Vitoss®, Alginate controls, and RGD-VKV-OVA; Data was calculated with mean ± SEM; statistics were done using one-way ANOVA; n = 5. *** egg white vs. sham, $p<0.001$

Examination of the implant 1 week after intraperitoneal implantation

One week after i.p. implantation, we examined the peritoneum for the gel. To determine whether immune cells infiltrated the implants, we performed an H&E staining of paraffin embedded gel sections (figure 3.14). As controls, we stained egg white (figure 3.12) and Vitoss® (figure 3.13) samples to compare the photomicrographs. Unfortunately, we were not able to embed the Alginate implants because they dissolved in the 7.5% paraformaldehyde during the fixation process. We know from the differential cell count (figure 3.3) that egg white causes a strong cellular response composed of macrophages and eosinophils, upon i.p. implantation. Figure 3.12 displays these two cells populations surrounding the implant, which can primarily be observed at the periphery of the material. Foreign body giant cells are barely present. Furthermore, most of the cells were not able to penetrate the outer barrier of the implant. In contrast, Figure 3.13 displays the Vitoss® implant 1 week after i.p. implantation. Again, we observe macrophages and eosinophils, within the implant. The Vitoss sponge-like structure made it easier for the cells to enter the

implant. Additionally, a large number of foreign body giant cells were observed surrounding the Vitoss implant. Lastly, the stained RGD-VKV section revealed a cellular response with macrophages and eosinophils surrounding the implant able to enter holes in the gel that might be a result of degradation while it was in the peritoneal cavity. However, a significantly higher number of macrophages and eosinophils were not observed in the differential cell counts compared to sham controls (figure 3.3) and, the low concentrations of IL-1ß, IL-4, and IL-5 do not suggest a significant increase in macrophages and eosinophils, compared with the sham controls.

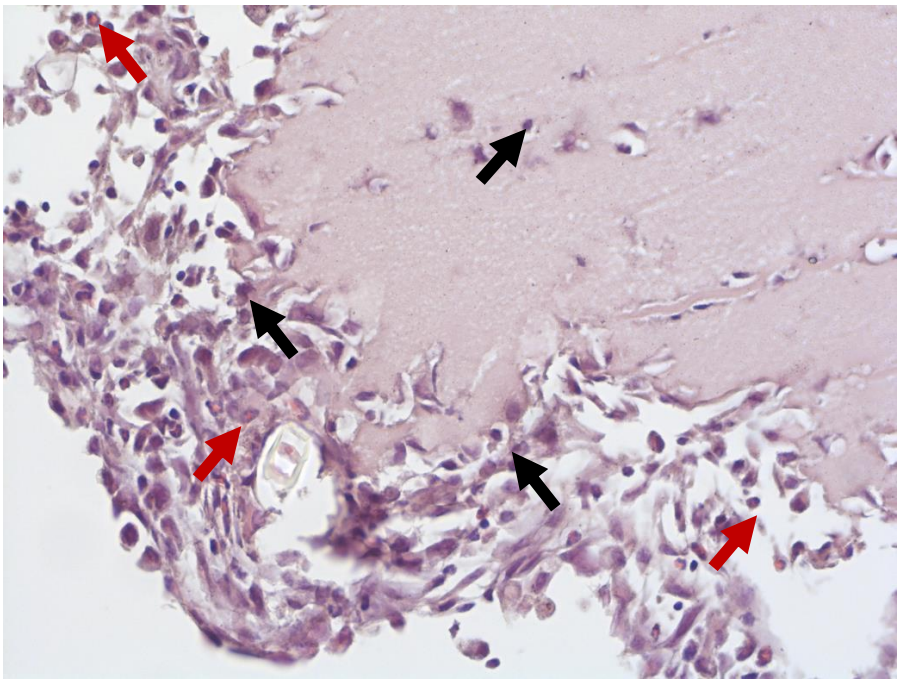


Figure 3.12. Picture of egg white, 1 week after i.p. implantation stained with H&E. Black arrows indicate macrophages, red arrows eosinophils. These cells can mostly be seen at the implants' border, but barely on the inside. Picture was taken using a 40x magnified light microscope.

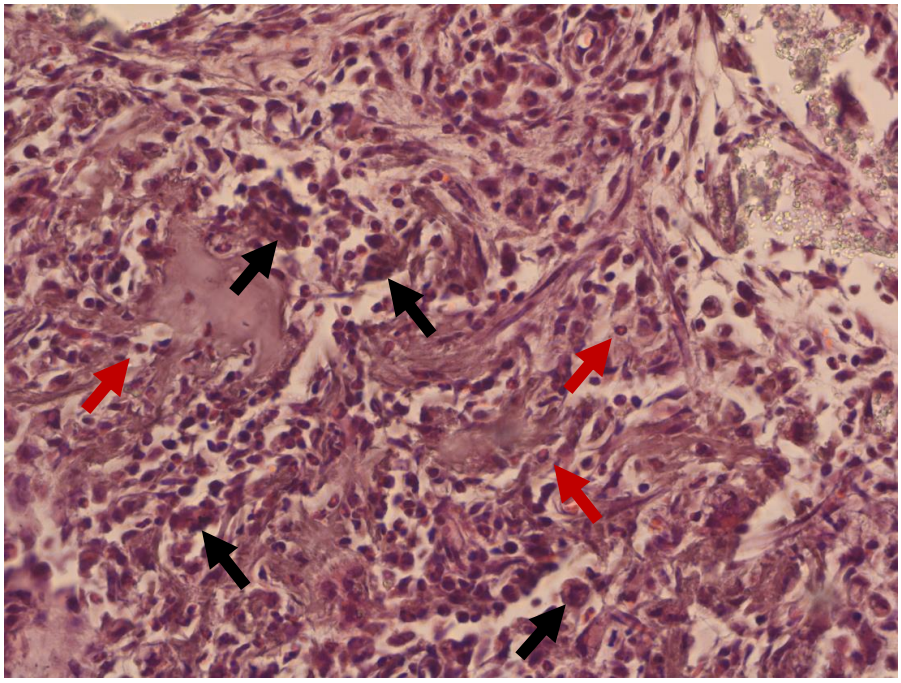


Figure 3.13. Picture of Vitoss®, 1 week after intraperitoneal implantation stained with H&E. Black arrows show fused macrophages, forming foreign body giant cells. Red arrows indicate eosinophils, infiltrating the material. Picture was taken using a 40x magnified light microscope.

