universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Identification of Interleukin-5 expression by stromal vascular cell derived macrophages in the murine white adipose tissue

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer.nat.)

Verfasser: **KELLER Thomas**

Martrikel-Nummer: **9808966**

Studenrichtung: **Molekulare Biologie (A490)**

Betreuerin / Betreuer:

Univ. Doz. DDr. Christoph J. Binder **Medical University Vienna**

Univ. Prof. Dr. Barbara Hamilton **University of Vienna**

Wien, am 29.03.2008

Die Endlosigkeit des wissenschaftlichen Ringens sorgt unablässig dafür, daß dem forschenden Menschengeist seine beiden edelsten Antriebe erhalten bleiben und immer wieder von neuem angefacht werden: die Begeisterung und die Ehrfurcht.

Max Planck (1858-1947),
deutscher Physiker (Quantentheorie),
1918 Nobelpreisträger

In der Gegenwart möchte ich nicht wissen was ich in der Zukunft über die Vergangenheit denke.

Thomas Keller

Abstract

Metabolic diseases like obesity are a major medical problem and the number of affected people is increasing dramatically. It has been shown that chronic inflammation mediated by macrophage infiltration during the course of obesity is a major hallmark of obesity associated diseases. Also, a variety of adipose tissue derived cytokines, adipokines, that modulate inflammation and metabolic processes in obesity have been identified in the past years. Atherosclerosis is one of the diseases associated with obesity. The anti-inflammatory cytokine IL-5 is expressed in human atherosclerotic lesions as well as in murine atherosclerotic lesions. Moreover, it has been shown that atherosclerosis is increased in IL-5-deficient LDLR-/- mice, demonstrating that IL-5 can act as an atheroprotective cytokine. I hypothesized that IL-5 is also expressed in the white adipose tissue, because mice on high fat diet exhibit high plasma IL-5 levels. Here, I identified stromal vascular cell derived macrophages (F4/80+ SVC) as source of interleukin-5 in the murine white adipose tissue. Furthermore, I found IL-5 but not IL-5Rα mRNA expression to correlate with weight increase in high fat diet fed mice. Moreover, pro-inflammatory stimuli such as LPS stimulation of cultured stromal vascular cells were found to induce IL-5 protein levels. Finally, genetic deficiencies of interleukin-5 resulted in differences in expression of metabolically important genes, suggesting a potentially protective function of interleukin-5.

Thus, my data identify il-5 as an adipose tissue derived cytokine and suggest that interleukin-5 may be added to the list of metabolically important adipokines.

Kurzfassung

Metabolische Erkrankungen wie Fettleibigkeit sind eines der größten medizinischen Probleme und die Anzahl der betroffenen Personen steigt dramatisch an. Darüber hinaus wurde gezeigt, dass die chronische Entzündung durch Infiltration von Makrophagen in das Fettgewebe während des Verlaufes der Fettleibigkeit ein Hauptmerkmal von Fettleibigkeit ist. In den vergangenen Jahren wurden viele vom Fettgewebe produzierte Zytokine, sogenannten Adipokine, die für die Entzündung und den Stoffwechsel im Fettgewebe verantwortlich sind identifiziert. Atherosklerose ist eine Erkrankung die mit Fettleibigkeit in Zusammenhang steht. Das entzündungshemmende Zytokine IL-5 ist sowohl in atheroskleotischen Läsionen des Menschen als auch der Maus exprimiert. Außerdem konnte gezeigt werden, dass Atherosklerose in IL-5/LDLR defizienten Mäusen erhöht ist, welches auf eine atheroprotektive Rolle hinweist.

Die Hypothese ist, dass IL-5 im weißen Fettgewebe exprimiert wird, weil bei Mäuse durch fettreiche Diet der IL-5 Spiegel im Plasma ansteigt.

In meiner Diplomarbeit konnte ich Makrophagen, die aus Stromazellen stammen (F4/80+ SVC) als Quelle von Interleukin-5 im weißen Fettgewebe der Maus nachgewiesen. Des weiteren konnte ich zeigen, dass die mRNA Expression im Fettgewebe von IL-5 aber nicht von IL-5Rα mit der Gewichtszunahme von Mäusen, die eine fettreiche Diet erhalten haben korreliert. Stimulation von in vitro kultivierten Stromazellen des Fettgewebes mit LPS führte zur Induktion von IL-5 Protein. Schlussendlich, genetische Defizienz von Interleukin-5 in Mäusen resultiert in Veränderungen der Expression von wichtigen Genen des Stoffwechsels. Dies deutet auf eine potentielle schützende Funktion von Interleukin-5 hin. Meine Daten deuten darauf hin, dass Interleukin-5 in die Liste der Adipokine aufgenommen werden soll.

Table of Contents

Table of Figures

Index of Tables

1. Abbreviations

ACRP30	Adipocyte complement-related protein 30kDa
Arp3	actin-related protein 3
β-ME	β-Mercaptoethanol
BSA	Bovine serum albumin
Btk	Bruton tyrosin kinase
cDNA	complementary DNA
CycB	cyclophilin B
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid, Titriplex III
F4/80	Macrophage Marker
FACS	Fluorescence Activated Cell Sorting
HPRT	Hypoxanthine-Guanine Phosphoribosyl Transferase
IL-5	Interleukin-5
IL-5Rα	Interleukin-5 Receptor α
JAK	Janus kinase
M-MLV	Moloney Murine Leukemia Virus
mRNA	messenger RNA
PBS	Phosphate Buffered Saline
RNA	Ribonucleic acid
RNasin	Ribonuclease Inhibitor
RPMI-1640	Roswell Park Memorial Institute 1640
RT	Reverse Transcriptase
SH2	src homology domain 2
SH3	src homology domain 3
SSCS	spleen single-cell suspension
SVC	stromal-vascular cells
TAE	Tris-Acetate-EDTA
Tris	Hydroxymethylaminoethane
WAT	white adipose tissue
TBS	Tris buffered saline
FAF	fatty acid free

2. Summary

IL-5 has been shown to act as an atheroprotective cytokine in a mouse model of atherosclerosis, because reconstitution of irradiated LDLR -/- mice with bone marrow from IL-5 deficient mice led to increased lesion size compared to reconstitution with bone marrow from wild-type mice [Binder et al. 2004]. This clearly demonstrates the impact of hematopoietic cell derived IL-5 for the development of atherosclerotic lesions.

I tested the hypothesis that adipose tissue may be a major source of secreted IL-5 during atherogenesis. Adipose tissue is one type of fibrous derived tissue and consists of different cell populations like adipocytes and stromal vascular cells (SVC). The SVC fraction is composed of preadipocytes, macrophages, endothelial cells, mast cells, and other cells. Importantly, obesity has been shown to be associated with an increased infiltration of macrophages.

During my diploma thesis I have established and evaluated a method to separate the white adipose tissue into its different compartments. The isolation was done by digestion with enzymes and subsequent separation with magnetic beads, which are coated with an antibody directed against F4/80 to sort out the macrophages.

I demonstrate that stromal vascular cells derived macrophages (F4/80+ SVC) express interleukin-5 mRNA in the murine gonadal white adipose tissue. Furthermore, high fat diets induce the IL-5 mRNA expression in the stromal vascular fraction compared to normal diet fed C57BL/6 mice. Interleukin-5 may be added to the list of adipokines.

3. Introduction

3.1. Metabolic syndromes

The metabolic set of symptoms or in short the metabolic syndrome is characterized by a set of metabolic risk factors in humans [American-Heart-Association 2007]. The metabolic syndrome emerges from defective metabolic processes in the body. One prime cause of the metabolic abnormalities is visceral obesity, which is characterized by excess fat storage in and around the abdomen [Matsuzawa 2006]. Pathologies of the metabolic syndrome are diabetes mellitus type 2, high blood pressure, decreased HDL cholesterol, elevated triglycerides and cardiovascular diseases (CVD) like atherosclerosis. CVD is the major cause of death in industrialized countries. The metabolic syndrome is one of the major upcoming medical problems and the number of affected people has dramatically increased in the last twenty years worldwide [UT-Southwestern-Medical-Center 2006].

3.1.1. Obesity, adipose tissue and inflammation

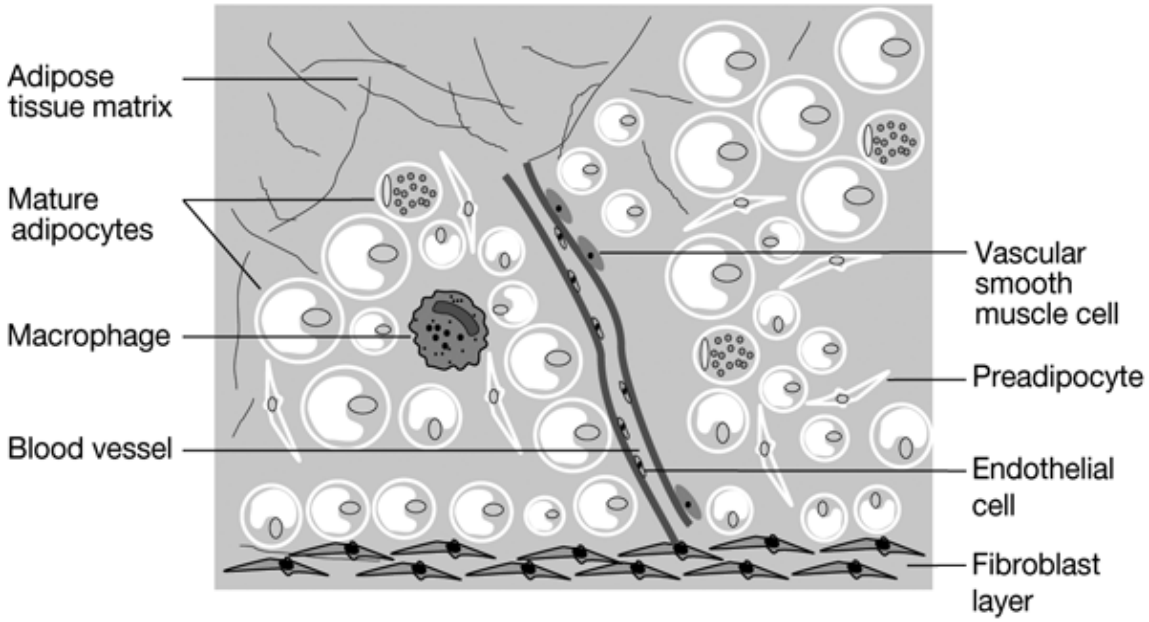
Obesity is the most common metabolic disease and it is affecting over 50% of the adult population [Wellen and Hotamisligil 2003]. Obesity and overweight are defined as an accumulation of excess body fat that may impair health. The body mass index (BMI) is a simple index that is commonly used in classifying obesity and overweight in adult populations and individuals. It is defined as the weight in kilograms divided by the square of the height in meters. The World Health Organization (WHO) defines "overweight" as a BMI equal to or more than 25, and "obesity" as a BMI equal to or more than 30. Obesity does not occur suddenly but may arise during life time. Nonetheless, increasing numbers of obese children recently have drawn attention of health care organizations. Generally, an imbalance between energy intake and energy expenditure is the fundamental cause of obesity and overweight [WHO 2006].

The serious health consequences that are associated with obesity increase progressively as the BMI increases. Raised body mass index is a major risk factor for chronic diseases such as cardiovascular disease, diabetes and some cancers (breast and colon) [Reaven 1997; WHO 2006; WHO 2006; American-Heart-Association 2007; MedlinePlus 2007]. In addition, metabolic and morphological alterations in other organs can be observed in obesity, including moderate peroxisome proliferator-activated receptors *γ* (PPAR*γ)* mRNA expression in skeletal muscle and lung, fat storage in liver (nonalcoholic fatty liver) or alterations in gastrointestinal motility [Moller and Berger 2003; Xing and Chen 2004; Jernas et al. 2006; Stienstra et al. 2006].

Adipose tissue is a complex and highly active metabolic endocrine organ. Many cytokines, adipokines (= adipose derived cytokines) and other factors, which are secreted from the adipose tissue, could be identified: For example: leptin, adiponectin, resistin, visfatin, adipsin, interleukin-6, tumor necrosis factor-alpha, retinol binding protein 4, and plasminogen activator inhibitor-1 [Hotta and Matsuzawa 2001; Steppan and Lazar 2004; Fantuzzi and Mazzone 2007; Lara-Castro et al. 2007]. The adipose tissue is primarily located under the skin (subcutaneous adipose tissue), but also around organs [Kershaw and Flier 2004]. Depending on the location one can distinguish different 'adipose depots', for example the murine gonadal white adipose retroperitoneal, mesenteric and inguinal adipose tissue [Oliver et al. 2003; Danireed 2006].

The main role of adipose tissue is to store energy in the fat [ScienceDaily 2004]. In mammals, two types of adipose tissue exist: white adipose tissue (WAT) and brown adipose tissue (BAT), which are also known as white fat and brown fat, respectively. Both tissue types are mainly built up by specialized cells, the so called adipocytes. Adipocytes of the white adipose tissue contain a large lipid droplet surrounded by a ring of cytoplasm and a flattened, crescent-shaped nucleus which is located at the periphery [Requena 2007]. Brown adipocytes have a higher content of cytoplasm than white adipocytes, with far-scattered lipid droplets. The nucleus is round and located centrally. The brown colour derives

from the large amount of mitochondria. Unlike white adipose tissue, which stores nutrients in form of triglycerides, brown adipose tissue consumes its nutrient stores to produce heat to warm the body. Brown adipose tissue is largely reduced in adults because the majority of mitochondria are abolished during lifetime [PP 2004]. Besides mature adipocytes, adult white adipose tissue contains preadipocytes, connective tissue, neuronal cells, microvascular endothelial cells pluripotent stem cells, and - very importantly - also immune cells such as macrophages. Adipose tissue exhibits an organized structure (**Figure 1**) [Schaffler et al. 2005].



.

Figure 1: Schematic representation of the white adipose tissue [Schaffler et al. 2005]

All cells other than adipocytes in the white adipose tissue are called stromal vascular cells (SVC) (**Figure 2**). The complex signaling among adipocytes and cells of the stromal vascular fraction is still not fully understood.

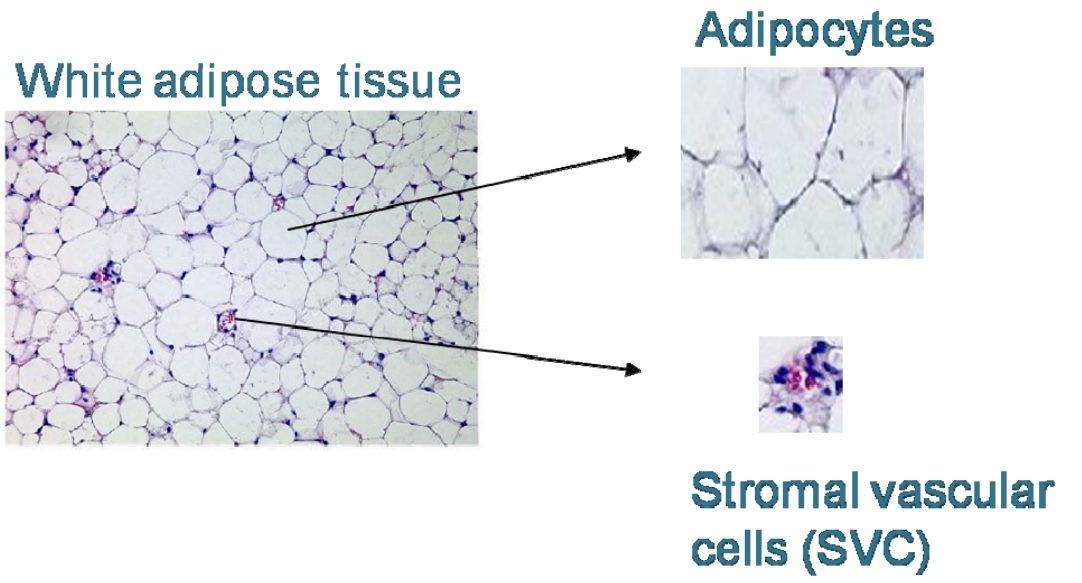


Figure 2: White adipose tissue contains two major cellular fractions. Adipocytes contain large lipid vesicles. Stromal vascular cell fraction is composed of many different cell types, modified [Ahima].

Preadipocytes have phagocytic capabilities and can trans-differentiate into macrophages [Charriere et al. 2003; Weisberg et al. 2003; Xu et al. 2003; Curat et al. 2004] but the function of macrophages in the adipose tissue is not described so far.

Both cell types, adipocytes and macrophages express common genes, such as *PPARγ* [Lee and Evans 2002]

Importantly, macrophage accumulation in white adipose tissue has been shown to be directly associated with obesity in both, human subjects and mice (**Figure 3**) [Weisberg et al. 2003].

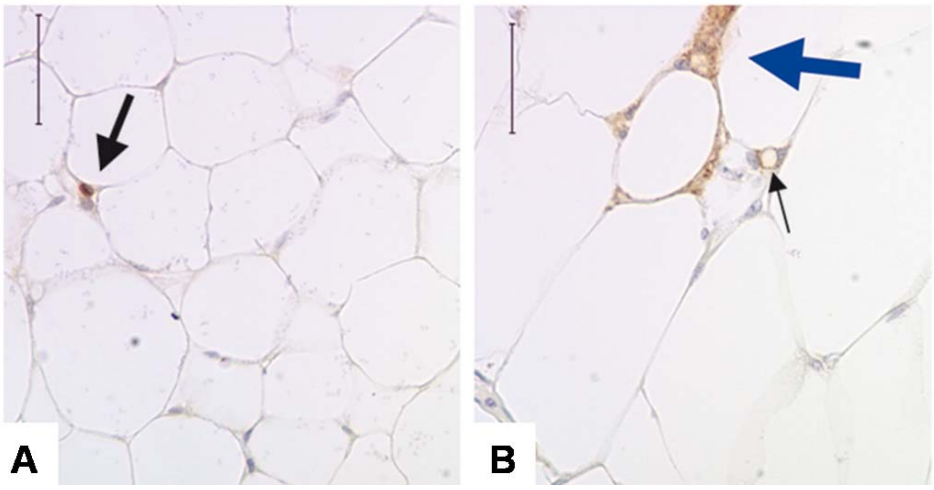


Figure 3: Macrophages in the white adipose tissue of C57BL/6J mice.
Lean male (A) and Lep-ob/ob male (B) mice. F4/80+ (black arrows) macrophages are stained brown. In lean animals (A), F4/80+ cells were small and not often seen in aggregates. More F4/80+ cells were observed in the severely obese Lep-ob/ob mice (B). In addition, obese animals contained aggregates of F4/80+ cells (large blue arrows) and a larger adipocyte cell size. Some macrophage aggregates contained small lipid-like droplets (small thin black arrow). Calibration mark = 40 µm. Modified after [Weisberg et al. 2003]

Adipose tissue derived macrophages of lean mice are typically small and distributed over the whole tissue, whereas in obese mice they form aggregates or surround adipocytes. The formation of macrophage aggregates in the white adipose tissue suggests an affinity for defective or necrotic adipocytes. Furthermore, adipose tissue derived macrophages are considered to be the main source to promote the low grade inflammation in obesity [Cancello et al. 2006].
The recruitment of macrophages during adipose tissue inflammation is partly dependent on the glycoprotein osteopontin (OPN), which is a major pro-inflammatory cytokine and plays amain important role in the cell-mediated immunity [Giachelli et al. 1998].
Activated macrophages secrete high levels of OPN which further leads to monocyte recruitment [Giachelli et al. 1998].

Monocyte chemoattractant protein-1 (MCP-1) or CCL2 is another important adipokine and is involved in macrophage infiltration of the white adipose tissue [Kanda et al. 2006].

3.1.2. Adipokines

White adipose tissue seems to play an active role in immunity and inflammation. A variety of pro-inflammatory and anti-inflammatory factors, as well as cytokines and chemokines, such as TNF-α, IL-6, monocyte chemoattractant protein 1 (MCP-1), and many others are secreted by adipose tissue and are called adipokines (**Figure 4**) [Laimer et al. 2002; Fantuzzi 2005; Trayhurn and Wood 2005].

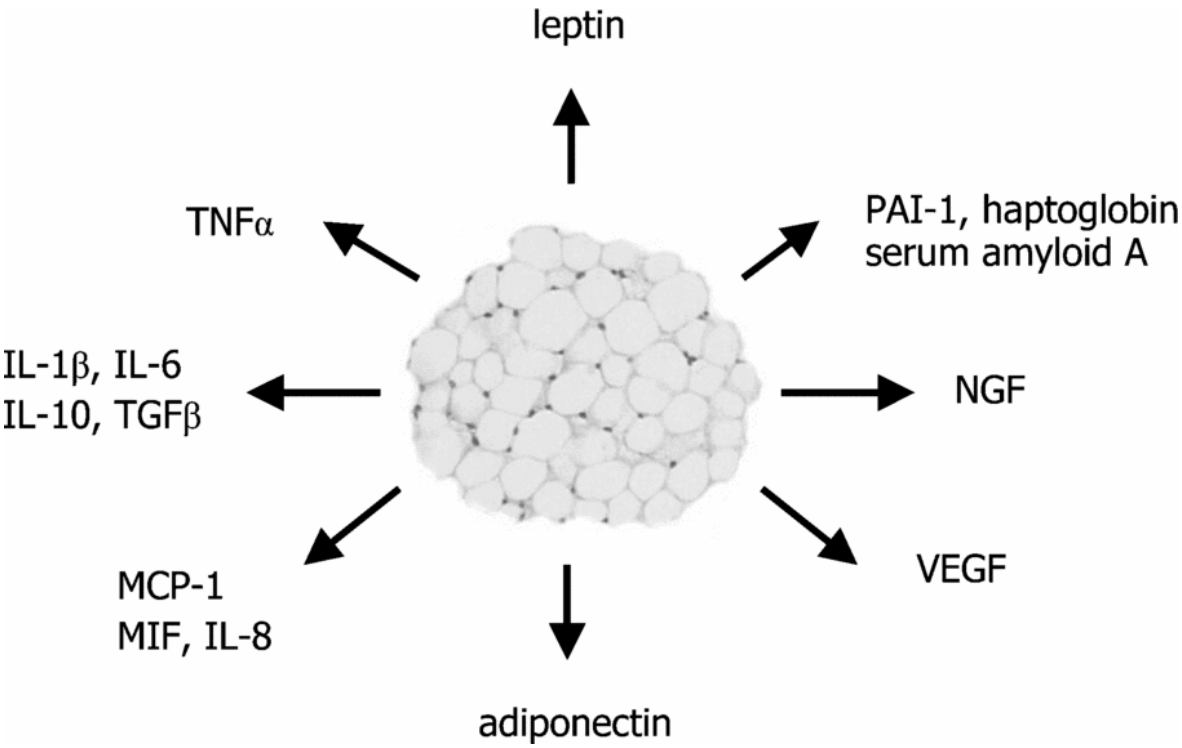


Figure 4: Adipose tissue derived chemokines

Proinflammatory molecules produced by adipose tissue have been implicated as active participants in the development of insulin resistance and in the increased risk of cardiovascular disease associated with obesity [Kershaw and Flier 2004; Fantuzzi 2005; Trayhurn and Wood 2005].