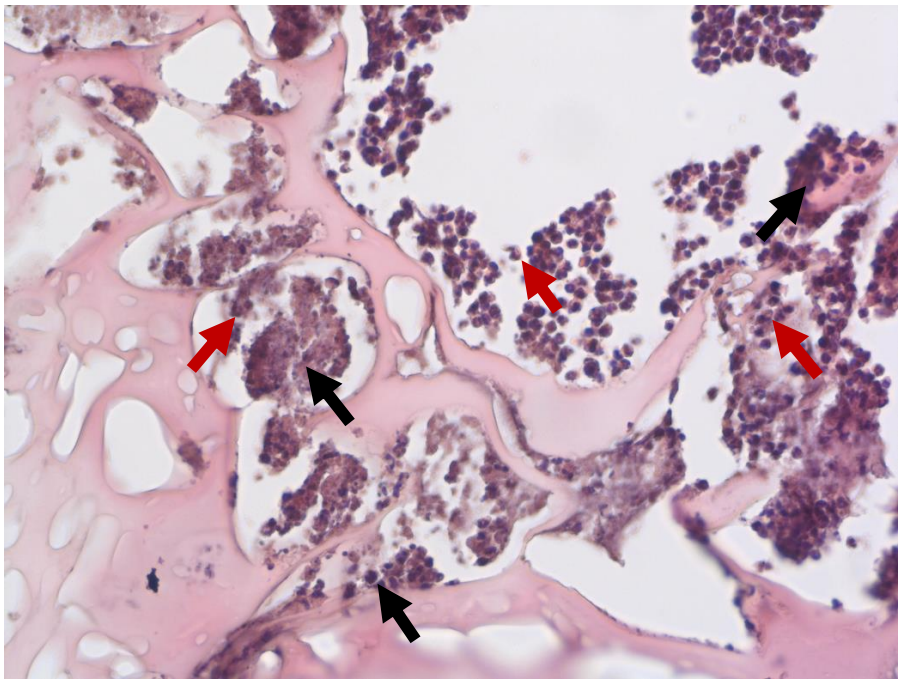


Figure 3.14. Picture of RGD-VKV gel, 1 week after intraperitoneal implantation stained with H&E. Black arrows show fused macrophages, forming foreign body giant cells. Red arrows indicate eosinophils, infiltrating the material. Most of the cells are around the implant, occasionally some were able to penetrate the outer border of the gel. Picture was taken using a 40x magnified light microscope.

4.2 Response to subcutaneous implantation of ELR-biogel

By measuring the IgG1 titer of the blood sera taken from BALB/c mice 3 weeks after s.c. implantation, we could determine whether there was a specific response to the polymers (figure 3.15). IgE measurement was performed, but no significant levels were detected for the gel and all control groups. The highest sera dilution used in the ELISA assay was 1:50. The highest titer of IgG1 was present in the samples from egg white-implanted mice, followed by RGD-VKV-OVA, and Vitoss®. As expected, Alginate triggered almost no IgG1 response and RGD-VKV induced RGD-specific antibodies, but in a lower titer than Vitoss®, egg white, and RGD-VKV-OVA sera. As negative controls, the sham sera was applied to all antigen-coated plates to exclude that there were unspecific IgG1 responses to the implants.

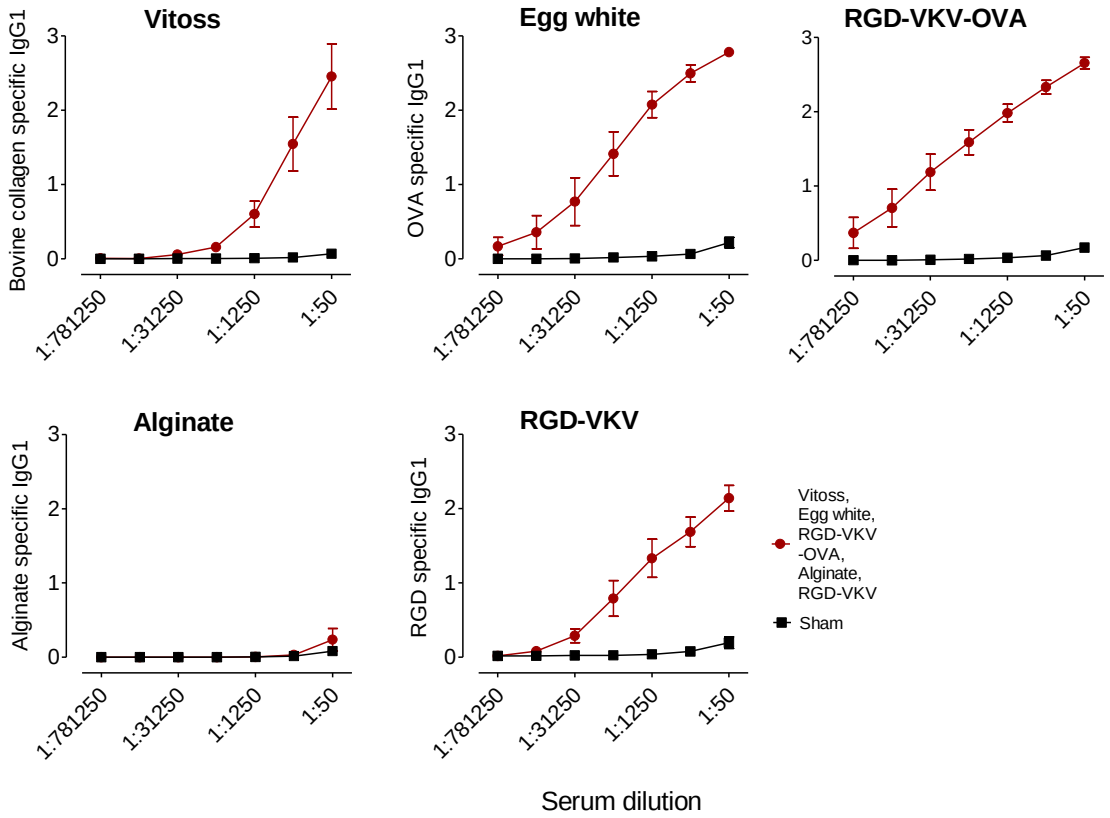


Figure 3.15. IgG1 titers of blood sera from BALB/c mice, 21 days after implantation with RGD-VKV, and sham and egg white controls, Vitoss®, Alginate, and, RGD-VKV-OVA. Data was calculated with mean ± SEM; n=5.

H&E is a staining method, to visualize inflammatory cells in tissue sections. The cell nuclei are stained dark blue, with Haematoxilyn, and their cytoplasm, pink, with Eosin Y. We used this method to determine whether inflammation at the site of implantation occurred and observed that the sham sections had no evidence of inflammation in the skin (figure 3.16.f). The same observation was made for the Alginate control (figure 3.16.d), RGD-VKV-OVA (figure 3.16.c), and RGD-VKV (figure 3.16.e) skin samples. Vitoss® (figure 3.16.b), and especially egg white (3.15.a), however, induced an intense inflammatory response at the site of implantation with Vitoss® inducing the highest number of foreign body giant cells. At a higher magnification, we observed that most of the infiltrating cells were macrophages, but there were also many eosinophils present.

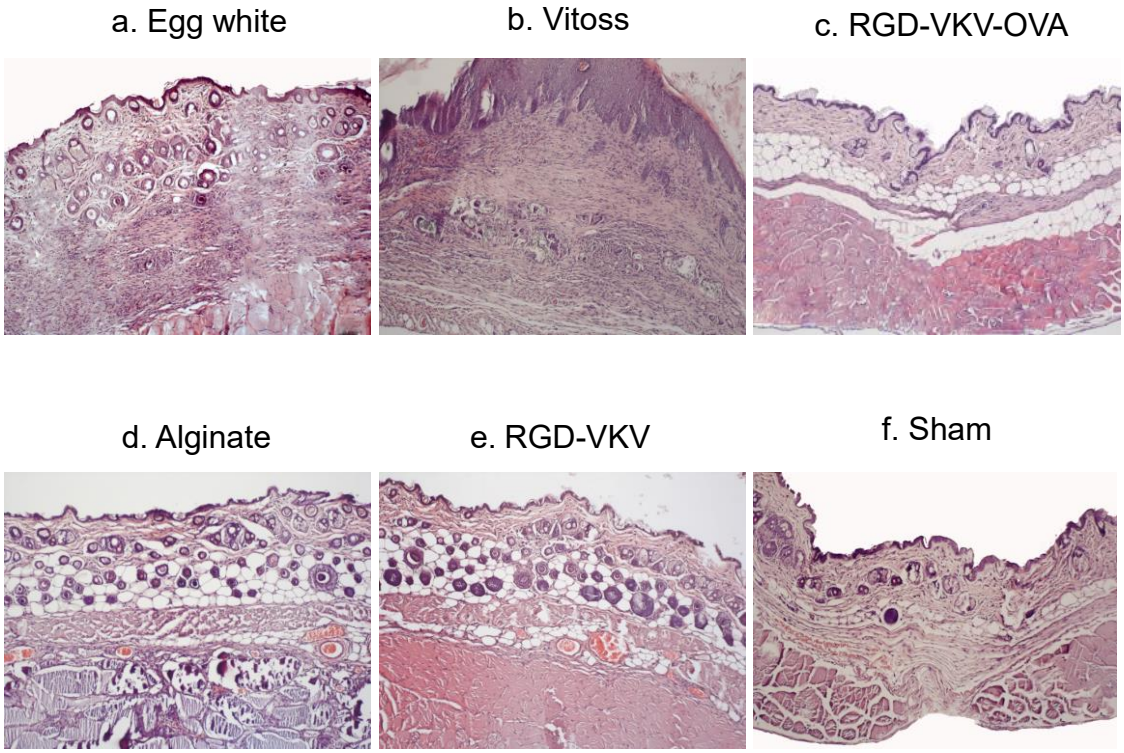


Figure 3.16. Skin histological sections, 3 weeks after implantation with egg white (a), Vitoss ® (b), RGD-VKV-OVA (c), Alginate (d), RGD-VKV (e) and sham control (f), stained with hematoxylin and eosin. Pictures were taken with a 10x magnified light microscope.

To examine the implant site for fibrosis, we performed a Masson's trichrome staining of the implant site tissue sections. This is a histochemical method stains

collagen blue with Anilin blue and inflammatory cells dark blue with Iron Hematoxylin, and cytoplasm pink, with Eosin Y. As demonstrated in Figure 3.17, even sham skin control tissue had mild fibrosis at the implant site, which is a process occurring during normal wound healing. In contrast, Vitoss® and egg white triggered severe fibrosis. Around the egg white, a fibrous capsule formed, which separated the implant from the surrounding tissue. In contrast, the RGD-VKV gel induced mild fibrosis, which we hypothesized that the implant would still be able to interact with the surrounding tissue, because no fibrous capsule was seen. Alginate (Fig 3.17d) also induced minimal formation of collagen fibers. RGD-VKV-OVA (Fig 3.17c) induced fibrosis, but not as severe as egg white.

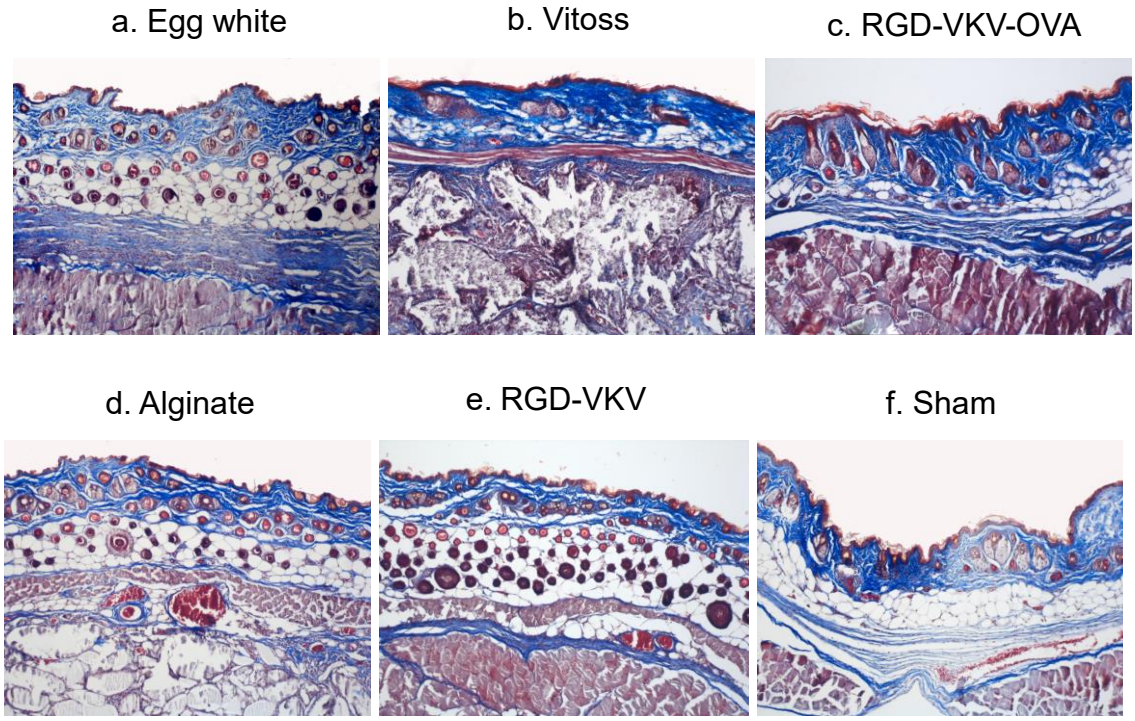


Figure 3.17. Skin histological sections, 3 weeks after implantation with egg white (a), Vitoss ® (b), RGD-VKV-OVA (c), Alginate (d), RGD-VKV (e), and sham (f), stained for collagen (blue), cytoplasm (pink), and nuclei (dark blue). Photomicrographs were taken with a 10x magnified light microscope.