Adipokines are involved in many different physiological processes such as haemostasis, blood pressure regulation, insulin sensitivity, lipid metabolism and angiogenesis [Fruhbeck et al. 2001; Trayhurn and Beattie 2001; Rajala and

Scherer 2003; Trayhurn and Wood 2004]. Moreover, during development of obesity and the change in adipose tissue mass, there is also a change in the composition of secreted adipokines [Weisberg et al. 2003]. The number of inflammation associated adipokines increases rapidly which indicates the important role of the white adipose tissue.

Inflammation in obesity is characterized by the increased production of inflammatory molecules like C-reactive protein (CRP), IL-18, macrophage migration inhibitory factor (MIF), monocyte chemoattractant protein-1 (MCP-1) and plasminogen activator inhibitor 1 (PAI-1) in the circulation [Trayhurn and Wood 2004]. High sensitivity measurements of CRP (hsCRP) are a marker for inflammation [Laimer et al. 2002; Panagiotakos et al. 2005]. And it has been shown that the reduction of body weight is associated with a significantly decrease in circulating CRP levels [Laimer et al. 2002; Esposito et al. 2003].
TNF-α has been shown to be an important mediator of insulin resistance during the state of obesity [Peraldi and Spiegelman 1998; Laimer et al. 2002].
One exception is the major adipose tissue derived hormone adiponectin which decrease during the state obesity and act as an anti-inflammatory molecule [Arita et al. 1999; Ouchi et al. 2000]
The secretion of inflammatory adipokines associated with a high BMI are important factors in the development of chronic inflammatory state in obesity [Yudkin 2003]
The consequences of inflammation in obesity are atherosclerosis, Type II diabetes and other metabolic syndrome associated diseases [Trayhurn and Wood 2005].

3.1.1. Atherosclerosis and Atherogenesis

Atherosclerosis is a disease that affects arterial blood vessels. It is a chronic inflammatory disease of the vascular wall and inflammatory processes occur at all stages of this disease [Ross 1986; Ross 1993; Ross 1999; Glass and Witztum 2001; Steinberg 2002]. Chronic inflammation is a pathological hallmark of atherosclerosis [Ross 1986; Capron 1989; Ross 1993; Ross 1999], and inflammatory processes are involved at all stages of this disease [von der Thusen et al. 2003]. Fatty streak is the first morphological change in the development of atherosclerosis. The fatty streak is followed by the formation of an established plaque, which is characterised by the accumulation of macrophage foam cells and a local chronic inflammation. The problematical plaque is characterised by the formation of a fibrous cap. The production of degradative enzymes can then lead to a burst of the fibrous cap, which allows the exposure of prothrombotic material leading to the formation of a thrombus through platelet aggregation [Stevens et al. 2005], which causes clinical events such as heart attacks or strokes.
Risk factors of atherosclerosis are high blood pressure, smoking, abdominal obesity, advanced age, male gender, diabetes, dyslipoproteinemia, high plasma homocysteine levels and in general an unhealthy pattern of life. Atherosclerosis is therefore the most common cause of illness and death in the western world.

3.1.2. Atherosclerosis & Immunity

Both, innate and adaptive immunity are involved in the pathogenesis of atherosclerosis. The communication between the innate and adaptive immune systems involves cell-cell interactions in relation to antigen presentation or proinflammatory and anti-inflammatory mediators such as cytokines or chemokines [Getz 2005]. IL-5 has recently been implicated in murine atherosclerosis.
The best way to study the biological role of IL-5 in atherosclerosis was to generate an IL-5 knockout mouse. IL-5 deficient mice were generated in the C57/BL6

background. Manfred Kopf and colleagues [Kopf et al. 1996] insert a neomycin resistance gene into the start of exon 3 in a codon for cysteine of the IL-5 gene which inhibited the dimerization and activity of IL-5 [Campbell et al. 1988; Takahashi et al. 1990]. IL-5 deficient mice had no observable signs of illness and were fertile [Kopf et al. 1996], but they have decreased numbers of eosinophils [Sferruzzi-Perri et al. 2003]. This mouse model is also particularly suitable to study the role of IL-5 in atherosclerosis.

It has been shown that the anti-inflammatory cytokine IL-5 is expressed in human atherosclerotic lesions [Frostegard et al. 1999; Schonbeck et al. 2002] as well as in murine atherosclerotic lesions [Binder et al. 2004]. Furthermore, atherosclerotic lesions are significantly increased in IL-5-deficient LDLR-/- mice compared to LDLR-/- mice (**Figure 5**) [Binder et al. 2004]

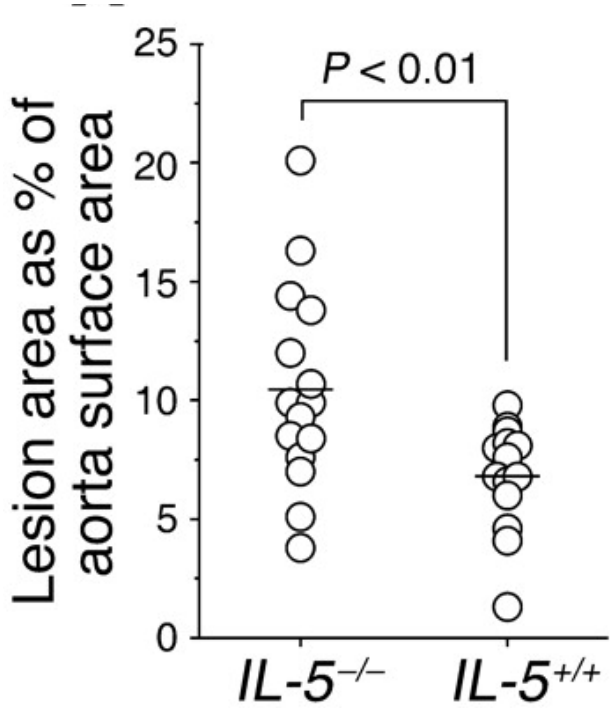


Figure 5: Increased atherosclerosis in IL-5–deficient LDLR–/– mice

IL-5 secretion has also been shown to stimulate B-1 cells. B-1 cells are B cells that express the cluster of differentiation marker 5 (CD5), and express the pentameric

immunglobulin M (IgM) in greater quantities than monomeric Immunglobulin G (IgG). B-1 cells constitutively express membrane-anchored interleukin 5 receptor [Moon et al. 2004; Binder 2006]. IL-5 could play an indirect regulatory role by stimulation of B-1 cells to produce protective natural IgM antibodies (**Figure 6**).

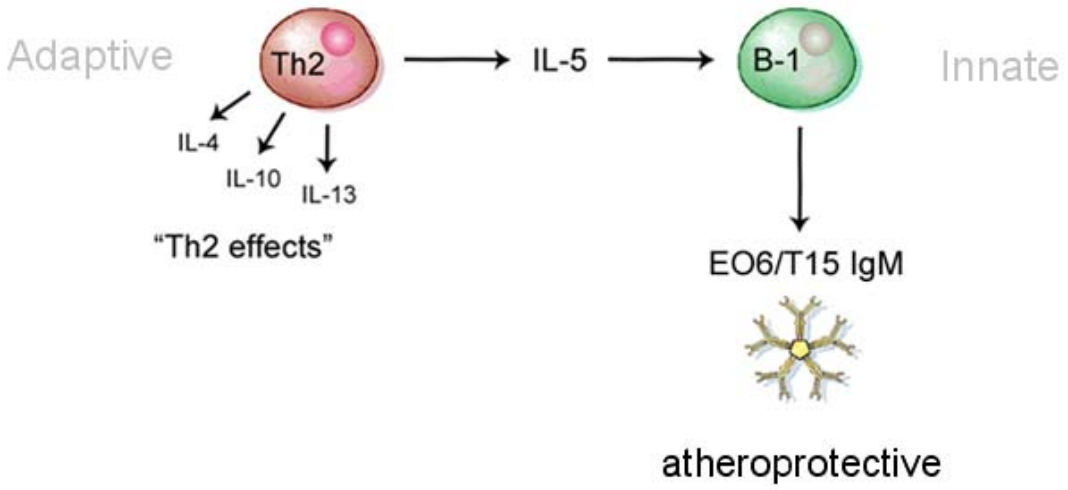


Figure 6: IL-5 links adaptive and innate immunity and activates an atheroprotective mechanism by secretion of natural IgM.

3.2. Interleukins and their receptors

Interleukins are a group of secreted immune system signaling molecules produced mainly, but not exclusively, by leukocytes. Interleukins initiate, mediate, and regulate immune responses and inflammatory reactions [Powrie and Coffman 1993]. Interleukins or cytokines are involved in nearly each phase of immunity and inflammation. Usually they are not stored in specialized vesicles, but they are synthesized after cellular activation. One released cytokine can act on different cell types, which allows different biological effects also called pleiotropism. On the other hand, different interleukins have the same functional effect which refers to the redundant effect of interleukins. In most cases, the release of one cytokine leads to the synthesis and action of many other cytokines. Their action is initiated by binding to specific surface receptors on target cells. Functional properties of interleukins can be divided into three categories: (1) Mediators and initiators of

innate immunity, (2) Mediators and initiators of adaptive immunity and (3) stimulators of hematopoiesis [Abbas et al. 2003]. Further, these specialized signaling molecules can be divided into two groups: the proinflammatory and anti-inflammatory cytokines. The proinflammatory property of cytokines results in their ability to produce tissue destruction and fever, whereas anti-inflammatory cytokines promote wound healing.

3.2.1. Interleukin-5

Interleukin-5 (IL-5) is a synonym of B-cell growth factor II, T-cell replacing factor, IgA enhancing factor and eosinophil differentiation factor [Takatsu et al. 1994].
IL-5 is mainly produced by activated T_H2-cells which are a subpopulation of T-cells. IL-5 plays a major role in differentiation of B-cells, in the recruitment, activation, persistence of eosinophils, and the production of immunoglobulin A (IgA) [Pazdrak et al. 1995]. Each cytokine binds to a specific cell-surface receptor. IL-5 interacts with the interleukin-5 Receptor. The binding leads to the activation of at least three different signaling pathways. First the activation of Ras Pathway and proto-oncogenes [Takaki et al. 1994], second the activation of Janus kinases (JAK) and Signal Transducer and Activator of Transcription (STAT) proteins [Weltman and Karim 2000] and third the activation of Bruton and Src-family tyrosine kinases [Sato et al. 1994]. IL-5 is crucially involved in many immunological processes and scientific publications about IL-5 are of substantial interest during the last 20 years in immunological research (**Figure 7**).

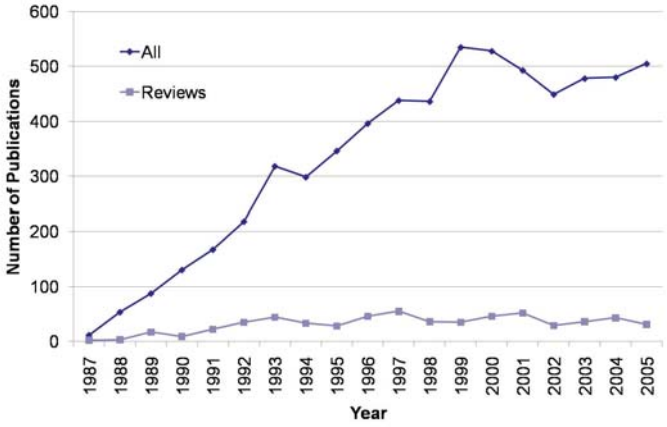


Figure 7: Scientific publications on Interleukin-5 found with NCBI search engine “Pubmed”. Pubmed search term: "IL-5" AND year[dp]

3.2.2. The Interleukin-5 gene

In humans, the IL-5 gene is located on chromosome 5q31 in a gene cluster together with IL-3, IL-4, IL-13 and granulocyte-macrophage colony stimulating factor (GM-CSF) [van Leeuwen et al. 1989]. In mice, the IL-5 gene is located on Chromosome 11 at location 29.2 cM [Lee et al. 1989]. The coding sequence of the murine and humane IL-5 gene consists of four exons. The mouse has a 738-base-pair (Alu-like repeat inserted) in the 3-prime untranslated region (UTR), which leads to a difference in mRNA size between human and mouse. The size of the murine mRNA is 1.6 kilo bases (kb) whereas that of human mRNA is 0.9 kb (**Figure 8**).

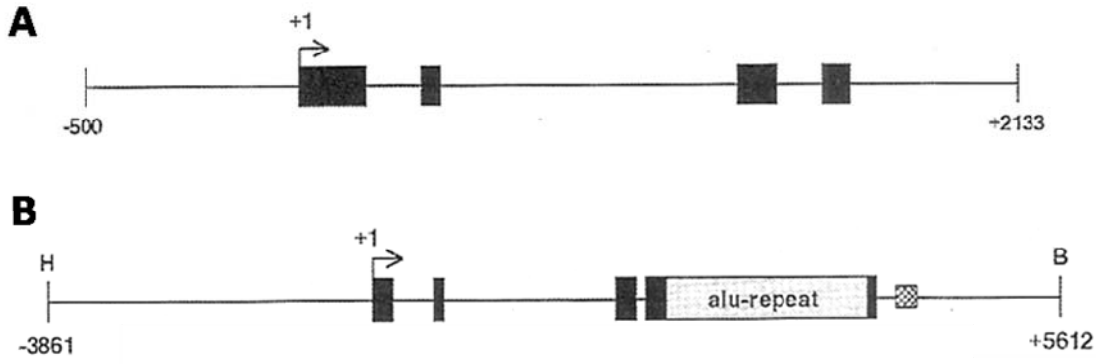


Figure 8: Schematic representation of the human (A) and murine (B) IL-5 gene.

Several studies have shown that expression of the IL-5 gene is regulated at the transcriptional level. Upstream of the IL-5 coding sequence a TATA-like and a CAAT-like transcriptional initiation site are located and several other positive and negative regulatory elements have been identified [Le Beau et al. 1989; Wang et al. 1993; Gruart-Gouilleux et al. 1995; Yamagata et al. 1995; Zheng and Flavell 1997].

3.2.3. Protein structure of Interleukin-5

IL-5 is a glycoprotein with a molecular mass of 40-45 kDa and forms a disulfide linked homodimer. The human IL-5 protein comprises 134 and the murine protein 133 amino acid residues, respectively and it includes a 19 amino acid signal peptide. Human and murine IL-5 proteins show 70 percent sequence homology which is dominantly located in the carboxyl-terminus (**Figure 9**).

```
P05113|IL5_HUMAN      MR-MLLHLSLLALGAAYVYAIPTEIPTSALVKETLALLSTHRTLLIANETLRIPVPVHKN 59
P04401|IL5_MOUSE      MRRMLLHLSVLTL--SCVWATAMEIPMSTVVKETLTQLSAHRALLTSNETMRLPVPTHKN 58
                      ** ******:*:*  : *:* . *** *::*****: **:**:** :***:*:***.***

P05113|IL5_HUMAN      HQLCTEEIFQGIGTLESQTVQGGTVERLFKNLSLIKKYIDGQKKKCGEERRRVNQFLDYL 119
P04401|IL5_MOUSE      HQLCIGEIFQGLDILKNQTVRGGTVEMLFQNLSLIKKYIDRQKEKCGEERRRTRQFLDYL 118
                      ****  *****:. *:.***:***** **:********** **:********..******

P05113|IL5_HUMAN      QEFLGVMNTEWIIES 134
P04401|IL5_MOUSE      QEFLGVMSTEWAMEG 133
                      *******.*** :*.
```

Figure 9: Alignment of the human and murine IL-5 amino acid sequence.
Colors: RED represent small, hydrophobic including aromatic, blue acidic, magenta basic, green hydroxyl, amine and other amino acids are gray. Consensus sequence: "*" = identical or conserved residues in all sequences in the alignment; ":" = indicates conserved substitutions; "." = indicates semi-conserved substitutions. Alignment was performed by Thomas Keller with ClustalW 1.83

This high degree of similarity explains the immunological cross-reactivity observed between human and mice IL-5 [Campbell et al. 1988]. It has been shown that IL-5 forms a cross-linked homodimer in an antiparallel arrangement (**Figure 10**) by a

disulfide bond between cysteine residues 44 and 86 [Minamitake et al. 1990; McKenzie et al. 1991; Milburn et al. 1993]. Reduction and alkylation of the two cysteins in recombinant hIL-5 or mutations in threonine residues lead to a biologically inactive monomer. The dimer formation via disulfide bonds is essential for the biological activity of IL-5. The variable, high molecular mass range observed for secreted IL-5 is explained by the heterogeneous addition of carbohydrates [Minamitake et al. 1990; Tominaga et al. 1990]. Mouse IL-5 (mIL-5) has an additional N-linked glycosylation at position Asn-55, which is not present in hIL-5 [Kodama et al. 1992].

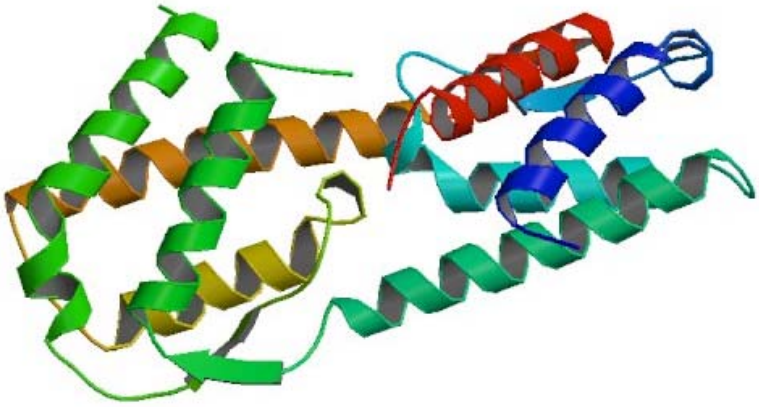


Figure 10: Crystal structure at 2.4 angstroms resolution of human Interleukin-5. Protein data bank ID: 1HUL

3.2.4. Biological function of Interleukin-5

Haematopoiesis is the formation of blood cells. The hematopoietic system can be divided into three major different lineages, the erythroid, the myeloid and the lymphoid system, respectively (**Figure 11**) [Karlsson 2006]. The cells of the erythroid line are the red blood cells which fulfill the oxygen transport. Granulocytes, megakaryocytes and macrophages belong to the myeloid lineage. These cells are involved in innate immunity, adaptive immunity and blood clotting. The lymphoid lineage is mainly composed of B- and T-cells.

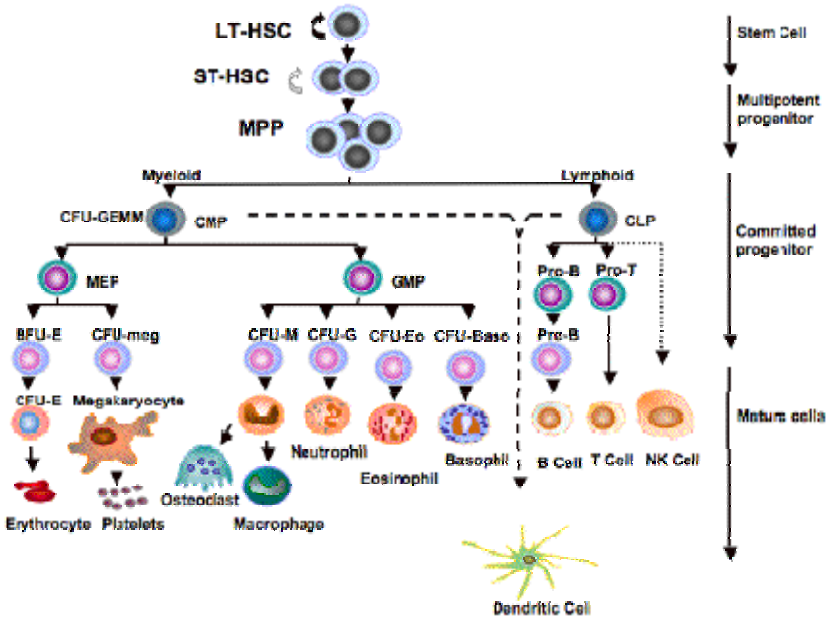


Figure 11: The hematopoietic system

The major function of B-cells is to produce and secrete antibodies against exogenous antigens. Four different subsets of B-cells have been distinguished by their function. These are plasma B cells – the antibody producing cell of the immune system, Memory B cells which derive from activated B cells and B-1 and B-2 cells. B-1 cells express CD5 on their surface and more IgM than IgG. The conventional B-cells are B-2 cells. Further all B-cells express a B-cell specific receptor called B-cell receptor (BCR) [Berland and Wortis 2002; Abbas et al. 2003].

The second important cell type of the lymphoid lineage is the T-cell. T-cells are produced in the thymus and play an important role in cell-mediated immunity of the adaptive immune system but do not recognize foreign antigens like B-cells. On the cell surface they express a specific receptor called the T cell receptor (TCR).

Th cells differentiate into two major subsets - Th1 and Th2-cells. The two subsets differ in their cytokine production and the way how they stimulate the immune system. Th1-cells play a role in cell-mediated immunity, whereas Th2 cells provide help for B cells which is essential for antibody-mediated immunity.

In mice, B-1 cells ($CD11b^+$ B-cells) isolated from the peritoneal cavity constitutively express the IL-5 Receptor complex and act in response to IL-5 to produce polyclonal IgM [Hitoshi et al. 1990; Rolink et al. 1990]. IgM is the first immunoglobulin expressed by mature B cells. The IgM antibody levels in serum are increased early during the course of an infection. Resting B-cells in spleen do not proliferate in response to IL-5, but IL-5 acts on certain activated B-cells to induce IgG1 and IgA production [Takatsu et al. 1994]. IL-5 induces proliferation, differentiation and maturation of eosinophilic precursors in bone marrow and expands the lifetime of eosinophils by delaying apoptotic death. IL-5 possesses eosinophil chemotactic activity, increases eosinophil adhesion to endothelial cells and enhances eosinophil effector functions [Clutterbuck et al. 1987; Yamaguchi et al. 1988; Yamaguchi et al. 1991; Sanderson 1992].

In chronic asthma, an inflammation of the bronchi, eosinophils and CD4+ T-cells are involved [Robinson et al. 1992; Corrigan et al. 1993]. Both cell types are the major source of IL-5, and IL-5 levels increase during course of acute asthma [Alexander et al. 1994]. Increased IL-5 mRNA expression in atopic asthma is confirmed [Humbert et al. 1997]. IL-5 also has major role in atopic dermatitis, a chronic inflammatory disease of the skin [Leung et al. 2004].

3.2.5. The Interleukin-5 Receptor

IL-5 binds to a specific heteromeric receptor which is called interleukin-5 Receptor (IL-5R). Based on its sequence homology, the IL-5R is a member of the class I cytokine receptors (hematopoietin-like). The class I cytokine receptors can be further divided into three subfamilies: The GM-CSF receptor subfamily, Interleukin-2 receptor subfamily and the Interleukin-6 receptor subfamily. The GM-CSF receptor subfamily contains the Receptors for GM-CSF, Interleukin-3 and Interleukin-5 (**Figure 12**) [Kuby 2006].

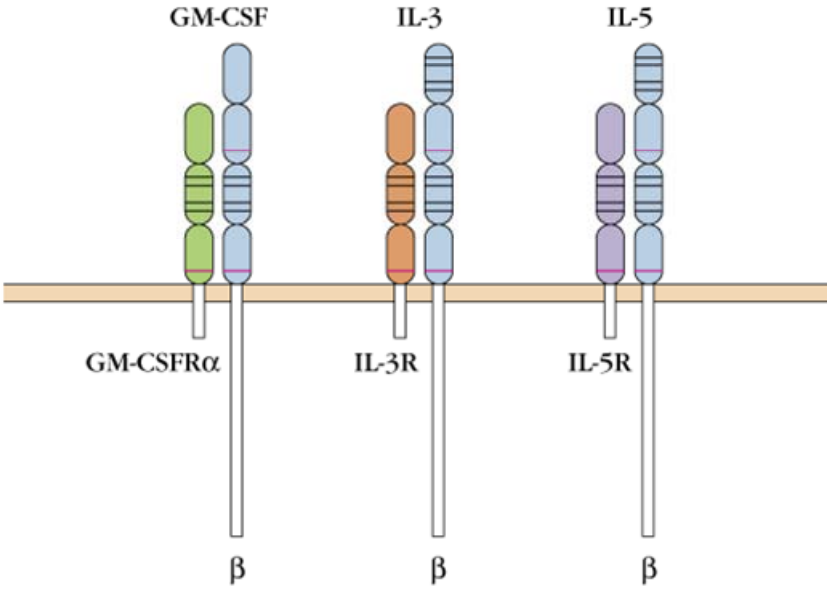


Figure 12: GM-CSF receptor subfamily

3.2.6. Molecular constitution of interleukin-5 receptor

The IL-5R complex is a heterodimer and consists of two polypeptide chains: an α-chain and a β-chain (**Figure 12**). Both chains contain an extracellular domain, a transmembrane domain and an intracellular domain. Special key motifs as two cysteine pairs and the so-called WSXWS (Trp-Ser-Xaa-Trp-Ser; where Xaa is any amino acid) motif are characteristic for this receptor subfamily/receptor [Bazan 1990]. The β-chain is shared between related receptors (IL-3, GM-CSF) in this subfamily and is denoted β-common (βc) subunit. The α subunit has specific ligand binding activity, whereas the βc subunit does not bind IL-5 by itself, but enhances the IL-5 binding. The intracellular domain of the βc subunit is essential for intracellular signal transduction [Takatsu et al. 1994]. The human IL-5 Receptor α (hIL-5Rα) gene is located on chromosome 3 in the region 3p26-p24 [Isobe et al. 1992], and the mouse IL-5 Receptor α (mIL-5Rα) gene can be found on the long arm of chromosome 6 [Tuypens et al. 1992]. The human IL-5Rα gene is composed of 14 exons [Perez et al. 2003] and the murine IL-5Rα gene of 13 exons, respectively (**Figure 14**) [Imamura et al. 1994; Bystrom et al. 2006].

The interleukin-5 receptor alpha precursor chain shows a high sequence homology between human and mice (**Figure 13**).

Figure 13: Alignment of the human and murine IL-5 receptor alpha amino acid sequence. Colors: RED represent small, hydrophobic including aromatic, blue acidic, magenta basic, green hydroxyl, amine and other amino acids are gray. Consensus sequence: "*" = identical or conserved residues in all sequences in the alignment; ":" = indicates conserved substitutions; "." = indicates semi-conserved substitutions. Alignment was performed by Thomas Keller with ClustalW 1.83

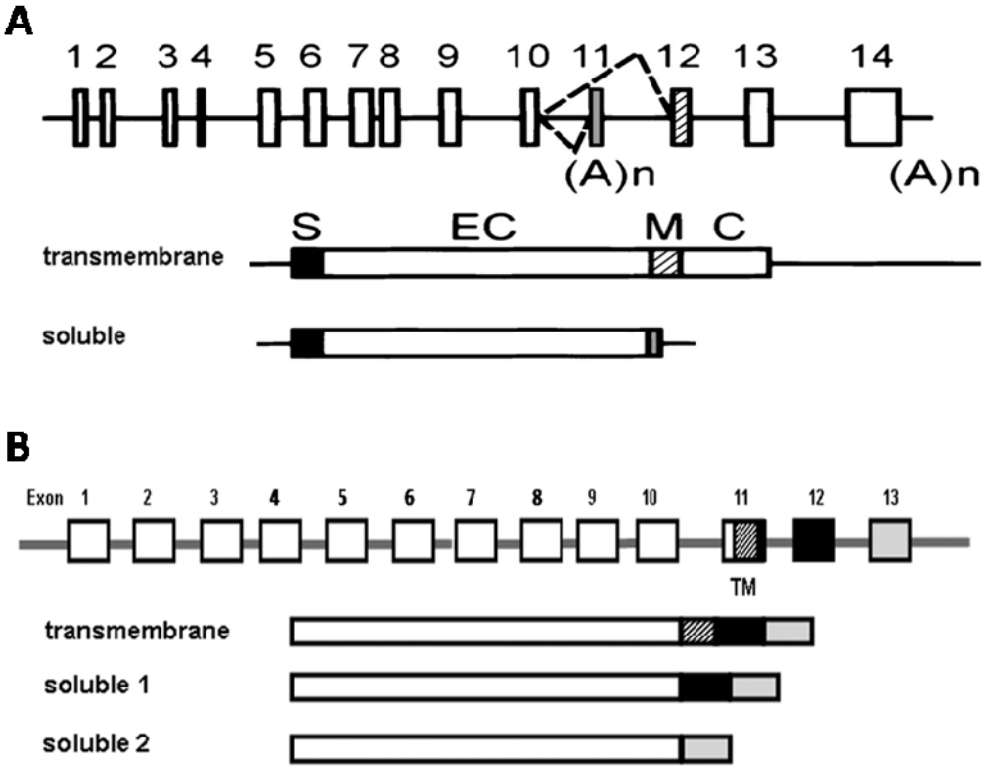


Figure 14: Schematic representation of the human (A) and murine (B) IL-5Rα exon structure. S, signal peptide; EC, extracellular domain; M, transmembrane domain; C, cytoplasmic tail; (A)n, Polyadenylation sites [Tavernier et al. 2000; Bystrom et al. 2006];

Human and mouse βc subunit gene loci are located at chromosomes 22q12.3-13.1 [Gorman et al. 1990; Shen et al. 1992].

Through alternative splicing three different transcripts are produced from the same IL-5Rα locus. In addition to the membrane-anchored receptor, two soluble transcripts can be detected [Tuypens et al. 1992]. In humans, alternative splicing at exon 11 leads to the soluble isoforms, while exon 12 encodes the transmembrane domain (**Figure 14A**). Differential splicing of the murine IL-5Rα exons 11–13 result in the transmembrane (TM) and soluble isoforms 1 and 2 (**Figure 14B**) [Bystrom et al. 2006].

These soluble receptor proteins can bind and neutralize secreted IL-5 by preventing interaction of the cytokine with the membrane-bound isoforms of the receptor α-chain [Kikuchi et al. 1994; Takatsu et al. 1994]. Only the membrane anchored form builds a complex with the common β-chain, which is required for

signalling. It also forms heterodimers with IL-3Rα and granulocyte-macrophage colony stimulating factor (GM-CSF) α-chain [Hara and Miyajima 1992] to build the IL-3 and GM-CSF receptors (**Figure 12**).

3.2.7. Interleukin-5 receptor signal transduction

Ligand binding is the first event in IL-5R signal transduction. Binding of IL-5 leads to a dimerization of the ligand specific α-chain and the signal transducing βc-chain and to the activation of at least three different signaling pathways. IL-5 induces the activation of 1) Janus kinases (JAK) and Signal Transducer and Activator of Transcription (STAT) proteins [Weltman and Karim 2000], 2) Ras pathway and proto-oncogenes [Takaki et al. 1994], and 3) Bruton and Src-family tyrosine kinases (**Figure 15**).

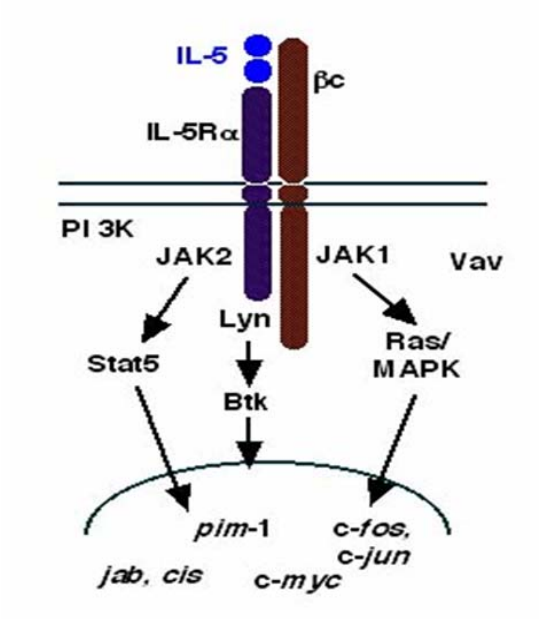


Figure 15: Interleukin-5 receptor signaling events. Binding of IL-5 to its specific receptor leads to the activation of at least three different signaling pathways [Meneki 2004]

After dimerization of the IL-5R, the intracellular domain of the α-chain and ßc-chain is tyrosine phosphorylated by associated JAK1 and JAK2 proteins. JAK proteins

contain a kinase domain and a kinase-like domain, but they have no src homology 2 or 3 domain (SH2, SH3). Members of this kinase family translate ligand binding to the membrane-anchored receptor with tyrosine phosphorylation of cellular proteins [Ihle 1995; Ihle 1995; Ihle and Kerr 1995]. Proteins which contain a SH2 domain bind to phosphorylated tyrosine residues. STATs possess SH2 domains and bind to phosphotyrosines at the intracellular receptor domain [Wakioka et al. 1999]. Further, JAK proteins phosphorylate STATs. This phosphorylation leads to the dimerization of STATs. In IL-5R signalling, especially STAT1 and STAT5 are involved. After dimerization, STAT proteins are activated and translocate from the cytoplasm to the nucleus and bind to specific DNA response elements in the promoter region of target genes to activate gene expression [Schindler 1999; Wakioka et al. 1999; Schindler et al. 2007].

4. Aim

My thesis was based on the observation that mice fed a high fat diet exhibited a substantial rise of interleukin-5 levels in plasma . And that interleukin-5 has been shown to function as an anti-inflammatory, protective cytokine in murine atherosclerosis [Binder et al. 2004].
Here, I hypothesized that interleukin-5 is prominently produced in the adipose tissue and that IL-5 expression is associated with obesity in mice.

The overall aim of the present study was to study interleukin-5 expression in the white adipose tissue as surrogate for the metabolically important adipose tissue in C57BL/6 mice on normal vs. high fat diet.

The key questions of my thesis were
(1) To demonstrate expression of IL-5 in white adipose tissue
(2) To unravel the exact cellular source of IL-5 in the adipose tissue
(3) To provide insights into the potential role of IL-5 in the white adipose tissue.

5. Materials and Methods

5.1. Antibodies and reagents

Monoclonal Anti-human/mouse IL5 Antibody-TRFK5
RnDSystems

Biotinylated Anti-mouse IL-5 Antibody-TRFK4
RnDSystems

Recombinant Mouse IL-5
RnDSystems;

Avidin-Fluorescein Isothiocyanate (Av-FITC)
BD Pharmingen;

Rat Anti-IL-5 Monoclonal Antibody, FITC Conjugated, Clone TRFK5
IMGENEX;

Anti-Mouse F4/80 Biotin conjugated
Caltag;

CD11b/Mac-1 (CR3
BD Pharmingen;

Purified anti-mouse CD16/32 (Fc-Block)
BD Pharmingen;

Red blood cell lysis buffer (ACK-buffer):
0.15 M NH4Cl, 10 mM KHC03, 0.1 mM Na2EDTA in distilled H20; pH 7-7.2

Krebs-Ringer HEPES (1L)
120 mM NaCl, 5mM KCl, 1,2 mM MgS04, 0,33 mM CaCl, 12 mM HEPES, 25 mM Glucose in distilled H20; pH 7.4
MACS-Buffer
2 mM EDTA, 0.5 % BSA in PBS

FACS buffer
2% BSA in PBS

Bewer thioglycolate medium (3%)
3% (w/v) Bewer thioglycolate medium is dissolved in H2O. Afterwards the solution was boiled and autoclaved. Finally the solution should be stored for 6 month.

5.2. Animals

Homozygous IL-5-/- mice on C56BL/6 genetic background and wild type C57BL/6 mice were kept under normal conditions at the animal facility at the Medical University of Vienna. Mice were obtained by heterozygous breeding of IL-5+/- mice. Figure 17 shows the genotype and gender distribution of 71 offspring's. The animals were allowed free access to water and standard chow or high fat diet under a 12 h light/dark cycle. Mice were fasted for 2 hours before sacrifice by CO_2 inhalation. For mice earmarking system see supplemental material 10.4.

For preparation of peritoneal exudate cells, 3ml of 3% Brewer thioglycollate media was injected into the peritoneal cavity. After 3 days, the mouse was sacrificed and peritoneal lavage with 10 ml 2%BSA/PBS was performed. The collected suspension contains the peritoneal exudate cells. Afterwards, the cells were washed 2 times with PBS and used for RNA isolation.

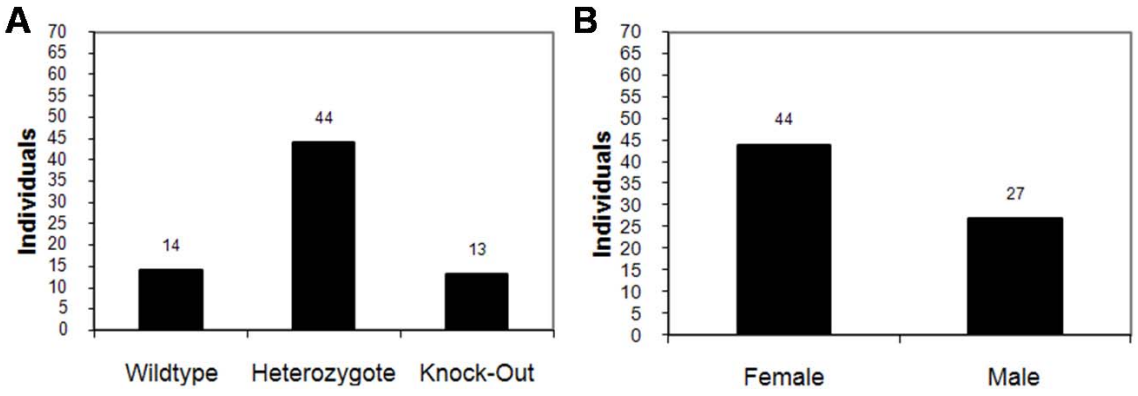


Figure 16: Mice breeding statistics of 71 mice. Heterozygous IL-5+/- mice on C56BL/6 genetic background was bred.

5.3. Preparation of primary cell suspensions

C57BL/6 mice were used for preparation of peritoneal exudates and splenocyte preparations. Peritoneal exudate cells (PEC) were isolated by peritoneal lavage with 10 ml ice-cold PBS (pH 7.4). For splenocyte preparations, spleens were removed after sacrifice by CO_2 inhalation. The spleens were passed through a cell strainer with 100 µm mesh size into a small culture plate (40mm x 10mm) using a sterile syringe plastic plunger to prepare a single-cell suspension. The mesh was rinsed twice with 2 ml PBS under sterile conditions. The cell suspension was then transferred into a new 15 ml falcon and kept on ice for 5 minutes to allow cell and connective tissue clumps settle on the bottom of the tube. Afterwards, the cell suspension in the supernatant was centrifuged at 300 g for 5 min at 4°C and washed with PBS followed by another step of centrifugation. Next, cells were resuspend in 5ml red blood cell lysis buffer (ACK-buffer:0.15 M NH4Cl, 10 mM KHC03, 0.1 mM Na2EDTA in distilled H20; pH 7-7,2) for 10min at room temperature. The cells were washed again with PBS and pelleted by centrifugation at 300 g for 5 min at 4°C. Finally, the pelleted splenocytes were resuspended in 10ml RPMI 1640 cell culture medium, supplemented with 10% fetal bovine serum, 50 µg/ml gentamycin and 2,5 µg/ml Fungizone. Cell viability was assessed with trypan blue exclusion.

5.4. Isolation of murine stromal vascular cell fraction and adipocytes of adipose tissue

Gonadal white fat pads from male or female C57BL/6 mice fed normal chow or a high fat diet were excised, and tissues were minced in a cell culture plate in PBS. The minced tissue was then transferred into a 50 ml tube and digested enzymatically. Briefly, 30 µg/ml Liberase Blendzyme 3 (Roche) and 50 U/ml DNase I (Fermentas) in Krebs-Ringer HEPES buffer was added followed by incubation in a shaking water bath at 37°C for 60 min (at 150 rpm in a 45° angle). Every 20 minutes cells were pipetted up and down to dissociate the tissue. The resulting cell suspension was passed through a 100 µm mesh size filter to generate a uniform cell suspension and then centrifuged at 20 g for 5 min. Afterwards, the infranatant was collected and centrifuged again at 300g for 5 min and the rest, the adipocytes were used for RNA isolation and The pellet contained the stromal vascular cells (SVC). Next the SVC were washed 2 times with PBS and treated with red blood cell lysis buffer (ACK-buffer:0.15 M NH4Cl, 10 mM KHC03, 0.1 mM Na2EDTA in distilled H20; pH 7-7,2) for 5 min at room temperature to destroy red blood cells. Finally, the SVCs were washed 2 times with PBS.

SVCs were cultured in 6-well cell culture plates in 1 ml RPMI 1640 supplemented with 10% fetal bovine serum, 50 µg/ml gentamycin and 2,5 µg/ml fungizone incubated at 37°C with 5% CO_2.

5.5. Measurement of Interleukin-5 levels by ELISA

Levels of IL-5 were measured by sandwich enzyme-linked immunosorbent assay (ELISA) in the supernatants of cultured stromal vascular cells. Either, with or without 1µg/ml LPS stimulation. Microfluor 2 white U bottom 96-well microtiter plates were coated with 50 µl monoclonal anti-human/mouse IL-5 antibody (clone: TRFK5, class: rat IgG1; RnDSystems) in TBS overnight at 4°C or 2 h at room temperature. Thereafter, wells were washed with PBS/EDTA to remove unbound

antibodies using an automated microplate washer (ELx405™, Bio-Tek), and wells were blocked with TBS containing 1%BSA for 30 minutes at room temperature. Plates were washed again, and 50 µl of cell culture supernatant or a dilution curve of recombinant IL-5 (Recombinant Mouse IL-5; RnDSystems) diluted in TBS/1%BSA were plated and incubated for 2 h at room temperature. Following another washing step, a biotinylated anti-mouse IL-5 (Clone TRFK4; RnDSystems) was added at a concentration of 150ng/ml for 1h at room temperature. Thereafter, plates were washed again and incubated with neutravidin alkaline phosphatase (AP) conjugate at room temperature for 1 hour. A final washing step was performed with water, followed by the addition of 25 µl/well chemiluminescent substrate LumiPhos (Aureonbio) 1:3 diluted in water. After 90 minute incubation at room temperature in darkness, plates were read using a chemiluminescent plate reader (Perkin Elmer Victor2). Values were retrieved as relative light units per 100ms and the concentration of IL-5 was calculated on a standard curve constructed with serial dilutions of recombinant mouse IL-5.

5.6. Protein quantification

The protein concentration of protein extracts was determined using a commercially available kit, the “Bicinchoninic Acid (BCA™) Protein Assay Kit” from Pierce (UK). A bovine serum albumin (BSA) was used to generate a standard curve.

5.7. Dead cell removal

The Dead Cell Removal Kit (Miltenyi Biotec) was used to remove dead cells according to the manufacturer’s recommendations.