After evaluating i.p. and 3 week s.c. data, we eliminated the RGD-VKV-OVA, and Vitoss controls because egg white was an excellent positive control, due to its induction of inflammation, IgG1 antibodies, and fibrosis. From this point on, we used egg white as the only positive control.

The 8 week subcutaneous implantation time point was used to investigate possible subchronic inflammation in response to the ELRs. Serum antibody titers were similar to the 3 week time point (figure 3.18). RGD-VKV, and egg white induced an antigen-specific IgG1 response upon implantation compared to no response in sham controls. Alginate did not induce an antigen-specific IgG1 response.

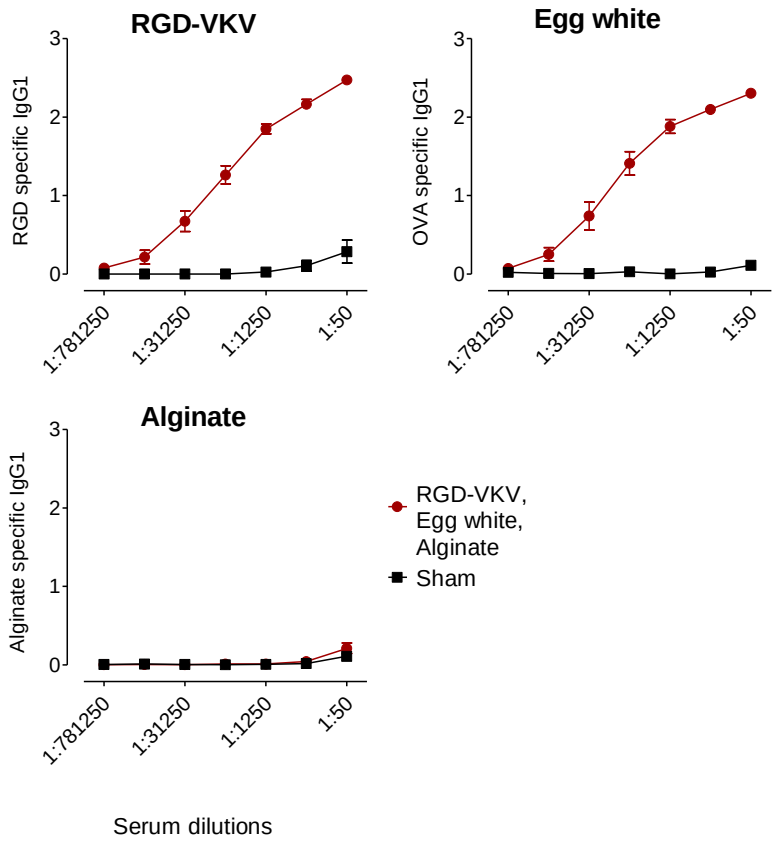


Figure 3.18. IgG1 titers of blood sera from BALB/c mice, 56 days after implantation with RGD-VKV and sham, egg white, and Alginate controls. Data was calculated in mean ± SEM; n=5

Fig 3.19 displays the 8 week s.c. histology data, which revealed results similar to the 3 week s.c. data (figure 3.16). H&E stained skin samples from mice implanted with egg white had many inflammatory cells in the tissue compared with sham controls whereas, Alginate and RGD-VKV did not induce inflammation.

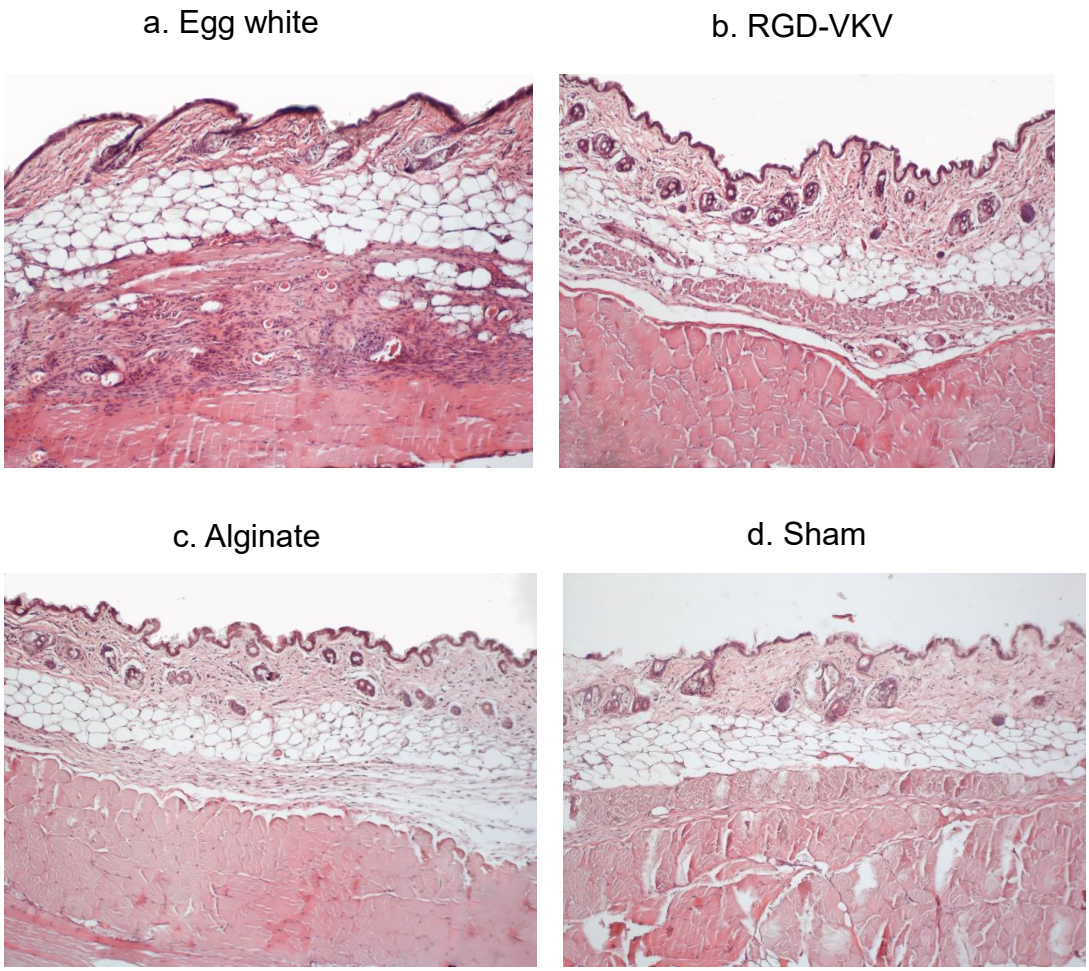


Figure 3.19. Skin histological sections, 8 weeks after implantation with egg white (a), RGD-VKV (b), Alginate (c), and sham (d), stained for hematoxylin (dark blue) and eosin (pink). photomicrographs were taken with a 10x magnified light microscope.