5.8. Magnetic bead cell sorting (MACS)

To separate macrophages from the remaining stromal vascular cell suspensions, magnetic bead cell sorting was employed. A stromal vascular cell suspension was

prepared from murine white adipose tissue as described above. Cells were washed once in PBS and centrifuged at 300g for 5 min. The supernatant was removed and the cell pellet was resuspended in 500 µl degased MACS-Buffer (2 mM EDTA, 0.5 % BSA in PBS). $5x10^5$ cells were then incubated with 2.5 µg/ml purified anti-mouse CD16/CD32 (0.5 mg/ml; BD Pharmingen) diluted in MACS-Buffer for 5 min at room temperature to block non-specific binding to Fcγ receptors. Subsequently, biotin-conjugated anti-mouse F4/80 antibodies were added a final concentration of 2 µg/ml to the cell suspension and incubated for 10 min at RT. Then, cells were washed in 5 ml MACS-buffer, and the pellet was resuspended in 80 µl MACS-buffer and 20 µl (for up to 10^7 cells/ml) avidin-coupled MACS micro beads. After 15 min incubation at 4°C, cells were washed again. The supernatant was removed, and the pellet resuspended in 500 µl MACS-Buffer for magnetic separation. The cell suspension was applied onto a pre-washed MACS LS positive selection column, which was placed in the magnetic field of a MACS separator. The flow through was collected as the F4/80 negative fraction. Subsequently, the MACS column was removed from the magnetic field and F4/80 positive cells were rinsed with 2 ml MACS buffer. Finally, the two cell fractions were washed and the cell viability was assessed by trypan blue exclusion.

5.9. Flow-cytometry

Surface expression of CD11b and F4/80 on stromal vascular cells was determined by flow cytometry. After isolation and separation as described above aliquots of 5x105 cells were resuspended in 100 µl FACS buffer (2% BSA in PBS) in micronic tubes. The tubes were centrifuged at 300 x g for 5 minutes, and cell pellets were resuspended in 50 µl FACS buffer containing APC conjugated anti-mouse-CD11b antibody (BD Bioscience) at a dilution 1:2000 (0.2 mg/ml) or FITC conjugated anti-mouse-F4/80 antibody (BD Bioscience) at a dilution 1:100 (0,1 µg/ml). After 30 minutes of incubation at 4 °C in the dark, 1 ml FACS buffer was added to each sample for washing and cells were centrifuged at 300 x g for 5 minutes. The

resulting pellets were resuspended in 500 µl of FACS buffer and analyzed on an FACS Calibur Instrument (Becton Dickinson) using software FCS Express 2.0.

5.10. Microscopy analyses

5.10.1. Immunofluorescence

Cells were washed in PBS and fixed in 100 µl of a 4% paraformaldehyde solution at 4°C for 10-20 min. After fixation cells were carefully washed in 1x PBS containing 0.1% saponin to permeabilize the cells. Blocking was performed 20 min at room temperature using a 1:5 dilution of goat serum. After blocking, cells were washed with PBS and then incubated with either FITC-conjugated anti-mouse IL-5 monoclonal antibody (Clone TRFK5; IMGENEX) or Biotin-conjugated anti-mouse F4/80 (Caltag). The 1st antibody was diluted in 1xPBS containing 0.1% saponin and incubated for 1h at room temperature. After incubation of the primary antibody cells were carefully washed three times with PBS. Afterwards, 0.5 µg/ml of the 2nd antibody, Avidin-Fluorescein Isothiocyanate (Av-FITC) diluted in PBS was applied for 1h at room temperature. Then, cells were washed again three times. Nucleic staining was performed using DAPI diluted 1:200000 in PBS for 1min at room temperature. Cells were washed one more time with PBS, diluted in100µl PBS, and spun onto glass slides at 400rpm using a cytospin centrifuge (Shandon centrifuge) for 2min , 5x10^4 cells (1x10^5 cells) in was performed. Finally, cover slips were mounted with “Vectra shield mounting medium”, and glass slides were stored at 2-8°C in the dark until analysis by fluorescent microscopy. Cells without staining (autofluorescence) and staining with 2nd antibody only were used as negative controls.

5.10.2. Haematoxylin & Eosin staining

After dissection, the white adipose tissue was rinsed three times with 1x PBS and fixed with 4% Paraformaldehyde or immediately frozen in liquid nitrogen. Stromal

vascular cells were stained after cytospin at 800rpm for 1 min. On the next day Haematoxylin & Eosin (HE) was performed after sectioning of the fixed, frozen tissue with an automatic slide stainer at the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Vienna.

5.10.3. Microscopy

Immunofluorescence analysis was performed on an upright Zeiss Fluorescence microscope (Axioskop 40FL). Axio vision Software was used for data acquisition and analyses.

5.11. Nucleic acid methods

5.11.1. Determination of nucleic acid concentrations

To quantify the amount of DNA or RNA, the optical density of probes was measured with a photometer (Bio-Rad) at a wavelength of 260 nm and 280 nm. For DNA (double strand) the OD260= 1 corresponds to a concentration of 50 µg/ml, for RNA the OD260= 1 corresponds to a concentration of 40 µg/ml. These formulas were used to determine the DNA or RNA concentration of a sample.

DNA:	Unknown (µg/ml) = 50 µg/ml x measured A_{260} x dilution factor
RNA:	Unknown (µg/ml) = 40 µg/ml x measured A_{260} x dilution factor

The ratio of OD260 nm versus OD280 nm describes the degree of purity of the nucleic acids. These should be ≥1.8 and should not exceed 2.0. Smaller ratios usually indicate contamination by protein remnants or organic compounds.

5.11.2. Genomic DNA preparation from mouse tissues

Mouse genomic DNA for genotyping was prepared from either tail or ear clips. Using a DNeasy tissue kit (Qiagen) following the manufacturer´s instructions.

5.11.3. Genotyping of mice

Genotyping of mice was performed by standard PCR. PCR reaction mix (Table 1), Primers for IL-5 genotyping (Table 2) and PCR conditions for genotyping (Table 3) are listed below.

	1 Reaction [µl]
10x Buffer	2,5
MgCl2 [25mM]	2
dNTP [25mM]	0,25
s/α PrimerMix [10pmol each]	1
"Hot Start" Taq	0,125
Aqua bidestillata	18,125
MasterMix	**24**
DNA	1
Total	**25**

Table 1: PCR reaction mix

Primer	Sequence (5´ to 3´)	Target element	Company
IL5KOSint2	TCC TGA TCC TCT TAC CTC AAC TTC C	Interleukin-5; Intron 2	JAX
IL5KOSneo	GAA CCT GCG TGC AAT CCA TC	Interleukin-5; neomycin cassette (disrupted Interleukin-5)	JAX
IL5KOASex3	GTC TAG CCC CTG AAA GAT TTC TCC	Interleukin-5; Exon 3	JAX

Table 2: Primers for IL-5 genotyping

		Temperature [°C]	Time [mm:ss]	
1	activation of hot start Taq	94	10:00	
2	denaturation	94	00:30	**40 Cycles**
3	annealing	60	00:30	
4	lengthening	72	00:30	
5	final lengthening	72	10:00	
6	cooling	4	∞	

Table 3: PCR conditions for genotyping were

The resulting fragment of 228bp indicates an IL-5 wild type mouse and fragment of 345bp a transgenic mouse. The insertion of a neomycin resistance gene results in the amplification of a bigger fragment in IL-5 deficient mice. A scheme of genomic situation is shown in Figure 16 and representative agarose gel electrophoresis of PCR products in Figure 17.

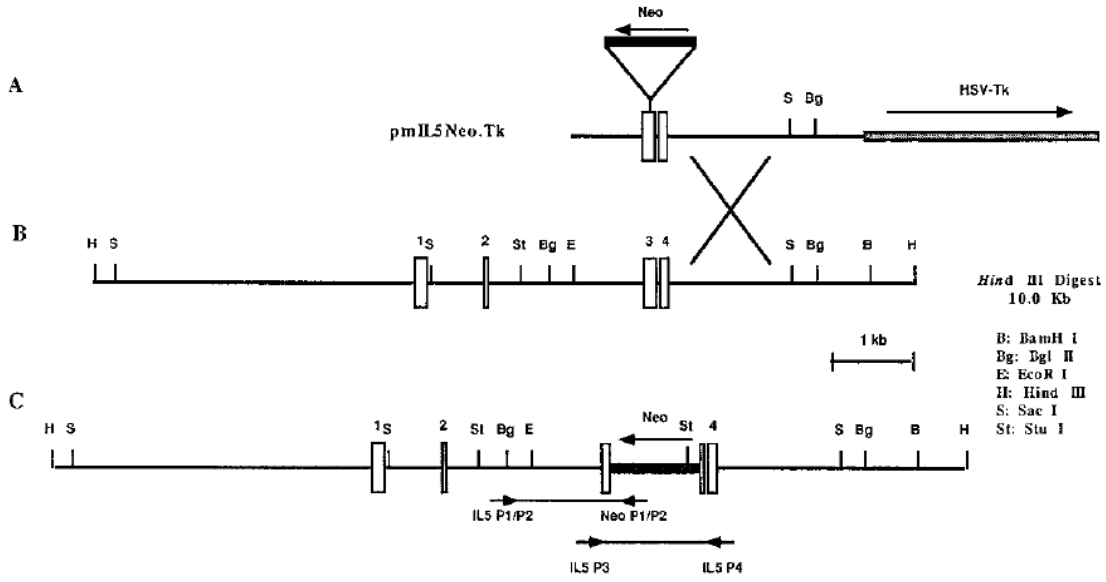


Figure 17: Scheme of the disruption of the murine IL-5 gene. Figure taken from [Kopf et al. 1996]

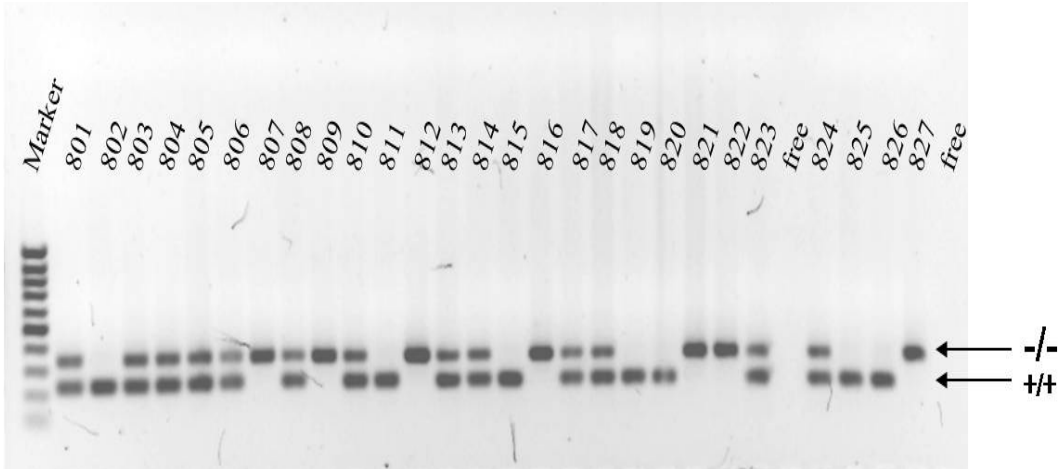


Figure 18: Agarose gel electrophoresis for the detection of IL-5 genotype. 228bp IL-5 wild type and 345bp knock-out mouse; both fragments indicate heterozygote mice 1.5% agarose gel.

5.11.4. Gel Electrophoresis

Agarose gel preparation as well as electrophoresis buffer was prepared using Tris-Acetate-EDTA (TAE) buffer solution (pH 8.0). 1.5 g agarose was added to 100 ml TAE to obtain a 1.5% w/v agarose gel. The PCR products were stained by 2 µl of 6x loading buffer (Fermentas). Amplicons together with 100 bp DNA marker (Fermentas) were separated on the gels using 120 Volt and 100 mA for 45 min. To visualize the DNA fragments, 0.5 µg/ml ethidiumbromide was added to the liquid agarose. Ethidiumbromide intercalates into the stacked bases of the DNA molecule and emits light when illuminated with ultraviolet light.

5.12. RNA techniques

5.12.1. Isolation of total RNA from stromal vascular cells and mouse tissue

Total RNA was isolated from the SVCs or from different organs using QIAGEN RNeasy mini spin columns according to the manufacturer's recommendations.

Cells were washed twice with PBS and lysed in 350 µl RNA lysis buffer. The lysate was mixed with equal volumes of 70 % ethanol and loaded onto the column. The column was spun at maximum speed for 15 seconds at RT. The column was then washed with RW1 (buffer provided by the manufacturer) and RPE (buffer provided by the manufacturer) buffer.RNA was eluted in 40 µl TE-buffer (QIAGEN) by centrifugation. RNA samples were stored at -80 °C.

5.12.2. Isolation of total RNA from white adipose tissue and adipocytes

The isolation of total RNA from white adipose tissue and adipocytes was performed with RNeasy Lipid Tissue Mini Kit from QIAGEN. For disruption and homogenization of the white adipose tissue a rotor-stator Ultra-Turrax homogenizer was used. In the presence of 1 ml QIAzol lysis Reagent (Qiagen) up to 1 g tissue was homogenized for 15 – 45 seconds. After 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes, 200 µl chloroform was added. Next the tube was mixed vigorously for 30 s to separate liquid and organic phase. The tubes were stored at room temperature for another 5 min. The homogenate was centrifuged at 12,000 x g for 15 min at 4°C. During centrifugation, the sample separates into 3 phases: an upper, colorless, aqueous phase containing RNA; a white protein containing interphase; and a lower, red, organic phase. For tissues with an especially high fat content, an additional, clear phase may be visible below the red, organic phase. The volume of the aqueous phase should be approximately 600 µl. The upper phase was transferred into a fresh RNAse-free tube without disturbing the interphase. 600 µl of 70% ethanol was added and mixed thoroughly by vortexing. Afterwards, 600 µl of the sample including any precipitate that may have formed were transferred into an RNeasy Mini Spin Column (QIAGEN) in a 2 ml collection tube and centrifuged at 8000 x g for 15 s at room temperature. To wash the RNeasy Mini Spin Column 700 µl Buffer RW1 (buffer provided by the manufacturer) was added and centrifuged for 15 s at 8000 x g. The washing procedure was repeated twice with Buffer RPE (buffer provided by the manufacturer). To elute the RNA, the RNeasy Mini Spin Column

was transferred to a new 1.5 ml collection tube. 40µl TE-buffer was added directly onto the RNeasy silica-gel membrane and centrifuge for 1 min at 10000 x g. RNA samples were stored at -80 °C until further analyses. RNA quality was assessed by UV spectroscopy.

5.12.3. Reverse Transcription - cDNA synthesis

For the synthesis of cDNA, total RNA was reversed transcribed using the murine moloney leukemia virus reverse transcriptase (M-MLV RT) from Invitrogen™. In brief, 1 µg of total RNA was incubated with random hexamer primers at 70°C for 10 min. Afterwards, 4 µl 5x first strand buffer, 2 µl dithiothreitol, 2 µl deoxyribonucleotide triphosphate [each 10mM], 0,5 µl ribonuclease Inhibitor (RNAsin) and 1µl M-MLV RT were added per reaction The temperature protocol is shown in Table 4

	Temp. [°C]	Time [hh:mm:ss]
1	25	0:05:00
2	37	0:45:00
3	42	0:15:00
4	70	0:20:00
5	4	∞

Table 4: RT-PCR temperature protocol

5.13. Real Time PCR

For quantitative assessment of specific mRNAs, real time PCR was performed. PCR product synthesis can be monitored by measuring the increase in fluorescence caused by the binding of the fluorescence dye SYBR Green. SYBR Green dye bind to double stranded DNA. Quantification of IL-5, IL-5Rα, F4/80, ACRP30, PPARγ, IL-10, MCP-1, LepR, HO-1, HPRT, CycB and Arp3 mRNA

expression was performed with the ABI Prism 7700 Sequence Detection System (Applied Biosystems) using SYBR Green I.

Primer	Sequence 5' - 3'
mIL-5_fwd	GAG ACG ATG AGG CTT CCT GTC
mIL-5_rev	TCCAATGCATAGCTGGTGATT
mIL-5R_fwd	TTCAAGTGAGAGCAGCTGTGAGC
mIL5R_rev	CACAATGAGATGCCATTCTACCCAG
mF480_fwd	CTTTGGCTATGGGCTTCCAGTC
mF480_rev	GCAAGGAGGACAGAGTTTATCGTG
mAcrp30_fwd	GCTCCTGCTTTGGTCCCTCCAC
mAcrp30_rev	GCCCTTCAGCTCCTGTCATTCC
mPPARγ_fwd	GCATGGTGCCTTCGCTGA
mPPARγ_rev	TGGCATCTCTGTGTCAACCATG
mIl10_fwd	GCCAGAGCCACATGCTCCTA
mIl10_rev	GTCCAGCTGGTCCTTTGTTTG
mMCP1_fwd	AAGAGGATCACCAGCAGCAG
mMCP1_rev	TCTGGACCCATTCCTTCTTG
mLepR_fwd	CACATCTGCCGGTGTGAGTT
mLepR_rev	GCAAACCTAAGGGTGGATCG
mHo1_fwd	AGGTCAAGCACAGGGTGACA
mHo1_rev	CATCACCTGCAGCTCCTCAA
mHprt_fwd	CGCAGTCCCAGCGTCGTG
mHprt_rev	CCATCTCCTTCATGACATCTCGAG
CycB_fwd	CAGCAAGTTCCATCGTGTCATCAAGG
CycB_rev	GGAAGCGCTCACCATAGATGCTC
mArp_fwd	GCCAATAAGGTGCCAGCTGCTG
mArp_rev	GAAGGAGGTCTTCTCGGGTCCTAG

Table 5: Primers for real time PCR

One reaction contained 10 µl iTaq™ SYBR Green Supermix with ROX, 0,5 µl forward and reverse primer mix [each 10mM], 7µl aqua bidest and 2,5 µl 1:5 diluted reverse transcribed RNA. PCR conditions are shown in Table 5.

	Temperature [°C]	Time [mm:ss]	
1	50	02:00	
2	94	10:00	
2	94	00:15	40 Cycles
3	60	00:15	
4	72	00:30	
5	20	02:00	

Table 6: Real time PCR temperature protocol

After the last PCR cycle the melting curves for the amplification products were measured from 60°C to 95°C. The results were analysed with the SDS software v1.9.1 (Applied Biosystems) and the software “dissociation curve” to analyze the melting curve. The quantification of the PCR product is based on the calculation of the fluorescence threshold value (CT-value). The CT-value indicates the PCR-cycle in which the reporter-fluorescence (SYBR green) surpasses the background-fluorescence. After the CT-value is achieved the kinetics of the amplification reaction should be exponential. The CT-values of different templates can be compared among each other (relative quantification) or with a CT-value of a known template concentration (e.g. after cloning the target gene into a vector and transfections of cells) using a standard curve (absolute quantification). Raw data C_T values above 35 were not used for calculation of the relative gene expression values, in all experiments. Here a relative quantification was performed. A Real-Time PCR Thermal Cycler Model 7700 was used (Perkin Elmer ABI Prism). Analysis was performed with the Sequence Detection Software (SDS).

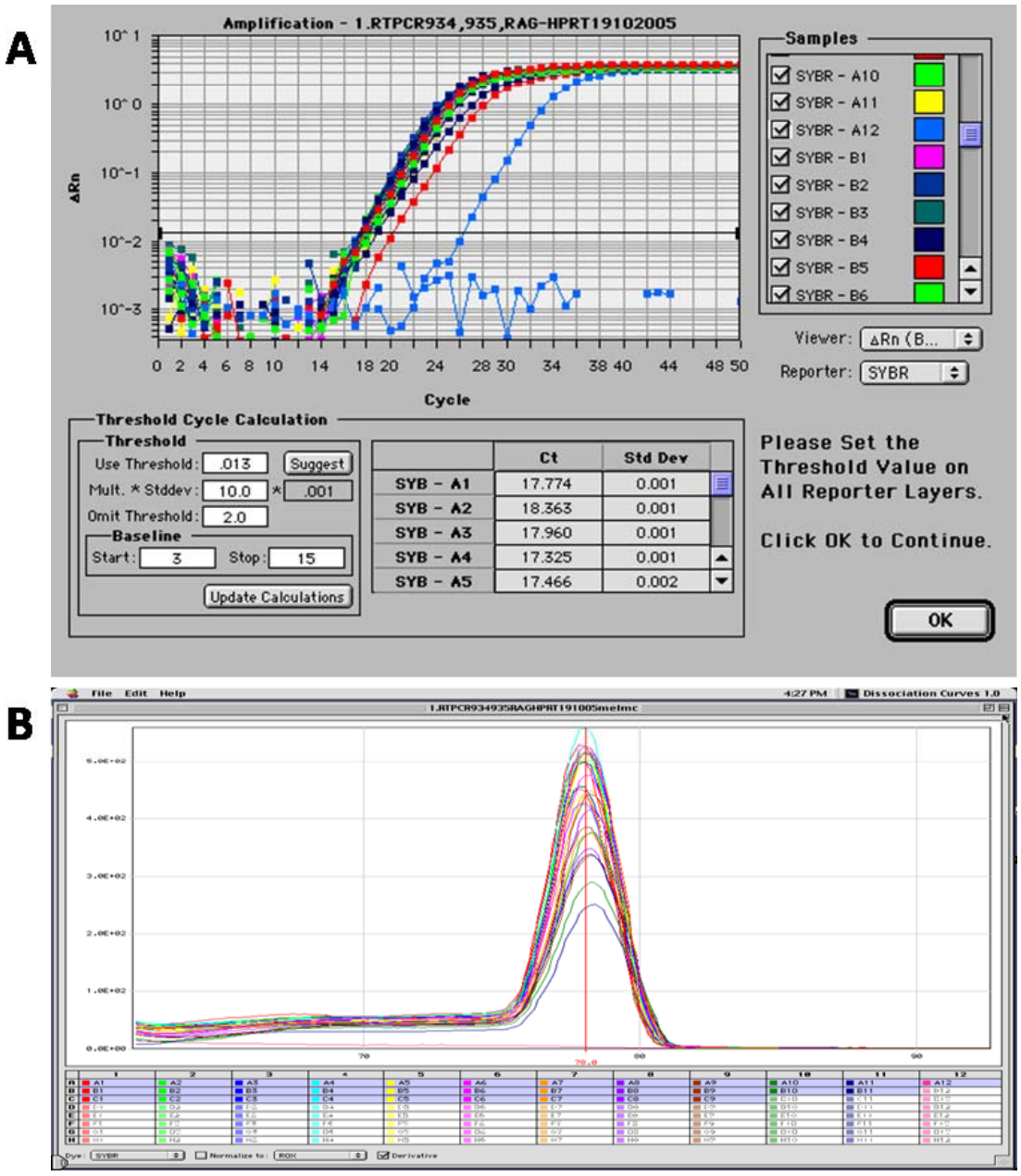


Figure 19: Screenshot of realtime quantification software. (A) increased fluorescence caused by the binding of the fluorescence dye SYBR Green during different PCR cycles. (Fluorescence as a function of time). (B) Control of the primer specificity by analysis of the melting temperature (Fluorescence as a function of temperature).