Masson's trichrome staining of 8 week s.c. tissue sections (figure 3.18) is consistent with the 3 week s.c. data. Egg white induced a high level of fibrosis (figure 3.20.a) compared with RGD-VKV, sham, and Alginate tissue sections which induced minimal collagen fibers at the implant site, which is a normal process of wound healing.

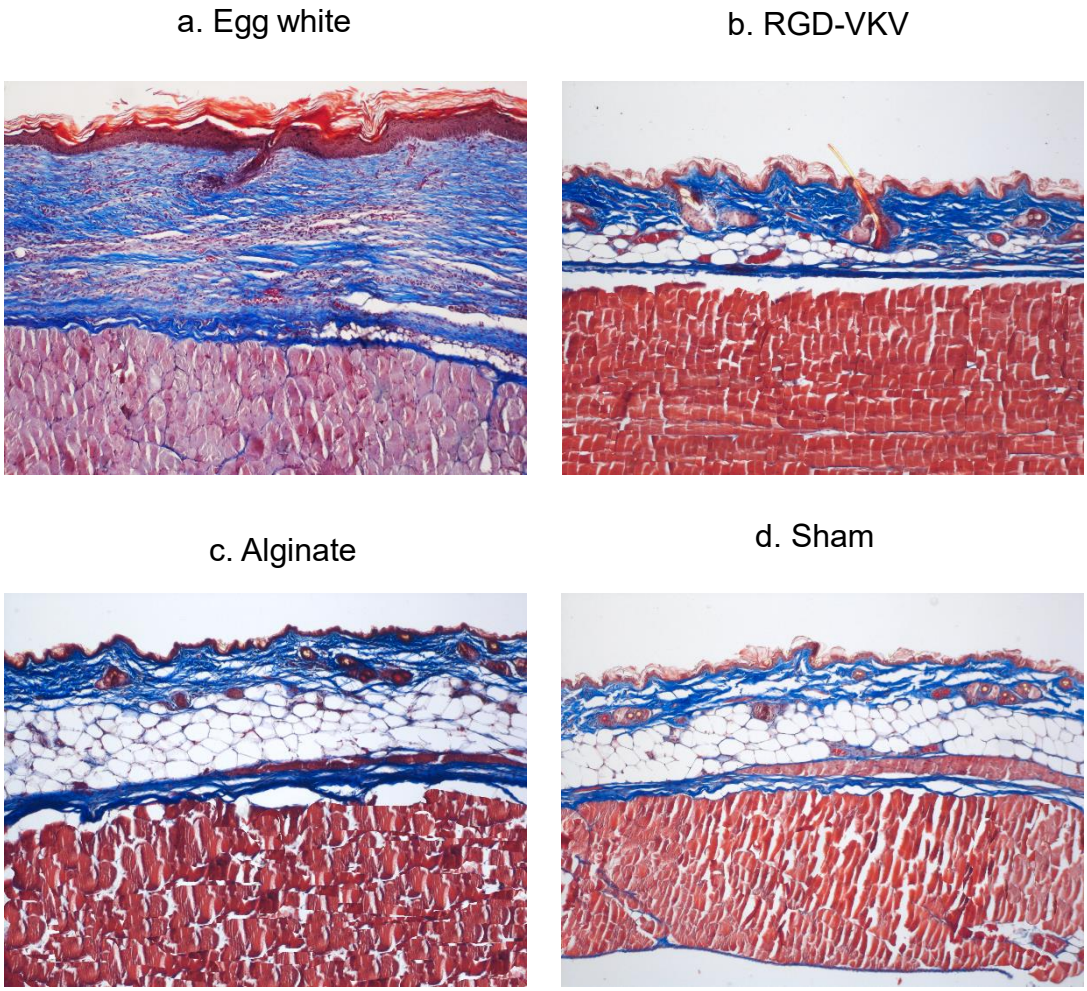


Figure 3.20. Skin histological sections, 8 weeks after implantation with egg white (a), RGD-VKV (b), Alginate (c), and sham control (d), stained for collagen (blue), cytoplasm (pink), and nuclei (dark blue). photomicrographs were taken with a 10x magnified light microscope.

4.2.1 Rechallenge of RGD-VKV implanted mice

Biogel s.c. implantation induced an RGD-specific antibody response, which is an undesired side effect that might cause problems with patients who may require more than one islet transplantation. Therefore, we investigated the response to a second gel implantation to determine whether a re-implantation would be potentially rejected due to an immunological memory response. A boost in antibody production after the second implant might be an indication of an allergic reaction to the material. However, re-implantation with RGD-VKV did increase the IgG1 titer compared to sham controls (figure 3.21). Therefore, we concluded that the Gel-specific IgG1is not inducing an allergic reaction to re-implantation.

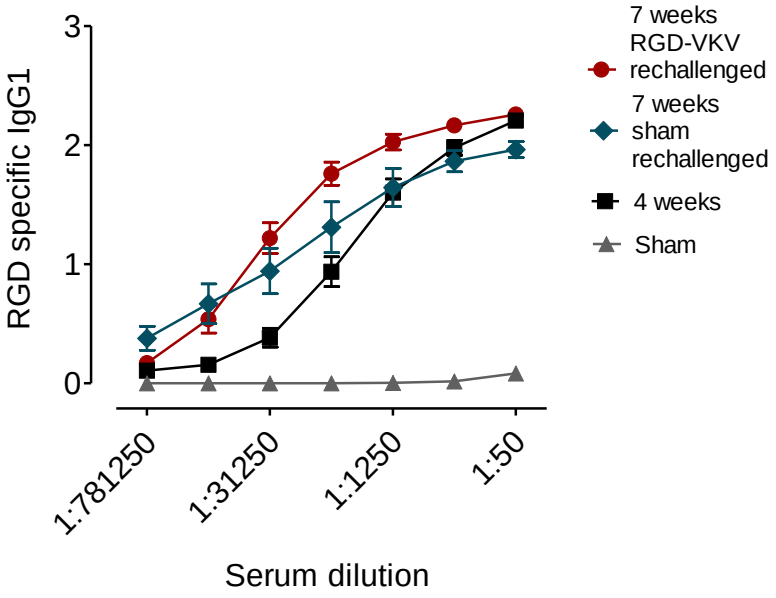


Figure 3.21. IgG1 titers of blood sera from BALB/c mice, 4 and 7 weeks after implantation. At 4 weeks, 6 mice were rechallenged with RGD-VKV, and 6 mice with sham surgery. sham data are from 4 weeks implanted mice. Data was calculated with mean ± SEM; n(4 weeks)=12, n(8 weeks)=6, n(sham)=5

Skin tissue sections of the implantation site, stained with H&E contained no inflammation 4 weeks after implantation (figure 3.22.a). Furthermore, skin reimplanted with gel (figure 3.22.b) in sham controls (figure 3.22.c) had no inflammatory cell infiltration into the skin. Therefore, we conclude that a second implantation of the ELR gel did not trigger inflammation. This data is consistent with the IgG1 measurement of the rechallenged mice.