6. Results

6.1. Interleukin-5 and Interleukin-5 Receptor α mRNA expression in different organs of mus musculus

Different tissues where dissected from normally fed healthy C57BL/6 wild type mice to analyze IL-5 and IL-5Rα mRNA expression in these tissues. Twelve week old mice were used to obtain PEC, liver, kidney, thymus, the small intestine, white adipose tissue, and the spleen. After tissue homogenization, RNA was isolated, reverse transcribed into cDNA and analyzed by real time PCR for expression of IL-5 and IL-5 Rα (**Figure 20**).

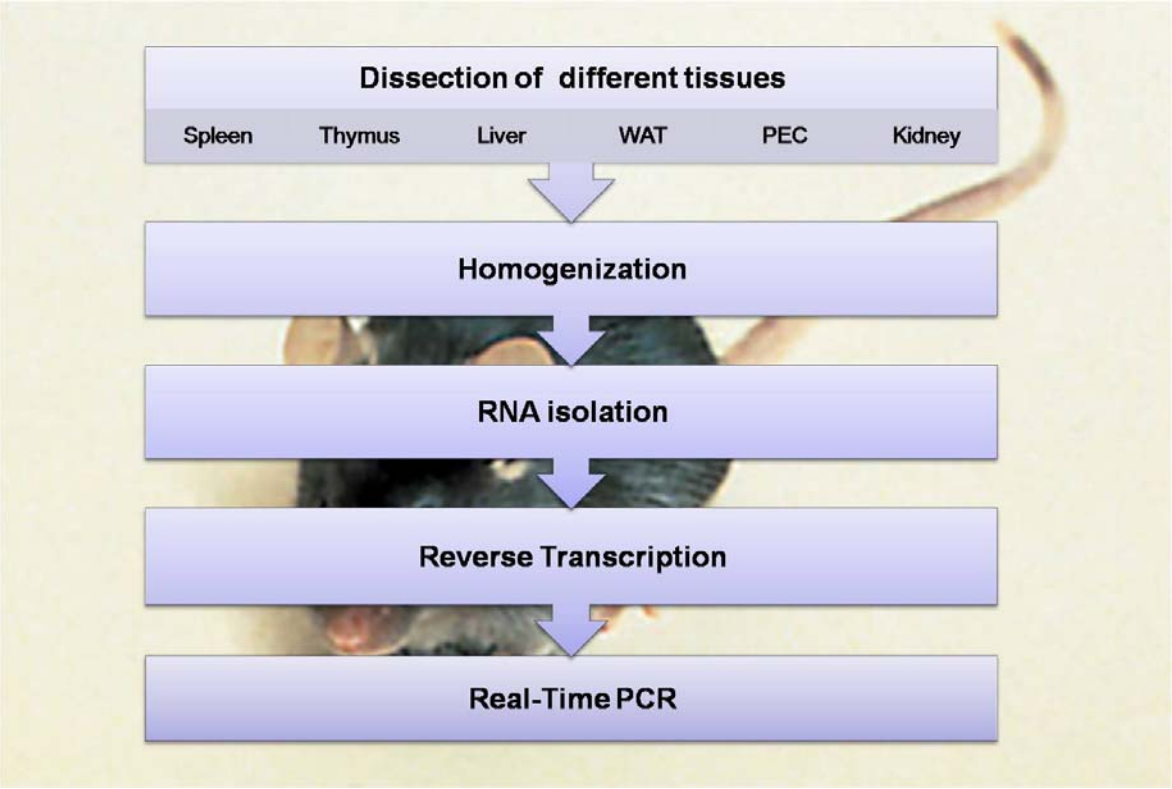


Figure 20: Flowchart of the murine organ screen

Thymus and spleen, which contain Il-5 expressing T-cells were used as positive control for IL-5 mRNA expression. In the case of IL-5Rα, peritoneal exudate cells (PEC) that are enriched in IL-5R expressing B-cells were used as positive control.

Analysis of the real time PCR data (**Figure 21**) clearly showed expression of interleukin-5 and interleukin-5 receptor α mRNA in whole gonadal white adipose tissue from healthy C57BL/6 wild type mice fed regular chow. This indicated that WAT could be a source of IL-5, and was the first time that IL-5 mRNA expression was detected in white adipose tissue.

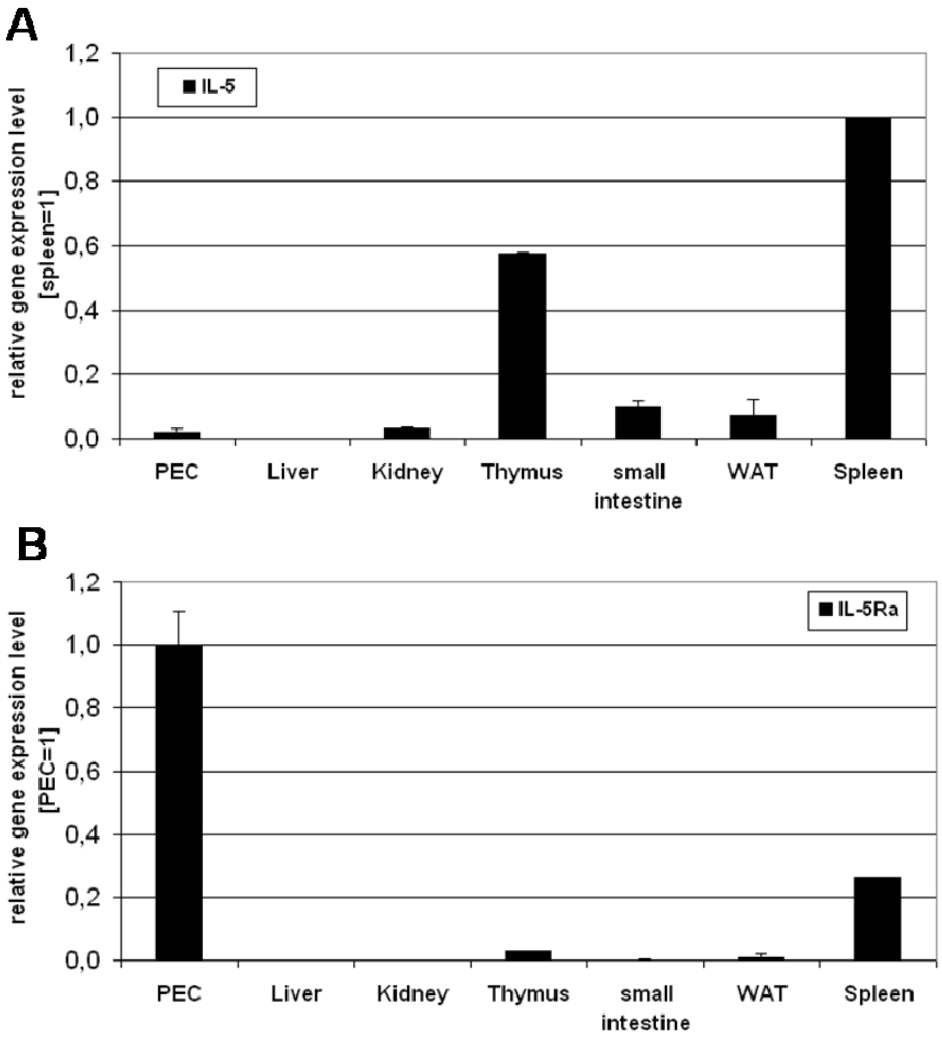


Figure 21: Interleukin-5 (A) and Interleukin-5 receptor α (B) mRNA expression in different organs. PEC, peritoneal exudate cells. 3 mice, male.

As expected, spleen and thymus displayed high IL-5 mRNA levels, whereas kidney, small intestine had moderate IL-5 mRNA expression.IL-5Rα mRNA was also found to be expressed in white adipose tissue, though at very low levels when compared to peritoneal exudate cells. Thus, white adipose tissue contains cells that express IL-5 (as well as the IL-5Ra).

6.2. Analysis of Interleukin-5 and Interleukin-5 Receptor α mRNA expression in different cell size fraction of the white adipose tissue

After identifying IL-5 and IL-5Rα mRNA expression in the white adipose tissue, I asked which fraction dependent on the cell size expressed interleukin-5 and interleukin-5 receptor α, as WAT contains many different cell types, including mature adipocytes, macrophages, pre-adipocytes, stem cells, and endothelial cells. For this purpose I digested the dissected white adipose tissue to obtain the different cellular fractions. First, I passed the resulting single cell suspension through a 100µm mesh-size cell strainer to obtain the >100µm large cell fraction (large adipocytes). Next, the flow trough was passed again through a 30 µm mesh size cell strainer to obtain a <30µm large cell fraction. After centrifugation of the <30µm fraction, I could distinguish floating lipid containing cells (<30µm supernatant) and pelleted cells (<30µm pellet). The cells in the 30µm mesh-size cell strainer were collected as the 30µm-100µm fraction (small adipocytes).

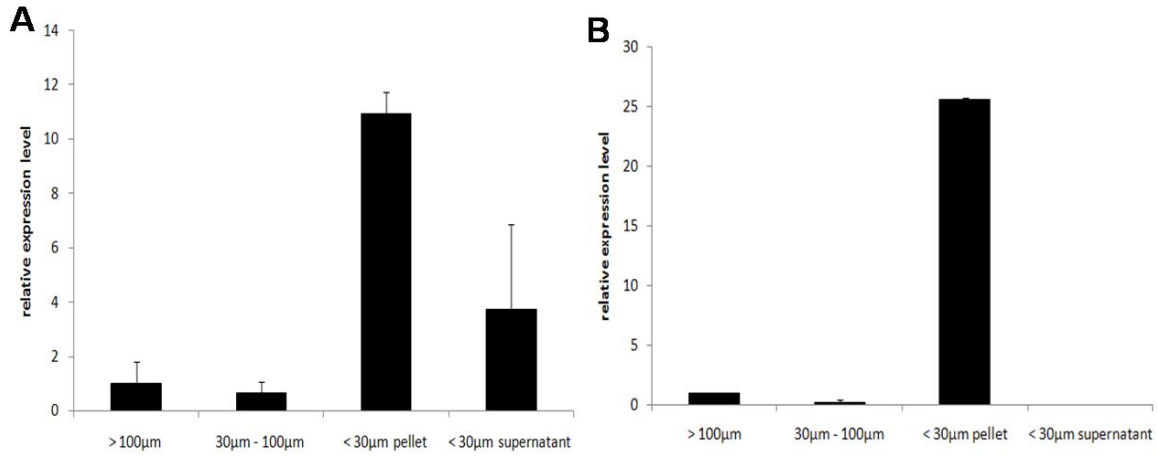


Figure 22: Interleukin-5 (A) and Interleukin-5 receptor α (B) mRNA expression in cell size dependent fractions. 2 mice, male.

As shown in **Figure 22**, the <30µm pellet is the major IL-5 and IL-5Rα mRNA containing cellular fraction. This fraction, which is commonly denoted as stromal vascular cell fraction (SVC) is mostly composed of cells of the connective tissue,

neuronal cells, microvascular endothelial cells, pluripotent stem cells and most importantly immune cells such as macrophages. After the first evidence of IL-5 and IL-5Rα mRNA expression in the <30µm size pellet, I went on to validate these results by direct isolation of adipocytes and SVCs from the dissected white adipose tissue. The white adipose tissue was digested and separated (see material and methods 5.4) and respective realtime PCRs were performed.

Analysis of the real time PCR data confirmed high levels of interleukin-5 mRNA expression in murine stromal vascular cells. In fact, compared to the whole WAT, IL-5 mRNA expression in the SVC fraction was enriched by the factor 12. Similarly, interleukin-5 receptor α mRNA expression was generally much higher in the SVC fraction than in the adipocyte fraction, though it was similar to the expression found in whole WAT (**Figure 23**).

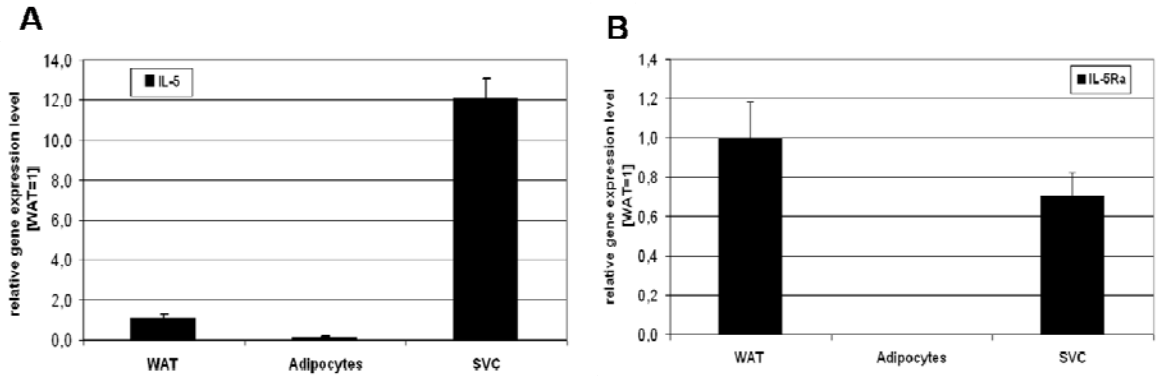


Figure 23: Interleukin-5 (A) and Interleukin-5 receptor α (B) mRNA expression in the white adipose tissue.

6.3. Immunofluorescence microscopy of murine stromal vascular cells of the white adipose tissue

It is crucial to verify real-time PCR results of gene expression on the protein level. Fluorescent dye labeled specific antibodies can be used to visualize antigens and their cellular distribution by immunofluorescence microscopy. To identify IL-5 expression by SVCs, I stained isolated SVCs with a fluorescently labeled antibody against mouse IL-5 and against F4/80, which is a macrophage marker.

Immunofluorescence microscopy of stromal vascular cells identified F4/80, IL-5 (**Figure 24**).

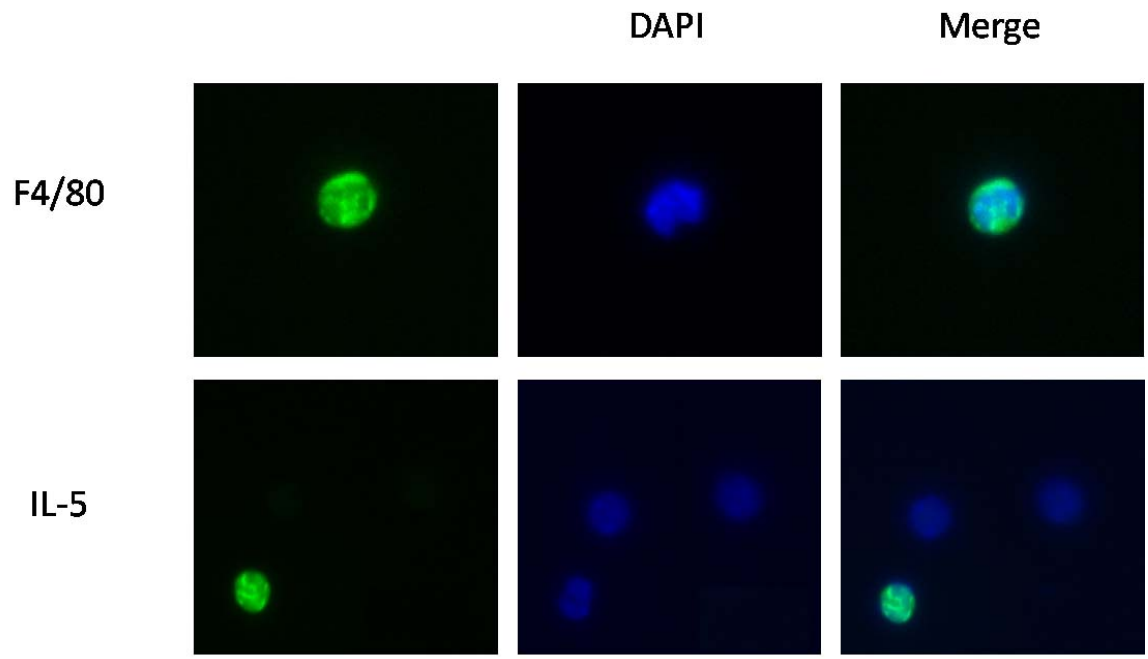


Figure 24: Immunofluorescence microscopy of stromal vascular cells

Further, the shape of the nucleus of IL-5 single stained positive cells displayed a horseshoe like structure as it is common for macrophages. Immunofluorescence microscopy of murine stromal vascular cells of the gonadal white adipose tissue confirmed the real-time PCR results on the protein level. I could demonstrate that IL-5 protein is expressed by some cells of the SVC fraction of the WAT.

6.4. In vitro culture of stromal vascular cells

To confirm the production of IL-5 by stromal vascular cells, SVC were not only used for RNA isolation but were also propagated in cell culture (**Figure 25**). The SVC fraction consists of many different cell types, for example preadipocytes, macrophages and microvascular endothelial cells. Analysis of conventional light microscopy images demonstrates the heterogeneity of this cell fraction.

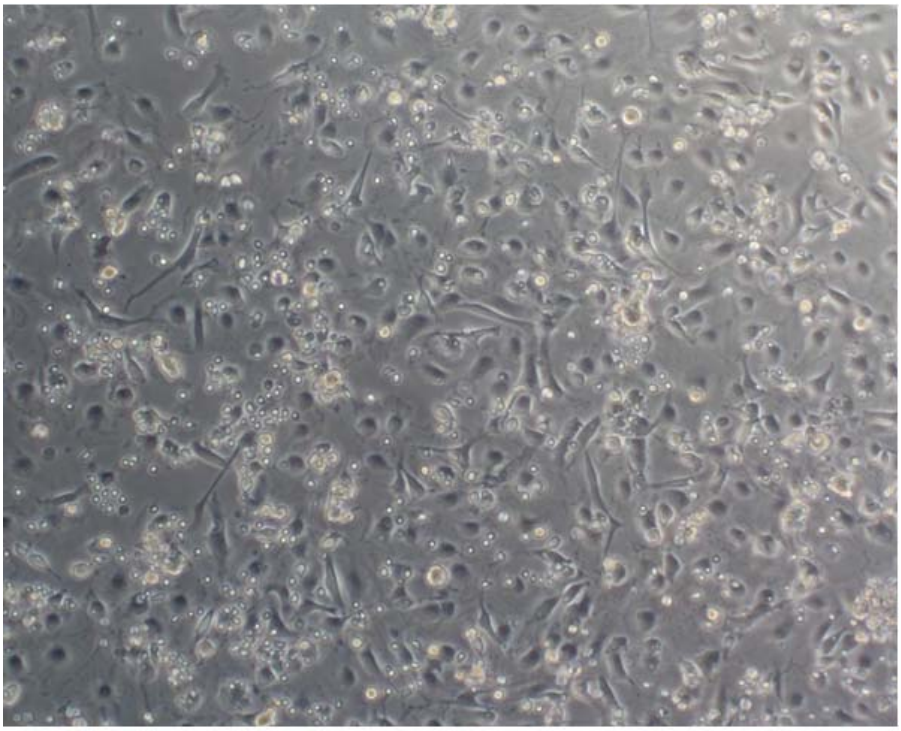


Figure 25: Bright field microscopy of primary cultured stromal vascular cells after 18h.

Among the viable cells, adherent cells as well as suspension cells were present after 18h in cell culture. A small amount of cells was dead, which were removed using a “dead cell removal kit” in further experiments to ensure a dead cell independent analysis. In this SVC fraction, adherent cells are typically considered to be macrophages. Macrophage accumulation is known to be associated with obesity and they represent a type of the stromal vascular cell fraction. Therefore, these cells were a chosen for further analysis.

We also performed hematoxylin eosin (HE) staining of primary isolated murine stromal vascular cells to identify cell types (**Figure 26**). Immediately after digestion the cells were washed, counted and prepared for cytospin. The cytospin was done by 800rpm for 1 min. But it was not so easy to distinguish between different cells in HE stained stromal vascular cells.

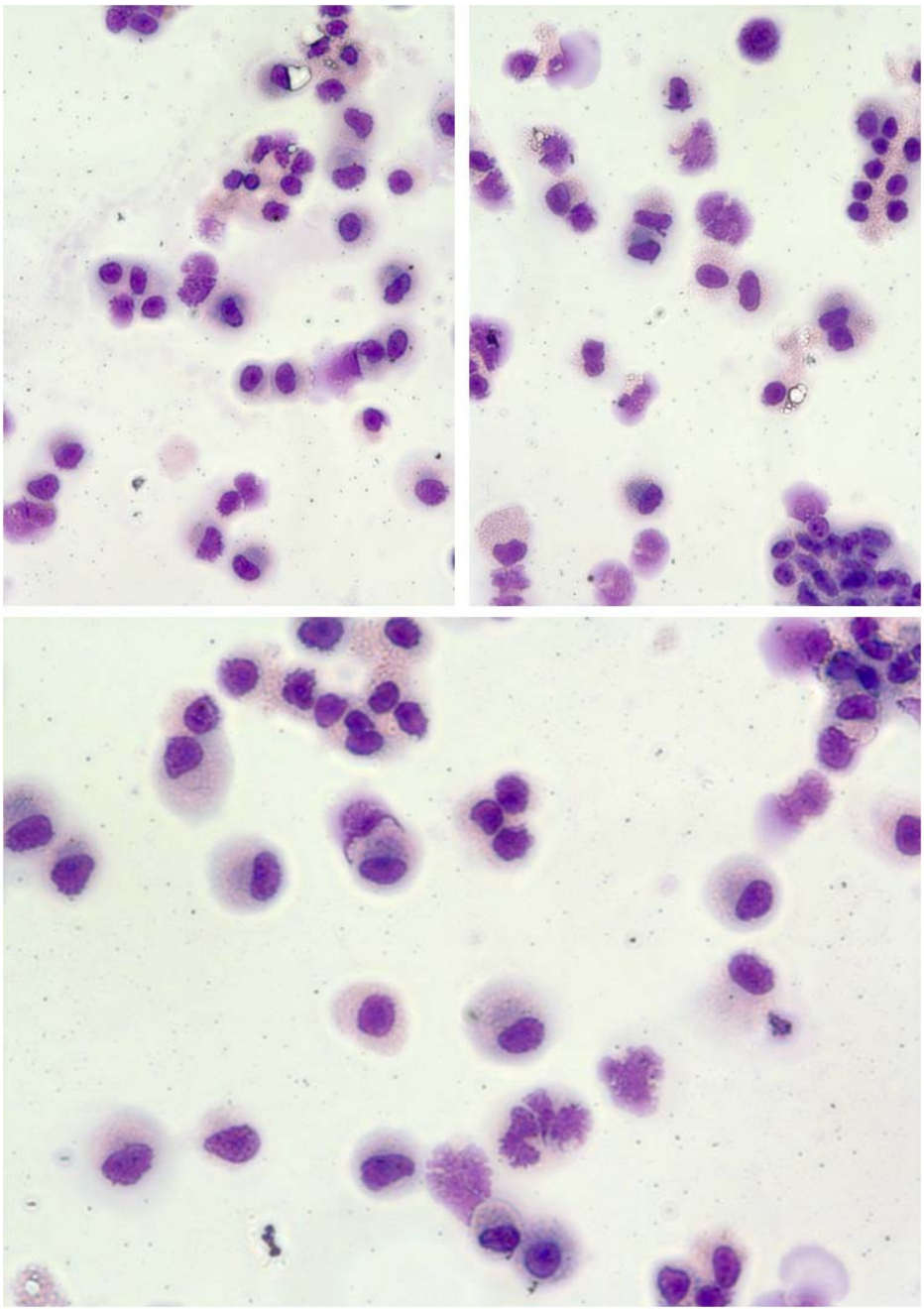


Figure 26: HE staining of primary isolated murine stromal vascular cells immediately after digestion.

6.5. Interleukin-5 protein level in cultured stromal vascular cells

To study the capacity of cultured stromal vascular cells to secrete IL-5 in response to an inflammatory stimulus, SVCs of normal diet fed mice were isolated and equal

cell numbers were cultured for 48h with and without addition of LPS (1µg/ml). IL-5 levels were determined at several time points in the culture supernatants by sandwich ELISA (**Figure 27**).

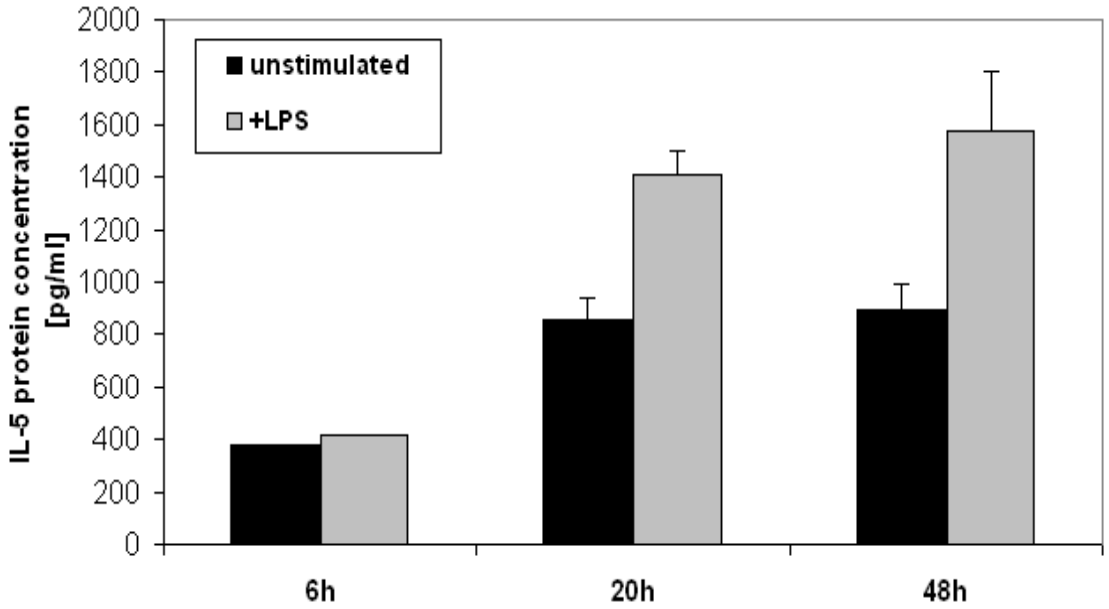


Figure 27: ELISA of Interleukin-5 protein levels in the supernatant of cultured stromal vascular cells at different time points

Surprisingly, cultured SVCs displayed significant spontaneous secretion of IL-5 even in the absence of any stimulus. In addition, LPS stimulation further increased IL-5 secretion in cultured stromal vascular cells. The level of IL-5 in the supernatant increased during the first 20h, whereas after 48h the secretion of IL-5 in the supernatant did not significantly change in both conditions, respectively. Importantly, these results show that SVC are a source of IL-5 protein and that inflammatory stimuli, such as LPS, can either directly or indirectly increase the IL-5 production by cells of the SVC fraction.

6.6. Effects of normal and high fat diet in C57BL/6 mice

Recent evidence demonstrates that obesity is also associated with a chronic inflammatory response. To investigate the possibility that obesity is associated with changes in IL-5 mRNA expression in the adipose tissue, we induced a high

fat, high cholesterol diet to C57BL/6 mice. The mice were allowed free access to water and high fat, high cholesterol diet under a 12 h light/dark cycle and the control group were fed with standard chow. The administration of high fat, high cholesterol diet starts after weaning the mice which was usually 3 weeks after the mice were born. Male mice were killed at the age of 8 weeks. After sacrifice, the body weight of the mice and of the isolated white adipose tissue was determined (**Figure 28**).

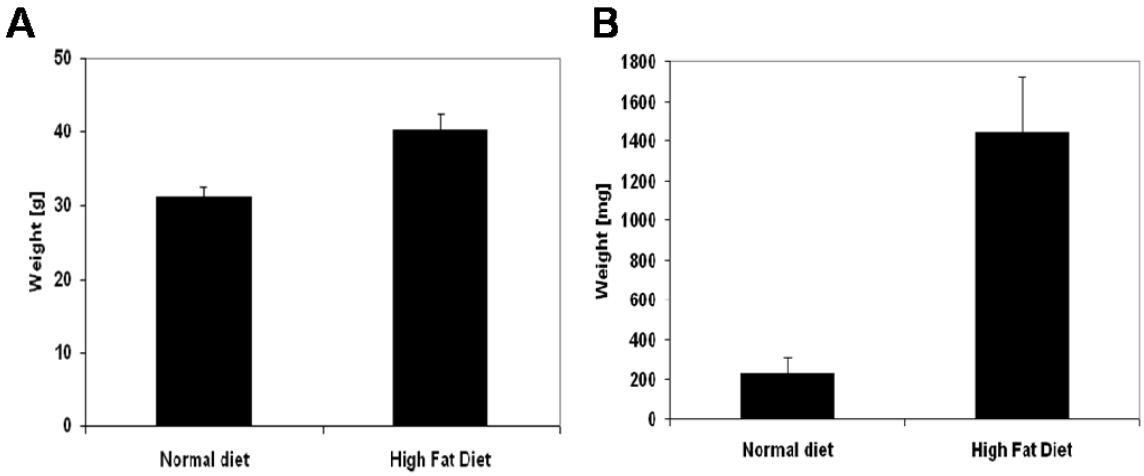


Figure 28: Body weight (A) and weight of the dissected white adipose tissue (B) of C57BL/6 wild type mice on normal (5 mice, male) and high fat diet (8 mice, male).

While after 8-9 weeks of high fat diet the body weight was 33% compared to mice fed regular chow, the weight of the white adipose tissue increased by 700%. The body weight to white adipose tissue weight ratio is shown in **Figure 29**.

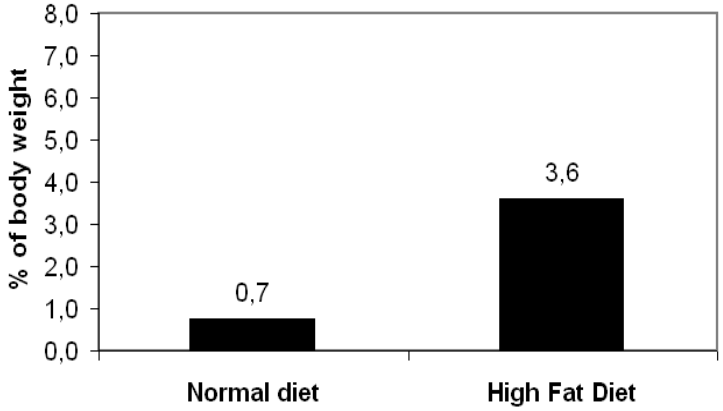


Figure 29: Body weight and white adipose tissue ratio in C57BL/6. C57BL/6 wild type mice on normal (5 mice, male) and high fat diet (8 mice, male).

As expected the administration of high fat, high cholesterol diet lead to a significant increase of the body weight to white adipose tissue ratio compared to normal diet fed mice.

6.7. HE staining of white adipose tissue from normal diet versus high fat diet fed C57BL/6 mice

I also performed microscopic analysis to investigate morphological differences between white adipose tissue from normal diet versus high fat diet fed C57BL/6 mice.

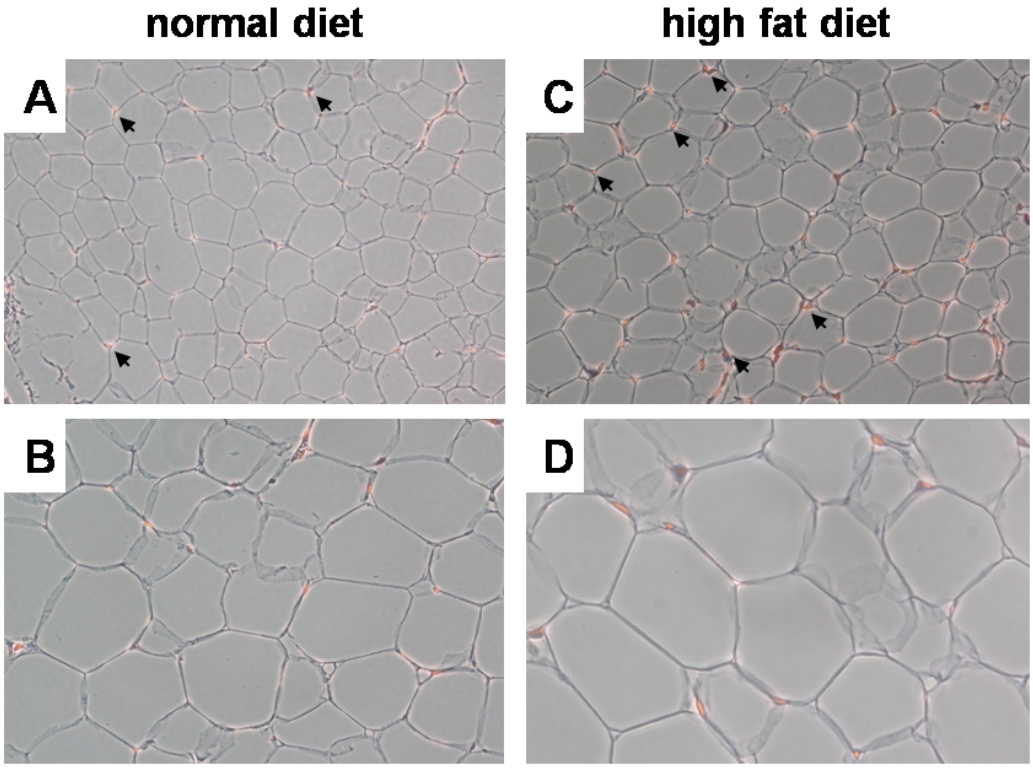


Figure 30: HE staining of the white adipose tissue. (A, C) 10x, (B, D) 20x magnification; arrow indicate SVC.

Consistent with previous reports [Weisberg et al. 2003], more SVCs in the white adipose tissue of high fat diet fed mice compared to normal diet fed mice (**Figure**

30) were found. Moreover, a marginally bigger adipocyte size in high fat diet fed mice was observed as well.

6.8. IL-5 and IL5Rα expression in WAT of normal and high fat diet fed mice

The expression of IL-5 and IL-5Rα mRNA expression in adipocytes and stromal vascular cells was analyzed by real time PCR (**Figure 31**). Male mice were sacrificed after 8 weeks on a high-fat diet at the age of 11-12 weeks.

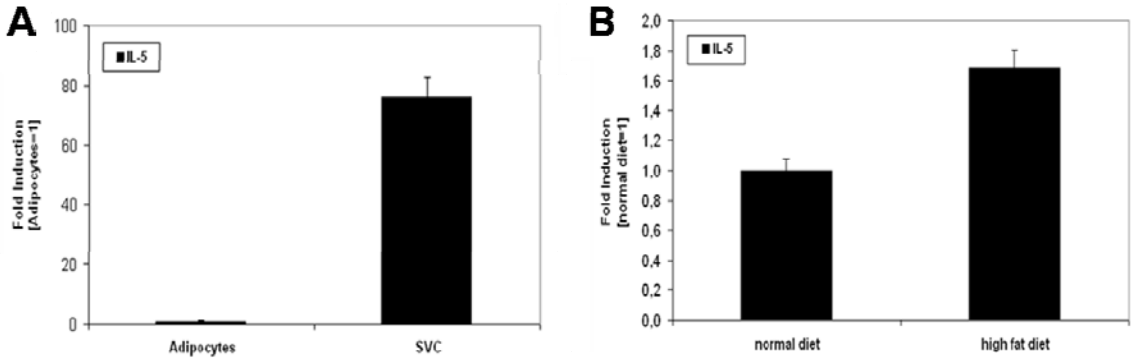


Figure 31: IL-5 mRNA expression in SVCs of normal (3 mice, male) and high fat diet (3 mice, male) fed mice (A). Comparison of the IL-5 mRNA expression in adipocytes and stromal vascular cells in high fat diet fed C57BL/6 mice (B).

As shown for the WAT of conventional mice, IL-5 expression was primarily associated with the SVC fraction of obese mice (**Figure 31B**). Moreover, IL-5 mRNA expression was significantly ($p=0.0000026$) increased in SVC of high fat diet administered mice compared to normal diet fed mice (**Figure 31B**), indicating an induction of IL-5 expression in response to high fat feeding and weight gain. In contrast, IL-5Rα mRNA expression did not change significantly (not shown).

6.9. Separation of SVC into F4/80+ and F4/80- cells

Because Weisberg and colleagues have shown that weight gain and obesity are associated with an increased macrophage infiltration of WAT, we aimed to isolate the macrophages of the SVC population and separated SVC into F4/80^{+} and F4/80^{-} cell types (**Figure 32A**) to determine the exact cellular source of IL-5 in the SVC fraction. Magnetic activated cell sorting (MACS) was used for separation and the quality of this procedure was controlled by real time PCR by identification of F4/80 mRNA in the F4/80^{+} and in the flow through of MACS separation, the F4/80^{-} cell population (**Figure 32B**).

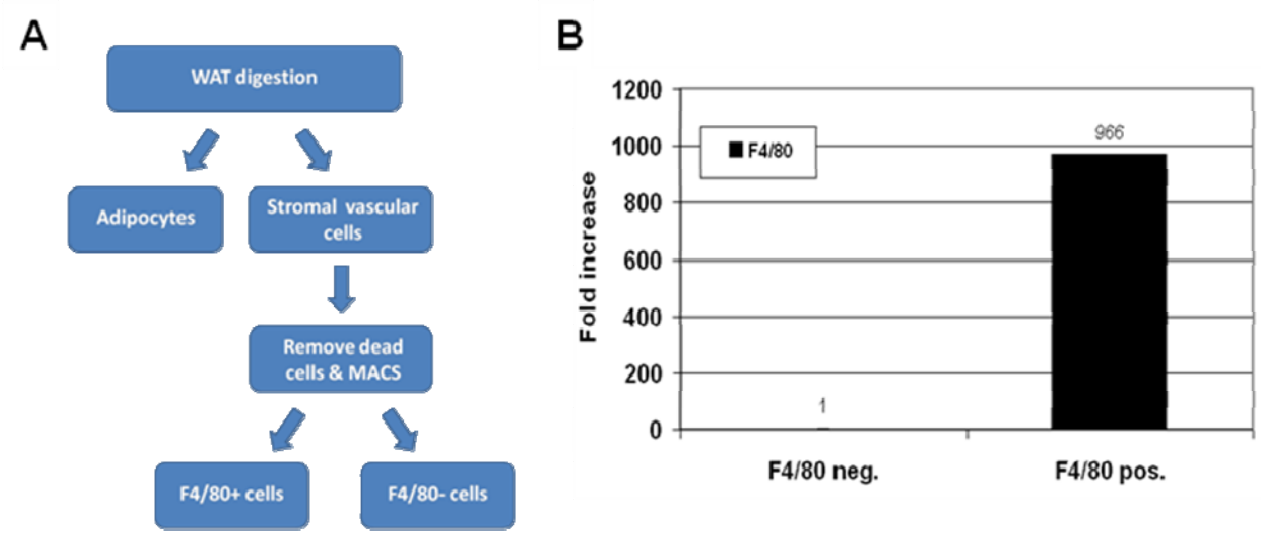


Figure 32: Schema of the separation of F4/80^{+} and F4/80- stromal vascular cells (A). F4/80 gene expression in F4/80+ and F4/80- MACS separated stromal vascular cells (B) derived from 4 mice.

My separation protocol led to highly pure F4/80^{+} versus F4/80^{-} cell populations. The F4/80 gene expression was enriched a 1,000-fold in the F4/80 positive compared to F4/80 negative fraction. Furthermore, this suggested that the gentle digestion procedure had no influence on F4/80 surface marker expression. This is important, because an intact F4/80 surface marker is necessary for magnetic beads mediated separation.

Upon cultivation of F4/80^{+} and F4/80^{-} bead sorted SVC, we could distinguish two different main cell populations in the F4/80^{-} cell fraction. Cultured F4/80^{-} fraction contained adherent and non adherent cells (**Figure 33**).

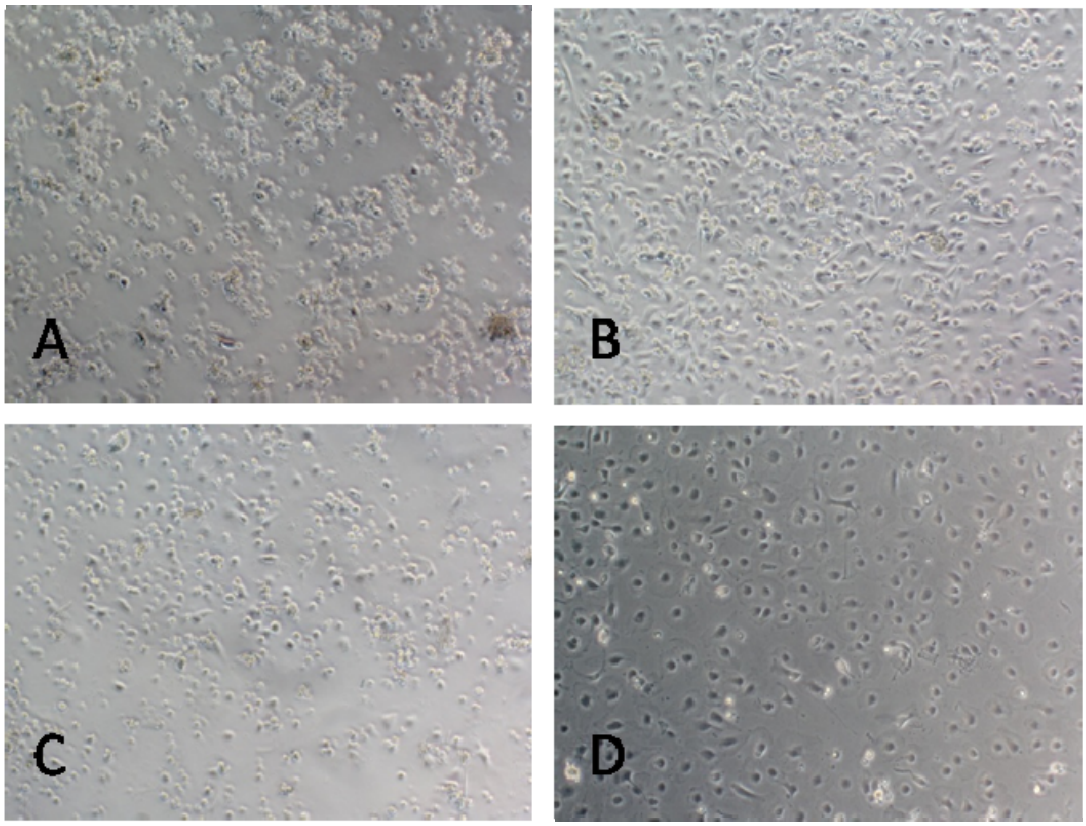


Figure 33: Bright field microscopy of stromal vascular cells. F4/80+ SVC fraction (A); F4/80- SVC fraction (B); F4/80- cells in suspension (C); F4/80- adherent cells (D); A-D:10x,18 h;

Primary isolated SVCs were viable after 18h at cell culture conditions. Interestingly, the F4/80^{+} SVC fraction - mainly macrophages - did not adhere after 18h in cell culture and formed cluster (**Figure 33A**). The F4/80^{-} SVC fraction contained suspension cells (**Figure 33B, C**) and adherent cells (**Figure 33B, D**). Further, in the adherent F4/80^{-} SVC fraction we observed cells with a round or a spindle shape structure. So we could also identify cells in suspension cells and adherent cells in the F4/80^{-} stromal vascular cell fraction, which likely represented fibroblasts.

The expression of the F4/80 antigen shifts during maturation and activation of macrophages. Another marker of macrophages is CD11b, which is also present on monocytes, NK cells and granulocytes. As additional quality control I analyzed the expression of CD11b in the F4/80^{+} versus F4/80^{-} MACS sorted cell populations by flow cytometry (**Figure 34**). As expected, there was a clear enrichment of CD11b positive cells within the F4/80 positive fraction.