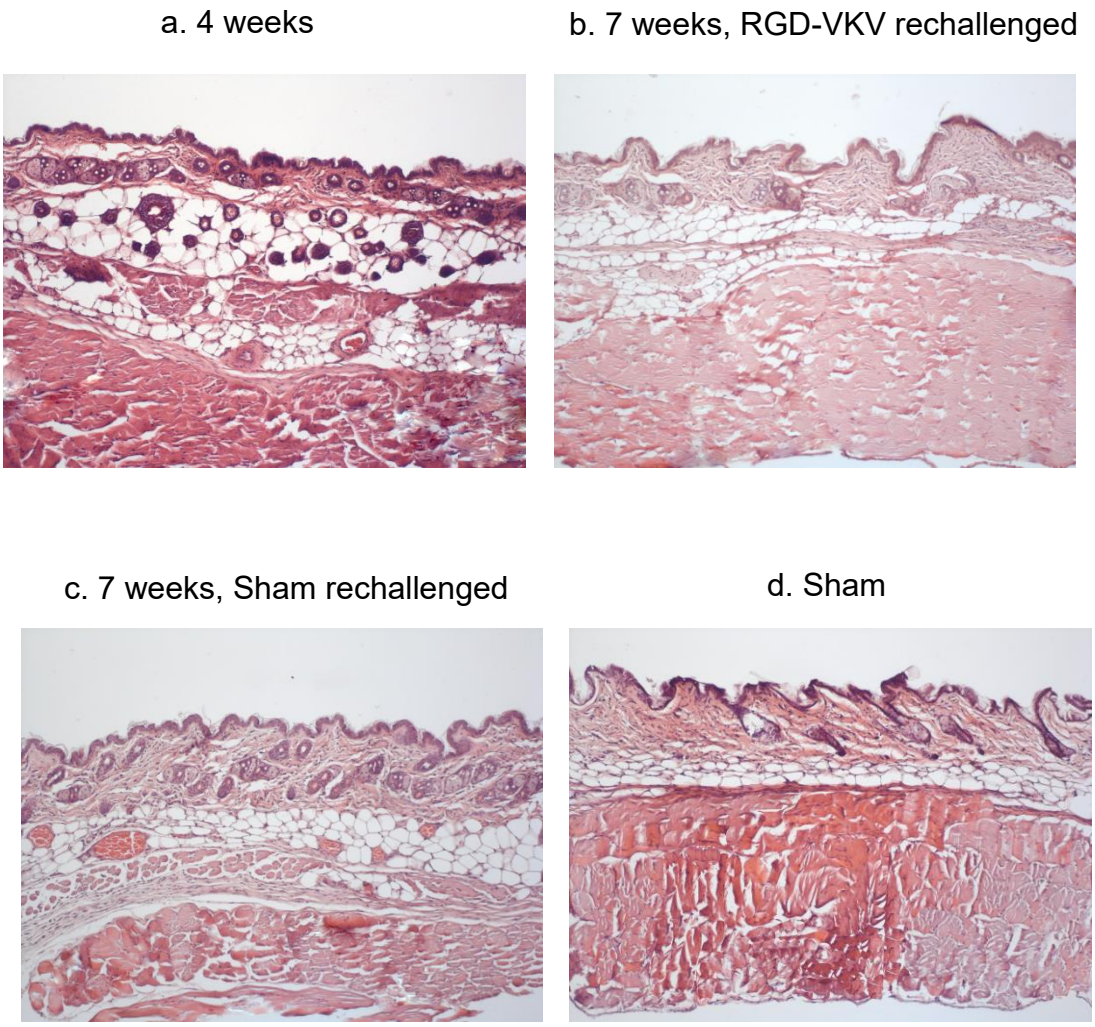


Figure 3.22. Skin histological sections, stained with Haematoxilyn and Eosin. **a.** 4 weeks after initial implantation of RGD-VKV gel. **b.** 7 weeks after initial implantation, rechallenged with RGD-VKV, after 4 weeks. **c.** 7 weeks after initial implantation, rechallenged with sham, after 4 weeks. **d.** sham, without rechallenge. Pictures were taken with a 10x magnified light microscope.

Skin histological sections, stained for collagen, showed no fibrosis 4, after initial challenge with the RGD-VKV gel (figure 3.23.a). Furthermore, skin reimplanted with gel (3.23.b), and rechallenged with sham surgery (figure 3.23.c), showed no difference in collagen formation. We suggest that a second implantation of RGD-VKV does not trigger a severe immune response, or the formation of a fibrous capsule.