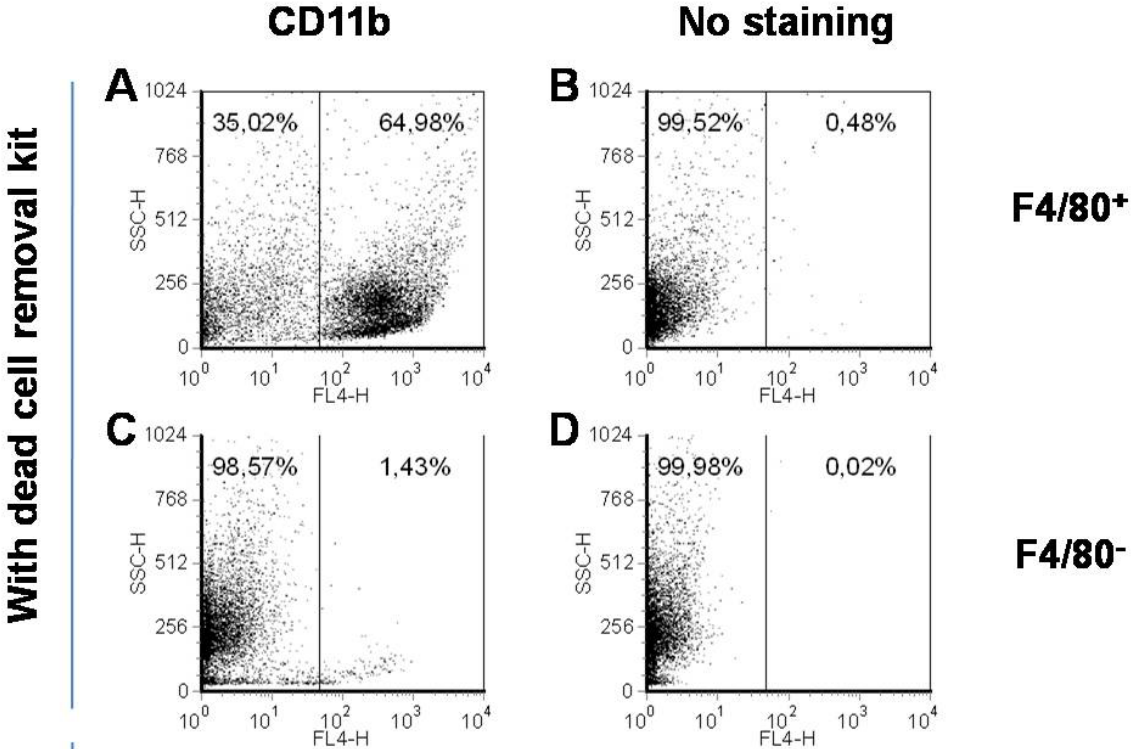


Figure 34: FACS analysis of the F4/80+ and F4/80- magnetic bead separated stromal vascular cells (A, B) F4/80+ and (C, D) F4/80- cells. The procedure was performed with using a dead cell removal kit.

However, only two-thirds of the F4/80^{+} cells were also CD11b^{+} (**Figure 34A**), suggesting differences in the cellular expression patterns between these two surface markers.

Following the verification of the purity of the F4/80^{+} and F4/80^{-} cell populations, I analyzed the IL-5 mRNA expression in both populations (**Figure 35**). As seen in Figure 36, there is a 2.5 fold enrichment of IL-5 expression in the F4/80^{+} cell population compared to total SVCs, which is not seen in the F4/80 – cell

population. Thus, in obese high fat diet fed mice IL-5 expression in WAT is predominantly associated with tissue infiltrating F4/80+ macrophages

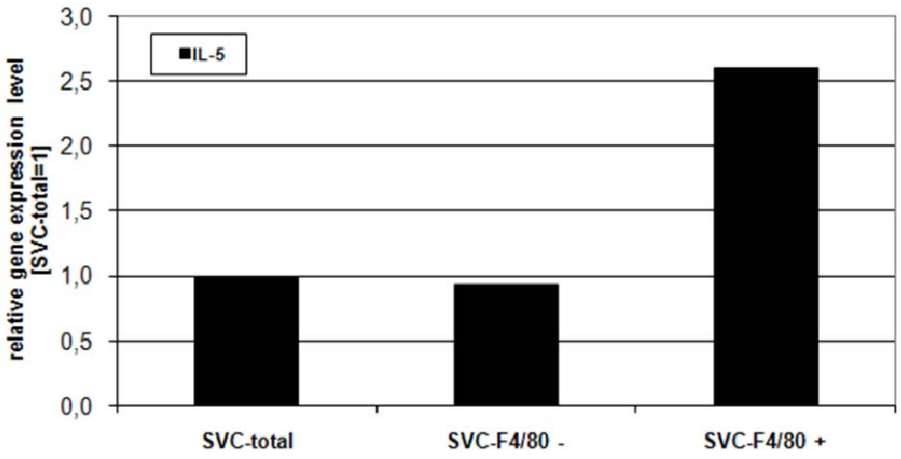


Figure 35: IL-5 mRNA expression in F4/80+ and F4/80- MACS separated stromal vascular cells (2 mice, male)

6.10. Cultured peritoneal macrophages of RAG deficient mice express Interleukin-5 in response to LPS

To further verify that macrophages have the capacity to express IL-5 mRNA, I isolated peritoneal macrophages from RAG deficient mice. RAG is the abbreviation for recombination activating gene [Villa et al. 2001]. There are two mature RAG proteins (RAG1 and RAG2). Both RAG proteins are essential for immunoglobulin (Ig) and T-cell receptor gene recombination. Therefore, RAG deficient mice have no mature T- and B-cells. Thus, using RAG deficient mice as source of peritoneal macrophages excludes the major cellular source of IL-5, namely Th2 cells. Peritoneal macrophages were enriched by inducing an acute inflammation (peritonitis) through thioglycollate injection into the peritoneal cavity. Peritoneal macrophages were isolated, washed and cultured for 18h with and without LPS. After 18h the cells were lysed and the RNA was extracted for real time PCR analysis (**Figure 36**).

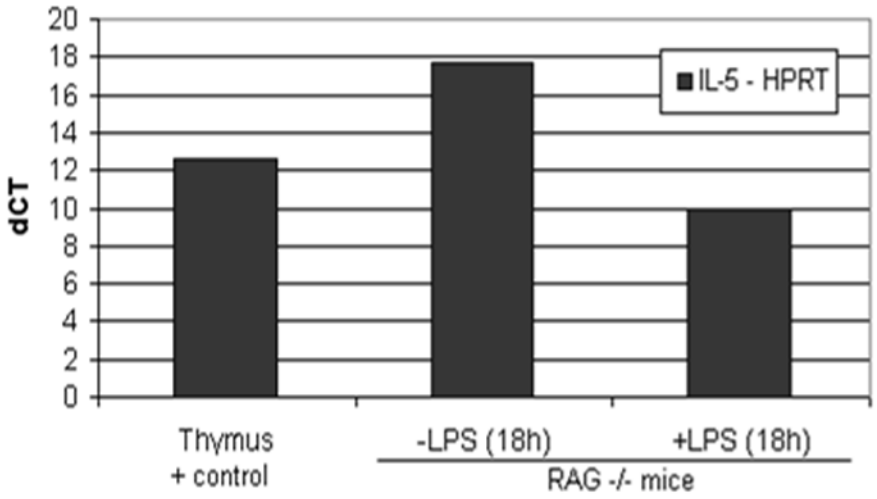


Figure 36: Thioglycollate elicited macrophages of RAG-/- mice express IL-5 in response to LPS

Indeed, I could demonstrate that macrophages have the capacity to make IL-5 in response to an inflammatory stimulus. On the other hand IL-5Rα mRNA expression was not detectable (not shown). These results suggested that LPS induces IL-5 mRNA expression in peritoneal macrophages and that macrophages are able to secrete IL-5.

6.11. Analysis of different genes in the stromal vascular cell population by real time PCR in IL-5 deficient and wt mice

Following the demonstration that macrophages, i.e. peritoneal macrophages in response to LPS or WAT-infiltrating macrophages secrete IL-5, I hypothesized that IL-5 may modulate expression of important other genes in the WAT. Therefore, I also analyzed the mRNA expression of obesity and inflammation associated genes in wild type mice compared to IL-5 deficient mice (**Figure 37**).

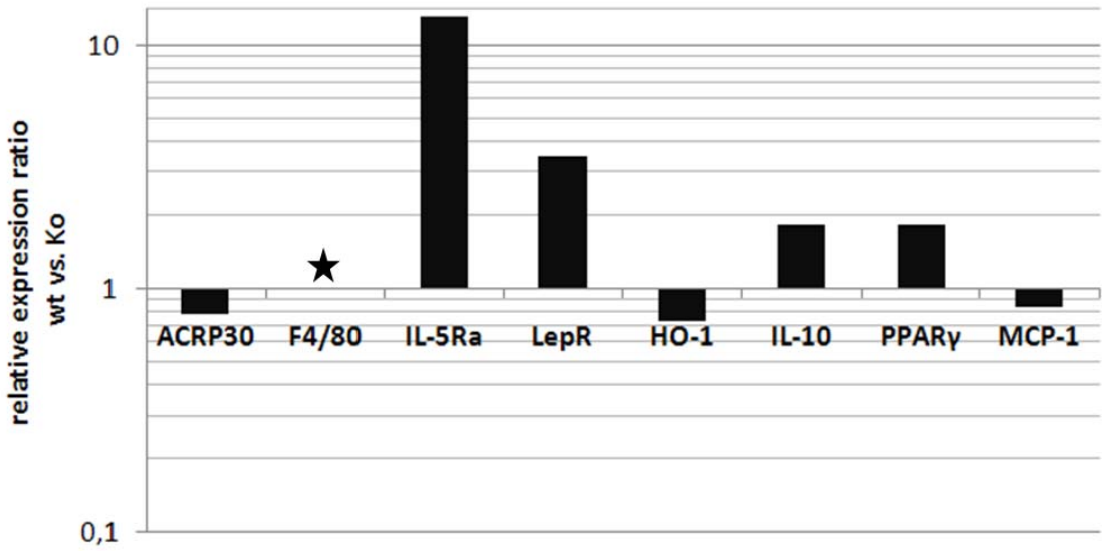


Figure 37: mRNA expression in SVC in wild type vs. IL-5 deficient mice.4 wt and 6 IL-5 deficient mice.

Values, bigger than 1 indicate an elevated gene expression in wt mice. The expression of IL-5Rα, the Leptin Receptor (LepR), IL-10 and PPARγ was decreased in IL-5 deficient mice compared to wild type mice.
Whereas adiponectin (ACRP30), Hemoxygenase-1 (HO-1) and MCP-1 is marginal up-regulated in IL-5 deficient mice. Interestingly, I did not observe any change in F4/80 mRNA expression. These data suggest that IL-5 may indeed influence expression of important genes in the WAT associated with obesity and the metabolic syndrome under physiological conditions (i.e. fed a regular chow).

7. Discussion

First, the Interleukin-5 and Interleukin-5 receptor α mRNA expression in seven different organs of C57BL/6 mice was analysed by real time PCR and it was demonstrated that interleukin-5 and interleukin-5 receptor α mRNA is expressed in the gonadal white adipose tissue in C57Bl/6 mice for the first time (**Figure 21**).

To verify these results, the white adipose tissue was digested and separated into two main fractions: adipocytes and stromal vascular cells. Interleukin-5 and interleukin-5 receptor α mRNA expression was clearly in the stromal vascular cell fraction (**Figure 23**).

Moreover, immunofluorescence microscopy was used to demonstrate that the IL-5 protein is expressed by some cells of the SVC fraction and not the adipocytes of the gonadal white adipose tissue (**Figure 24**). Interestingly, the shape of the nucleus of IL-5 positive cells is similar to macrophages.

The stromal vascular fraction contains macrophages, and obesity is associated with an infiltration of macrophages [Weisberg et al. 2003].
Based on this, I separated the SVC into $F4/80^+$ and $F4/80^-$ cell types by MACS and identify $F4/80^+$ cells of the stromal vascular cell fraction by real time PCR as the interleukin-5 positive fraction. F4/80 is a cell surface marker specific for macrophages.

To confirm the production of IL-5 by stromal vascular cells, primary isolated murine stromal vascular cells were propagated in cell culture and were visualized by hematoxylin eosin (HE) staining (**Figure 26**).

The supernatant of primary isolated cultured murine SVC were used for IL-5 ELISA. The IL-5 level in the supernatant increased dramatically during the first 20h. This result verifies that SVCs are able to secrete IL-5 and respond direct or indirect to LPS with an increased IL-5 production (**Figure 27**). In addition, I show

that thioglycollate elicited peritoneal macrophages of RAG deficient mice express IL-5 mRNA in response to LPS (**Figure 36**).

Next, I investigated the possible changes on IL-5 mRNA expression in obese mice. For this purpose we induce obesity, by administration of a high fat, high cholesterol diet to C57BL/6 wild type mice. The diet significantly increased the body weight and especially the white adipose tissue weight.

In this thesis, I could demonstrate that IL-5 but not IL-5R mRNA expression correlates with body weight and increases following feeding a high fat diet fed mice (**Figure 31**). HE staining of white adipose tissue from normal diet versus high fat diet fed C57BL/6 mice (**Figure 30**) was performed to investigate the diet induced enrichment of stromal vascular cells, especially macrophages. The infiltration of macrophages in the white adipose tissue was described by Weisberg et al. [Weisberg et al. 2003].

Finally, I analyzed the mRNA expression of metabolically important genes (e.g.MCP-1, LepR, PPARγ) in interleukin-5 deficient mice compared to wild type C57BL/6 mice. The differences in mRNA expression suggesting a protective role of interleukin-5 (**Figure 37**).

The pathological hallmark of metabolic diseases like obesity is a chronic inflammation and further alterations of the metabolic activity or the secretion of soluble molecules like cytokines. Cytokines which were secreted by the adipose tissue are also called adipokines. Another important issue is the infiltration of macrophages which is increased in obesity [Weisberg et al. 2003]. Macrophages provide important immunological functions.

Based on my results, IL-5 may be added to the list of adipokines, potentially protective function, because several metabolically relevant genes are deregulated in interleukin-5 deficient mice. For example, monocyte chemoattractant protein-1 (MCP-1) is decreased in IL-5 deficient mice. MCP-1 plays a role in the recruitment of monocytes which results in an accumulation of macrophages in the white adipose tissue. So, increased level of Interleukin-5 could prevent the infiltration of macrophages. And this could lead to a decline of the chronic inflammation in the metabolic active white adipose tissue.

Other metabolically active genes like peroxisome proliferator activated receptor γ (PPARγ) and leptin receptor are also decreased in IL-5 deficient mice. PPARγ is a member of the nuclear receptor family and play a major role in adipose tissue development, whereas leptin plays a important role in energy uptake and expenditure [Harris 2000; Rayner and Trayhurn 2001; Trayhurn and Beattie 2001]. The decrease of leptin receptor (LepR) in IL-5 deficient mice underlines the potential protective property of interleukin-5.

Further evidence of a metabolically protective role of IL-5 comes from experiments performed by Koza and colleagues. They performed DNA Microarray analysis of the white adipose tissue from C57BL/6J inbred mice with low and high weight gain [Koza et al. 2006]. Epididymal fat biopsies of 7 weeks old mice, before the high-fat diet and at the age of 15 weeks, after 8 weeks on a high-fat diet was analysed. Based on their weight gain following the high fat diet, individual mice were identified as low- and high-weight gainers for analysis, respectively. The analysis of the available microarray data showed differences in the expression of IL-5.

Interestingly, high weight gainers displayed very low, whereas low gainers high IL-5 expression (**Figure 38**). In other words, increased levels of IL-5 expression in mice prevents diet induced obesity in mice of the same genetic background. This microarray results corroborate our data and further indicate a protective role of interleukin-5.

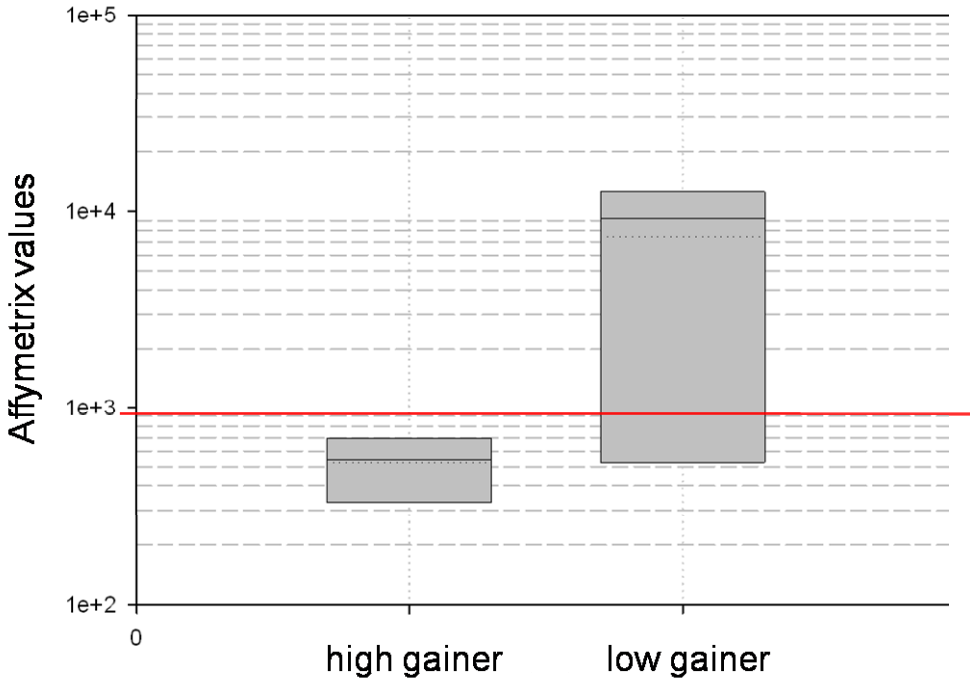


Figure 38: IL-5 box plot in low and high gainer genetic identically C57BL/6 mice. Below red line Affymetrix signal absent above present [Koza et al. 2006]

Obese mice have more interleukin-5 because the white adipose tissue contains more macrophages. On the other hand more macrophages secrete more interleukin-5 and should inhibit adipose tissue formation. In this context or physiological stage the highly metabolically white adipose tissue has a mechanism to prevent the increase of adipose tissue formation. However, the protective property of interleukin-5 is may only be relevant at early stages of obesity or adipose tissue development.

In this respect it would be interesting to analyse the role of interleukin-5 in the white adipose tissue of ob/ob deficient mice, because these mice gain much more body weight than C57BL/6 mice on a high fat diet.

In summary, the present study identifies stromal vascular cells derived macrophages (F4/80+ SVC) as source of interleukin-5 in the murine gonadal white adipose tissue. Furthermore, I could demonstrate that IL-5 mRNA expression in the stromal vascular fraction is increased in high fat diet compared to normal diet

fed C57BL/6 mice. Genetic deficiency of interleukin-5 results in differences in expression of metabolically important genes, suggesting a potentially protective function of interleukin-5. Interleukin-5 may be added to the list of adipokines.

8. Acknowledgements

I was supported by many people during my diploma thesis. In particular I like to express my gratitude to:

First I offer my sincerest thankfulness to my supervisor, **Doz. Dr. Dr. Christoph Binder**. Christoph has supported me all the way through my diploma thesis with his endurance, enthusiasm and knowledge offered me the possibility and apportionment to this thesis. He is the best supervisor I can imagine.

I'm very thankful to **Prof. Dr. Oswald Wagner**, the head of the department at the Institute of Medical and Chemical Laboratory Diagnostics (KIMCL) of the Medical University of Vienna, AKH, his kindly guidance, very interesting discussion during the journal club and kindly character.

My thanks, and greetings, goes also **Prof. Dr. Dr. Harald Esterbauer** kindly helpfulness in all situations and great and interesting discussions

I also want to thanks to the **whole team of the Clinical Institute of Medical and Chemical Laboratory Diagnostics** (KIMCL) for the unbelievable working atmosphere and helpfulness.

Special thanks also to **Prof. Dr. Friedrich Propst**, from the Department of Molecular Cell Biology, University of Vienna and **Prof. Dr. Herbert Strobl**, from the Institute of Immunology, Medical University of Vienna.

Last, but absolutely not the least sincere thanks to my **family** and **all my friends** and **Barbara Haigl** for their great support during this time.