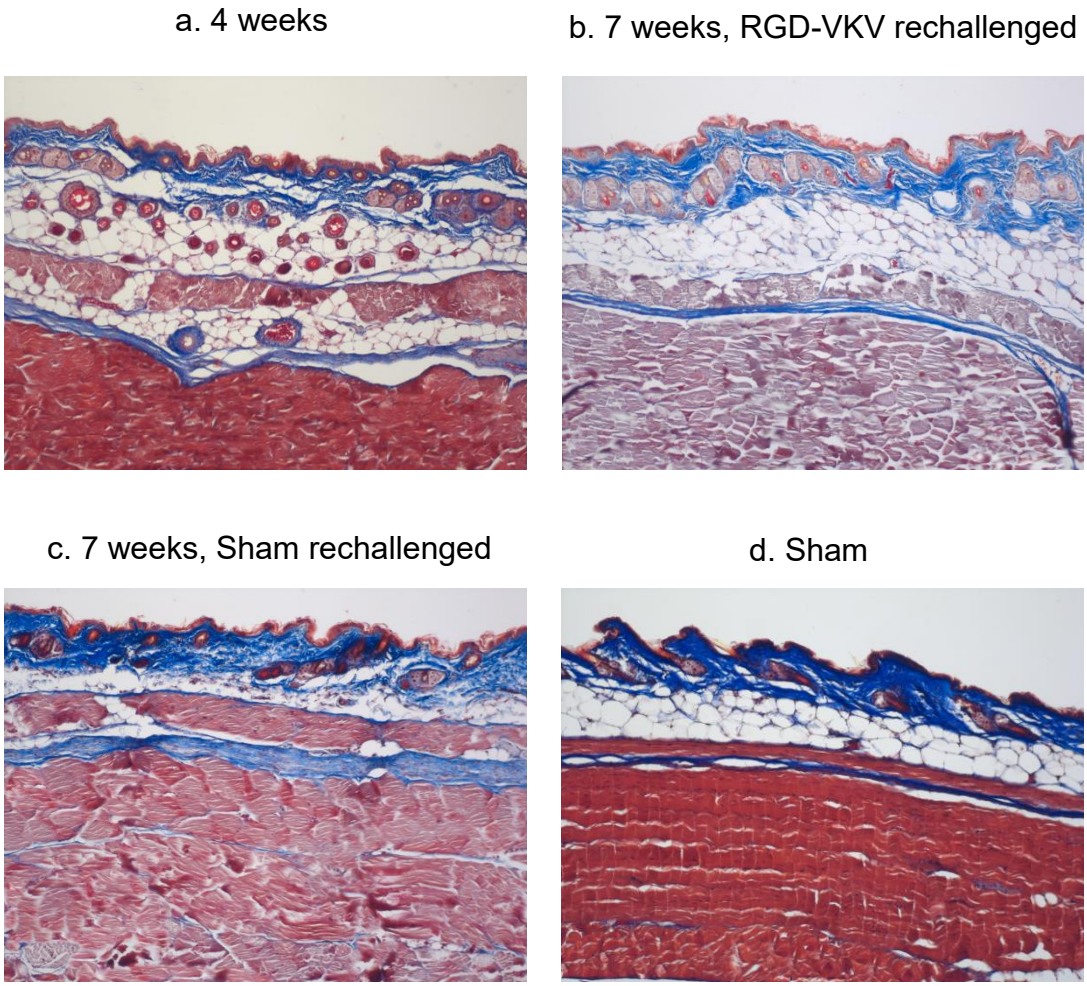


Figure 3.23. Skin histological sections stained with a Masson's trichrome kit. **a.** 4 weeks after initial implantation of RGD-VKV gel. **b.** 7 weeks after initial implantation, rechallenged with RGD-VKV after 4 weeks. **c.** 7 weeks after initial implantation, rechallenged with sham after 4 weeks. **d.** sham, without rechallenge. Pictures were taken with a 10x magnified light microscope.

5 Discussion

Diabetes mellitus is a complex metabolic disease, which can affect many organs, and reduce a patients' life quality, and life span dramatically. It has been attempted to cure type 1 diabetes by transplanting pancreatic beta cells before. Apart from conventional insulin injections, an autonomous and long-lasting insulin therapy would be a great breakthrough in the history of medicine. A promising approach is, to coat the cells with an inert material, to avoid the implants' rejection by the host immune system. To our knowledge, this is the first study, which uses a tailored, semi-artificial biogel, which resembles the structural protein collagen, to coat islet cells. The ELR polymers should provide immune stealth, and assure the proper interaction with the host's metabolism, to restore euglycemia. The remarkable feature of our coating polymers is that they neither trigger inflammation, and cytokine production upon intraperitoneal implantation (Fig. 3.3., 3.8., 3.9., 3.10., 3.11.), nor inflammation, a foreign body response, or fibrosis after subcutaneous implantation (Fig. 3.16., 3.17.). Nevertheless, the study has revealed that the RGD-VKV gel causes an elevated IgG1 titer in our test subjects. Whether the material gets degraded, still needs to be investigated more closely.

These findings concur with a study of M.Gürtler et al.[66], who coated human embryonic stem cells (SC-β cells) with modified alginate. Further, they transplanted them intraperitoneally into C57BL/6J mice, treated with streptozotocin, which is a mouse model for type 1 diabetes. Their results show that there is no foreign body response, or fibrosis, around the alginate implant. However, the euglycemic state of the mice could only be achieved for 174 days, afterwards the insulin production declined. Whether insulin production can be sufficient for a long period of time, still needs to be determined for our implants. Insulin production of the transplanted islets is meant to be controlled by the patients' blood glucose level. As a response to rising blood glucose, the cells will produce insulin, and stop, once the levels have normalized. To test, whether the RGD-VKV coated islets are able restore normal blood glucose levels, they can be implanted in C57BL/6J mice, treated with streptozotocin, to investigate the

insulin production[66]. One main limitation would be that the islets cannot respond to the patients' need of insulin, or that the insulin production can only be guaranteed for a short period of time. Furthermore, the right amount of cells in an implant needs to be determined for the patients. Additionally, the optimal location to place the implant, needs to be found. These critical factors still need to be determined for our study, to prove that the implant could provide a successful insulin therapy, in type 1 diabetes patients.

In order to assure long cell viability, host interaction has to be guaranteed by the semipermeability of the coat. We assume that the approach of *M. Gürtler et al*. using alginate as a coating material, is beneficial in one sector: Alginate is a carbohydrate, produced by brown algae, which has been found to be inert in several implantation studies, including ours. Importantly, our studies reveal that there is no IgG1 response towards the hydrogel (Fig. 3.15, 3.18). On the contrary, the RGD-VKV implants induce an RGD-specific IgG1 response, which could be a problem in the future. The findings of *M. Gürtler et al*. show that the coated islet cells decline their insulin production over time. Due to these limitation, a patient might need more than one implant during his/ her lifetime. If there is an IgG1 response towards the first implant, a second implantation might cause an elicitation of antibodies, or even a life-threatening allergic reaction. To investigate this further, we reimplanted RGD-VKV challenged BALB/c mice, with the same amount of gel, 4 weeks after the initial implantation. To investigate, if the antibody titer showed a boost, we analyzed the sera, and skin histological sections, 7 weeks after the initial surgery. This experiment has revealed that a rechallenge of s.c. implanted mice, does not cause an elicitation of IgG1 antibodies (Fig. 3.21.), a more severe inflammation (Fig. 3.22), or fibrosis (Fig 3.23) of the skin. Nevertheless, an allergic reaction cannot be excluded, since the period between the first and the second challenge might not have been long enough. The IgG1 response could still be against the first implant, since the half-life of IgG1 is 3 weeks[50]. Further experiments with a longer phase of recovery, after the first challenge, have to be done in the future. Moreover, our IgE measurements revealed that the ELR gel does not trigger the production of the allergy-related immunoglobulin. Nevertheless, we cannot exclude antigen-specific IgE

production. One explanation of the negative finding is that our assay was not able to detect the antibody. A likely explanation would be the low abundance of IgE compared to other serum antibodies, especially IgG1. As our IgG1 measurements show, the serial dilution of 1:3125, still contained a detectable amount of antibody (figure 3.18). IgE is the least abundant immunoglobulin, with a mean serum level of only 3x10-5 mg/ml^{-1}, while IgG1 has a mean serum level of 9 mg/ml^{-1} [50]. Because ELISA was the assay to determine the antibody titer, the binding of IgG1, instead of IgE, to the coated antigen, is very likely. Consequently, detection is impossible. More research in this area is necessary, to determine, if the RGD-VKV gel induces an IgE response.

Another limitation in our study could be that once the polymer is wrapped around islet cells, the immunogenicity of the implant might change. The ELR polymers are designed to exclude all immune cells and antibodies, but be permeable for insulin, nutrients, and oxygen. In the H&E stained gel pictures of the RGD-VKV gel (Fig. 3.14), we observed mainly macrophages and eosinophils at the border of the gel. We suggest that they are not able to migrate inside the gel, because they were not detected in these areas. The significance of this finding is unclear, because once the biogel is wrapped around the islets, its integrity might change and show different results. Also a complete encapsulation of the implant is undesired, because insulin, glucose, oxygen and nutrients should cannot pass the barrier anymore. Further studies in this field would be of interest, to prove the semi-permeability of the ELR coat.

Unexpectedly, our histology data indicates that the RGD-VKV biogel gets degraded after 3 weeks of s.c. implantation suggesting that the material might not be stable for a long period of time. This is a common obstacle in biomaterial studies. *Y. Chen et al.* used the approach to coat islet cells with the polyphenol tannic acid linked to poly(N-vinylpyrrolidone)[42]. Their coating material does not induce any immune response and has no effect on the islets' viability. Furthermore, insulin secretion of coated cells did not decline. Unfortunately, the implants were stable for only seven days *in vitro*, which was the main limitation in their study. To assure that our RGD-VKV coated islets provide euglycemia, over

a long period of time, the stability *in vivo* has to be investigated more closely. There is strong evidence that the gel gets degraded during the first three weeks of s.c. implantation. The gel could not be detected macroscopically between the skin and the peritoneum at the day of the assessment. Nevertheless, there are several reasons why we failed to see the implant in the histology pictures. It could be lost during the process of tissue fixation, before the embedding of the tissue, or during the process of staining. These methods expose the samples to several chemicals, which might cause the gel to dissolve or detach from the glass slides. However, we failed to see the gel underneath the skin, which is why we assume that it was already degraded at this time point. After 7 days of i.p. implantation, the gel was still found in the peritoneal cavity (Fig. 3.1). As our i.p. cell count data indicates, the macrophage numbers show no significant rise compared to sham (Fig 3.4). Additionally, the IL-1β concentration in the peritoneal lavage fluids are not higher compared to sham. A foreign body response would cause a proliferation of macrophages, driven by IL- β, to form foreign body giant cells. Furthermore, the 3 and 8 weeks histology pictures show no gel in the tissue (figures 3.16, 3.19). H&E stained skin samples do not show any inflammation, or foreign body giant cells, at the site of implantation. Nevertheless, the gel was lost. Tissue macrophages attempting to phagocyte the gel would remain in the skin and could be seen in the photomicrographs. Our conclusion is that the gel, in its native form, might be instable *in vivo*. To investigate whether the gel was degraded by macrophages, we are planning to subcutaneously implant a fluorescent gel into mice. The gels possess the same properties and structures of our conventional RGD-N_3 and VKV-cyclo gels, but they are linked to a fluorescent probe that can be excited at an appropriate wavelength. We will use the same experimental method as in our previous s.c. experiments and assess the mice at 1 day, 1, 2, and 3 weeks. Finally, we will look for a fluorescent signal in the skin, with a fluorescent microscope. If the gel is truly phagocytized by macrophages, the cells will emit a signal. By looking at the histology at different time points, we can determine how long it takes for the implant to be degraded. If the gel does not get removed by macrophages, we can conclude that the gel is not stable *in vivo*. Nevertheless, that same experiments have to be done with the

coated islets to determine whether the stability of the material is altered once it is wrapped around the cells.

Another finding of our study suggests that egg white is the best positive control for our experiments so far. It is of protein nature, induces a huge amount of inflammation (Fig. 3.2., 3.16.), an IgG1 response (Fig 3.15), fibrosis (3.17), and the production of IL-2 (Fig. 3.9.), IL-4 (Fig 3.10.), and IL-5 (Fig. 3.11.). RGD-VKV-OVA did not fulfill these requirements. The gel did neither induce an inflammatory response upon i.p. and s.c. implantation (Fig. 3.2, 3.16), nor fibrosis (figure 3.17.c), or cytokine production (figures 3.8 – 3.11). Nevertheless, it showed an elevated IgG1 titer compared to sham (figure 3.15). We hypothesize that the ovalbumin, which was dissolved in PBS, might not have been taken up by the gel. A new approach with hydrated, cross linked ovalbumin will be tested as possible positive control, in the future. Vitoss is discarded as a positive control even though it induces a high amount of inflammation (Fig. 3.2., 3.16.), fibrosis (Fig. 3.17b), cytokine (Fig. 3.8 – 3.11) and IgG1 response (Fig. 3.15). The type 1 collagen foam is not the best positive control for our studies. It does not resemble the structure of a gel and is therefore not optimal for comparison to the other groups (Fig. 3.12. -3.14.).

Another factor that has to be considered is that our study uses data form mouse models. Unfortunately, this does not assure that the human immune response will react the same way. We know for a fact that human and mouse immune systems show differences. Gene expression analyses of immune cells shows an 80% similarity between these two species[67]. The differentiating 20% result in differences, concerning receptor signaling, cell maturation, cytokine expression etc. For example, the cell composition of blood plasma shows a discrepancy. Healthy mice contain a higher lymphocyte, than neutrophil count. In humans, neutrophils are present at a higher amount, than lymphocytes[67]. Another difference can be seen in the expression of Fc receptors and the subclasses of immunoglobulins. Concerning the IgG1 response towards the RGD-VKV gel, this could be a relevant factor once the implants are tested in humans.

5.1 Conclusion

Our studies reveal that there is no cellular response upon i.p. implantation and no inflammation or fibrosis after s.c. implantation in female BALB/c mice. So far, the RGD-VKV gel looks promising as a coating material for pancreatic islet cells to treat type 1 diabetes. Nevertheless, there is an IgG1 response towards the RGD polymer. If a patient might need multiple implants during his/her life time, the development of an immunologic memory might be a huge risk. To assure that the re-implantation of the same polymers will not cause an allergic response, further experiments have to be done. Furthermore, our experiments demonstrate that egg white is a better positive control, than Vitoss. A huge advantage of the ELR polymers is that they resemble the structural protein elastin to make the coat immune stealth and are additionally open for modifications. Apart from an RGD domain, which is important for cell adhesion, other properties could be added to the basic structure. For example, to assure an optimal interaction with the host, factors that thrive angiogenesis could be added to the polymer. A good access to the blood stream is substantial for sensing and reacting to the patients' blood glucose level. Several questions remain to be resolved, in particular, the immune reaction to the islet cells coated with ELRs and its' stability *in vivo*. These factors have to be investigated in the future, to discover whether our tailored ELR coat provides euglycemia in type 1 diabetes patients.

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