9. Curriculum Vitae

Name	**Thomas KELLER**
Address	Austria, 1130 Vienna, Maxingstrasse 22-24
E-mail	ThomasMKeller@gmx.at
Birth date	02 July 1977
Place of birth	Vienna (Austria)
Citizenship	Austria
Marital Status	single

Education

1983 - 1987	Elementary school (Vienna /Austria)
1987 - 1991	Secondary school (Vienna /Austria)
1991 - 1997	HTBLA – communications engineering (Vienna /Austria) Matura
1997 - 1998	Civilian service – Vienna General Hospital (Vienna /Austria)
1998 - 2001	Study of Biology, University of Vienna
2001 -	Study of Molecular Biology; specialization: Neuroscience, Cell Biology and Immunology (University of Vienna, Vienna Bio Center, Austria)
2005 - 2006	Masters degree student; Molecular biology Supervisor: Doz. Dr. Dr. Christoph Binder Medical University of Vienna, Austria
2007 -	Institute of Clinical Pathology – Medical University of Vienna

Practical Training

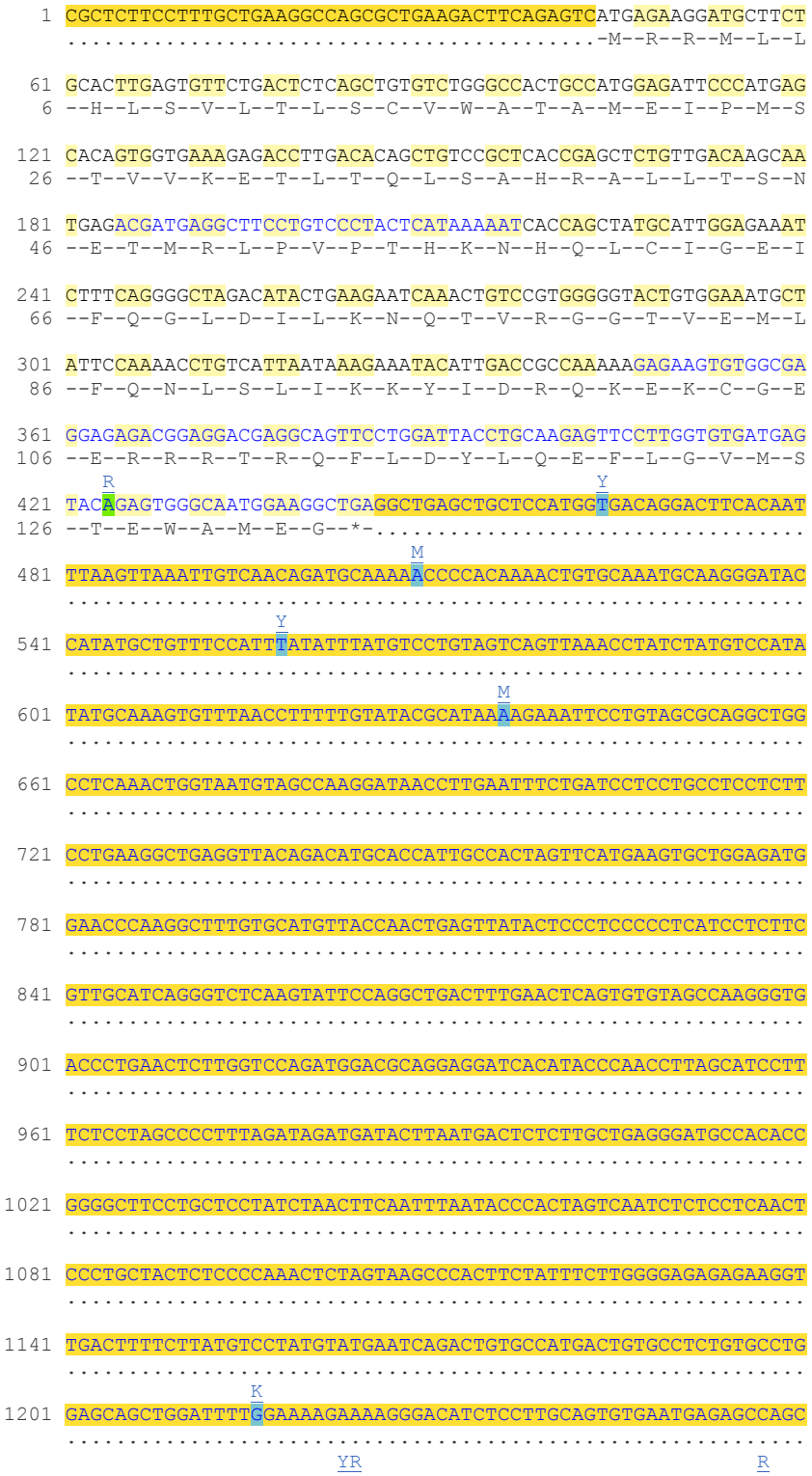
01.09.2001 – 25.08.2002	Research Group – Prof. Dr. Herbert Strobl Institute of Immunology, Medical University of Vienna
26.08.2002 – 20.09.2002	Research Group – Prof. Dr. Friedrich Propst Department of Molecular Cell Biology University of Vienna
05.07.2004 – 30.07.2004	Research Group – Prof. Dr. Roland Foisner Department of Medical Biochemistry Medical University of Vienna
20.12.2004 – 31.12.2004	Research Group – Univ. Doz. Dr. Gerhard Fritsch and Univ. Doz. Dr. Michael Dworzak Children Cancer Research Institute - Vienna Biology of stem cells and immunological diagnostics
14.02.2005 – 18.02.2005	Research Group – Univ. Doz. Dr. Phil. Peter Ambros Children Cancer Research Institute - Vienna
21.03.2005 – 06.05.2005	Research Group – Prof. Dr. Dr. Harald Esterbauer Vienna General Hospital
10.01.2005 – 20.01.2005	Tutor: „In Silico - Bioinformatik“ College of higher education, Vienna
14.03.2005 – 18.03.2005	Tutor: „EDV in der Molekularbiologie“ University of Vienna

Scientific Conferences

Apr 2007	1st International AllergoOncology Symposium, Vienna
July 2007	32nd FEBS Congress – Molecular Machines, Vienna
Jan 2008	Workshop on QRISP - Lymphangiogenomics Database for Integrated Bioinformatics Analyses, Sweden
Mar 2008	Gordon Research Conference: Molecular Mechanisms In Lymphatic Function & Disease, Ventura, CA, United States

10. Supplement

10.1. mIL-5 Transcript sequence

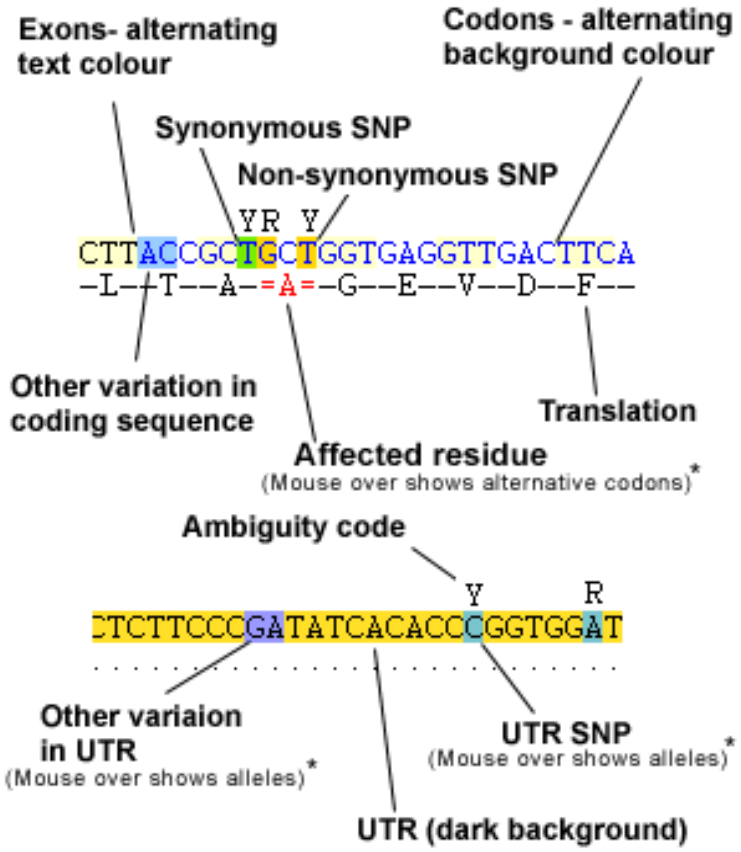


```
1261 CACATGCTGGGCCTTACTTCTCCGTGTAACTGAACTTAAGAAGCAAAGTAAATACCACAA
     ............................................................
                                        M
1321 CCTTACTACCCCATGCCAACAGAAAGCATAAAATGGTTGGGATGTTATTCAGGTATCAGG
     ............................................................
                                                  K
1381 GTCACTGGAGAAGCCTCCCCCAGTTTACTCCAGGAAAAACAGATGTATGCTTTTATTTAA
     ............................................................

1441 TTCTGTAAGATGTTCATATTATTTATGATGGATTCAGTAAGTTAATATTTATTACAACGT
     ............................................................

1501 ATATAATATTCTAATAAAGCAGAAGGGACAACTC
     ..................................
```

10.2. mIL-5Rα Transcript sequence

```
                        Y     S
   1 ATGGTGCCTGTGTTACTAATTCTTGTGGGAGCTTTGGCAACACTGCAAGCTGACTTACTT
   1 -M--V--P--V--L--L--I--L--V--G-=A=-L-=A=--T--L--Q--A--D--L--L-

  61 AATCACAAAAAGTTTTTACTTCTACCACCTGTCAATTTTACCATTAAAGCCACTGGATTA
  21 -N--H--K--K--F--L--L--L--P--P--V--N--F--T--I--K--A--T--G--L-

 121 GCTCAAGTTCTTTTACACTGGGACCCAAATCCTGACCAAGAGCAAAGGCATGTTGATCTA
  41 -A--Q--V--L--L--H--W--D--P--N--P--D--Q--E--Q--R--H--V--D--L-

 181 GAGTATCACGTGAAAATAAATGCCCCACAAGAAGACGAATATGATACCAGAAAGACTGAA
  61 -E--Y--H--V--K--I--N--A--P--Q--E--D--E--Y--D--T--R--K--T--E-

 241 AGCAAATGTGTGACCCCCTTCATGAAGGCTTTGCAGCTAGCGTGAGGACCATTCTGAAG
  81 -S--K--C--V--T--P--L--H--E--G--F--A--A--S--V--R--T--I--L--K-

 301 AGCAGCCATACAACTCTGGCCAGCAGTTGGGTTTCTGCTGAACTCAAAGCTCCACCAGGA
 101 -S--S--H--T--T--L--A--S--S--W--V--S--A--E--L--K--A--P--P--G-
                   R
 361 TCTCCTGGAACCTCGGTTACGAATTTAACTTGTACCACACACACTGTTGTAAGTAGCCAC
 121 -S--P--G--T--S--V--T--N--L--T--C--T--T--H--T--V--V--S--S--H-

 421 ACCCACTTAAGGCCATACCAAGTGTCCCTTCGTTGCACCTGGCTTGTTGGGAAGGATGCC
 141 -T--H--L--R--P--Y--Q--V--S--L--R--C--T--W--L--V--G--K--D--A-

 481 CCTGAGGACACACAGTATTTCCTATACTACAGGTTTGGTGTTTTGACTGAAAAATGCCAA
 161 -P--E--D--T--Q--Y--F--L--Y--Y--R--F--G--V--L--T--E--K--C--Q-

 541 GAATACAGCAGAGATGCACTGAACAGAAATACTGCATGCTGGTTTCCCAGGACATTTATC
 181 -E--Y--S--R--D--A--L--N--R--N--T--A--C--W--F--P--R--T--F--I-
                      R
 601 AACAGCAAAGGGTTTGAGCAGCTTGCTGTGCACATTAATGGCTCAAGCAAGCGTGCTGCA
 201 -N--S--K--G--F--E--Q--L--A--V--H--I--N--G--S--S--K--R--A--A-
                            S
 661 ATCAAGCCCTTTGATCAGCTGTTCAGTCCACTTGCCATTGACCAAGTGAATCCTCCAAGG
 221 -I--K--P--F--D--Q--L--F-=S=--P--L--A--I--D--Q--V--N--P--P--R-

 721 AATGTCACAGTGGAAATTGAAAGCAATTCTCTCTATATACAGTGGGAGAAACCACTTTCT
 241 -N--V--T--V--E--I--E--S--N--S--L--Y--I--Q--W--E--K--P--L--S-
               Y
 781 GCCTTTCCAGATCATTGCTTTAACTATGAGCTGAAAATTTACAACACAAAAAATGGTCAC
 261 -A--F--P--D--H--C--F--N--Y--E--L--K--I--Y--N--T--K--N--G--H-

 841 ATTCAGAAGGAAAAACTGATCGCCAATAAGTTCATCTCAAAAATTGATGATGTTTCTACA
 281 -I--Q--K--E--K--L--I--A--N--K--F--I--S--K--I--D--D--V--S--T-

 901 TATTCCATTCAAGTGAGAGCAGCTGTGAGCTCACCTTGCAGAATGCCAGGAAGGTGGGGC
 301 -Y--S--I--Q--V--R--A--A--V--S--S--P--C--R--M--P--G--R--W--G-

 961 GAGTGGAGTCAACCTATTTATGTGGGAAAGGAAAGGAAGTCCTTGGTAGAATGGCATCTC
 321 -E--W--S--Q--P--I--Y--V--G--K--E--R--K--S--L--V--E--W--H--L-
```

```
                      Y
1021 ATTGTGCTCCCAACAGCCGCCTGCTTCGTCTTGTTAATCTTCTCACTCATCTGCAGAGTG
 341 -I--V--L--P--T--A--A--C--F--V--L--L--I--F--S--L--I--C--R--V-
          Y
1081 TGTCATCTATGGACCAGGTTGTTTCCACCGGTTCCGGCCCCAAAGAGTAACATCAAAGAT
 361 -C--H--L--W--T--R--L--F--P--P--V--P--A--P--K--S--N--I--K--D-
                                                        R
1141 CTCCCTGTGGTTACTGAATATGAGAAACCTTCGAATGAAACCAAAATTGAAGTTGTACAT
 381 -L--P--V--V--T--E--Y--E--K--P--S--N--E--T--K--I-=E=--V--V--H-

1201 TGTGTGGAAGAGGTTGGATTTGAAGTCATGGGAAATTCCACGTTTTGA
 400 -C--V--E--E--V--G--F--E--V--M--G--N--S--T--F--*-
```

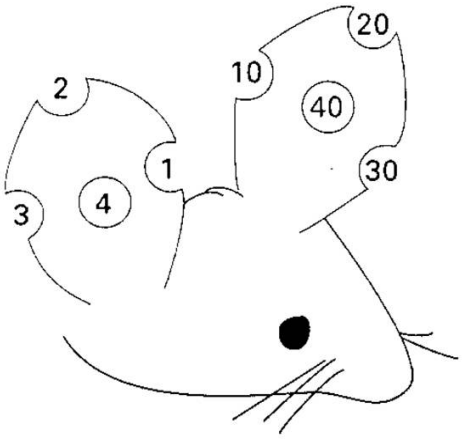


http://www.ensembl.org/index.html

10.3. mouse cage card design

			Cage#
Investigator			
Species			
Strain			
Gender	I.D.	Born	Weaned
Comments			

10.4. Mice earmark system



5 = 1 + 4
6 = 2 + 4
7 = 3 + 4
8 = 1 + 2
9 = 2 + 3

50 = 10 + 40
60 = 20 + 40
70 = 30 + 40
80 = 10 + 20
90 = 20 + 30

11. References

Abbas, Lichtman, et al. (2003). Cellular and Molecular Immunology.

Ahima, R. S. "white adipose tissue." from http://www.med.upenn.edu/ahimalab/Ahima_res_images/Slide5.jpg.

Alexander, A. G., J. Barkans, et al. (1994). "Serum interleukin 5 concentrations in atopic and non-atopic patients with glucocorticoid-dependent chronic severe asthma." Thorax **49**(12): 1231-3.

American-Heart-Association. (2007). "Obesity and Overweight." AHA Scientific Position, from http://www.americanheart.org/presenter.jhtml?identifier=4639.

American-Heart-Association. (2007). "What is the metabolic syndrome?" from http://www.americanheart.org/presenter.jhtml?identifier=4756.

Arita, Y., S. Kihara, et al. (1999). "Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity." Biochem Biophys Res Commun **257**(1): 79-83.

Bazan, J. F. (1990). "Structural design and molecular evolution of a cytokine receptor superfamily." Proc Natl Acad Sci U S A **87**(18): 6934-8.

Berland, R. and H. H. Wortis (2002). "Origins and functions of B-1 cells with notes on the role of CD5." Annu Rev Immunol **20**: 253-300.

Binder, C. J. (2006). "Immunity and Atherosclerosis." from www.cemm.oeaw.ac.at/downloads/CeMM-PrintableVersion0603.pdf.

Binder, C. J., K. Hartvigsen, et al. (2004). "IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis." J Clin Invest **114**(3): 427-37.

Bystrom, J., K. D. Dyer, et al. (2006). "Interleukin-5 does not influence differential transcription of transmembrane and soluble isoforms of IL-5R alpha in vivo." Eur J Haematol **77**(3): 181-90.

Campbell, H. D., C. J. Sanderson, et al. (1988). "Isolation, structure and expression of cDNA and genomic clones for murine eosinophil differentiation factor. Comparison with other eosinophilopoietic lymphokines and identity with interleukin-5." Eur J Biochem **174**(2): 345-52.

Cancello, R., J. Tordjman, et al. (2006). "Increased infiltration of macrophages in omental adipose tissue is associated with marked hepatic lesions in morbid human obesity." Diabetes **55**(6): 1554-61.

Capron, L. (1989). "[Pathogenesis of atherosclerosis: an update on the three main theories]." Ann Cardiol Angeiol (Paris) **38**(10): 631-4.

Charriere, G., B. Cousin, et al. (2003). "Preadipocyte conversion to macrophage. Evidence of plasticity." J Biol Chem **278**(11): 9850-5.

Clutterbuck, E., J. G. Shields, et al. (1987). "Recombinant human interleukin 5 is an eosinophil differentiation factor but has no activity in standard human B cell growth factor assays." Eur J Immunol **17**(12): 1743-50.

Corrigan, C. J., A. Haczku, et al. (1993). "CD4 T-lymphocyte activation in asthma is accompanied by increased serum concentrations of interleukin-5. Effect of glucocorticoid therapy." Am Rev Respir Dis **147**(3): 540-7.

Curat, C. A., A. Miranville, et al. (2004). "From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes." Diabetes **53**(5): 1285-92.

Danireed. (2006). "Adipose Tissue." from http://www.answers.com/topic/adipose-tissue.

Esposito, K., A. Pontillo, et al. (2003). "Effect of weight loss and lifestyle changes on vascular inflammatory markers in obese women: a randomized trial." Jama **289**(14): 1799-804.

Fantuzzi, G. (2005). "Adipose tissue, adipokines, and inflammation." J Allergy Clin Immunol **115**(5): 911-9; quiz 920.

Fantuzzi, G. and T. Mazzone (2007). "Adipose tissue and atherosclerosis: exploring the connection." Arterioscler Thromb Vasc Biol **27**(5): 996-1003.

Frostegard, J., A. K. Ulfgren, et al. (1999). "Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines." Atherosclerosis **145**(1): 33-43.

Fruhbeck, G., J. Gomez-Ambrosi, et al. (2001). "The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation." Am J Physiol Endocrinol Metab **280**(6): E827-47.

Giachelli, C. M., D. Lombardi, et al. (1998). "Evidence for a role of osteopontin in macrophage infiltration in response to pathological stimuli in vivo." Am J Pathol **152**(2): 353-8.

Glass, C. K. and J. L. Witztum (2001). "Atherosclerosis. the road ahead." Cell **104**(4): 503-16.

Gorman, D. M., N. Itoh, et al. (1990). "Cloning and expression of a gene encoding an interleukin 3 receptor-like protein: identification of another member of the cytokine receptor gene family." Proc Natl Acad Sci U S A **87**(14): 5459-63.

Gruart-Gouilleux, V., P. Engels, et al. (1995). "Characterization of the human interleukin-5 gene promoter: involvement of octamer binding sites in the gene promoter activity." Eur J Immunol **25**(5): 1431-5.

Hara, T. and A. Miyajima (1992). "Two distinct functional high affinity receptors for mouse interleukin-3 (IL-3)." EMBO J **11**(5): 1875-84.

Harris, R. B. (2000). "Leptin--much more than a satiety signal." Annu Rev Nutr **20**: 45-75.

Hitoshi, Y., N. Yamaguchi, et al. (1990). "Distribution of IL-5 receptor-positive B cells. Expression of IL-5 receptor on Ly-1(CD5)+ B cells." J Immunol **144**(11): 4218-25.

Hotta, K. and Y. Matsuzawa (2001). "[Molecular mechanism in the development of the complications associated with obesity--the physiological and pathological role of adipocytokines]." Nippon Rinsho **59**(3): 481-6.

Humbert, M., C. J. Corrigan, et al. (1997). "Relationship between IL-4 and IL-5 mRNA expression and disease severity in atopic asthma." Am J Respir Crit Care Med **156**(3 Pt 1): 704-8.

Ihle, J. N. (1995). "Cytokine receptor signalling." Nature **377**(6550): 591-4.

Ihle, J. N. (1995). "The Janus protein tyrosine kinases in hematopoietic cytokine signaling." Semin Immunol **7**(4): 247-54.

Ihle, J. N. and I. M. Kerr (1995). "Jaks and Stats in signaling by the cytokine receptor superfamily." Trends Genet **11**(2): 69-74.

Imamura, F., S. Takaki, et al. (1994). "The murine interleukin-5 receptor alpha-subunit gene: characterization of the gene structure and chromosome mapping." DNA Cell Biol **13**(3): 283-92.

Isobe, M., Y. Kumura, et al. (1992). "Localization of the gene encoding the alpha subunit of human interleukin-5 receptor (IL5RA) to chromosome region 3p24-3p26." Genomics **14**(3): 755-8.

Jernas, M., J. Palming, et al. (2006). "Separation of human adipocytes by size: hypertrophic fat cells display distinct gene expression." Faseb J **20**(9): 1540-2.

Kanda, H., S. Tateya, et al. (2006). "MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity." J Clin Invest **116**(6): 1494-505.

Karlsson, S. (2006). "Hematopoietic Stem Cells: Regulation of Self-Renewal and Lineage Choice." Molecular Medicine and Gene Therapy, from http://www.molmed.lu.se/HSC_regulation.htm.

Kershaw, E. E. and J. S. Flier (2004). "Adipose tissue as an endocrine organ." J Clin Endocrinol Metab **89**(6): 2548-56.

Kikuchi, Y., M. Migita, et al. (1994). "Biochemical and functional characterization of soluble form of IL-5 receptor alpha (sIL-5R alpha). Development of ELISA system for detection of sIL-5R alpha." J Immunol Methods **167**(1-2): 289-98.

Kodama, S., T. Endo, et al. (1992). "Characterization of recombinant murine interleukin 5 expressed in Chinese hamster ovary cells." Glycobiology **2**(5): 419-27.

Kopf, M., F. Brombacher, et al. (1996). "IL-5-deficient mice have a developmental defect in CD5+ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses." Immunity **4**(1): 15-24.

Koza, R. A., L. Nikonova, et al. (2006). "Changes in gene expression foreshadow diet-induced obesity in genetically identical mice." PLoS Genet **2**(5): e81.

Kuby (2006). Immunology 6e.

Laimer, M., C. F. Ebenbichler, et al. (2002). "Markers of chronic inflammation and obesity: a prospective study on the reversibility of this association in middle-aged

women undergoing weight loss by surgical intervention." Int J Obes Relat Metab Disord **26**(5): 659-62.

Lara-Castro, C., Y. Fu, et al. (2007). "Adiponectin and the metabolic syndrome: mechanisms mediating risk for metabolic and cardiovascular disease." Curr Opin Lipidol **18**(3): 263-70.

Le Beau, M. M., R. S. Lemons, et al. (1989). "Interleukin-4 and interleukin-5 map to human chromosome 5 in a region encoding growth factors and receptors and are deleted in myeloid leukemias with a del(5q)." Blood **73**(3): 647-50.

Lee, C. H. and R. M. Evans (2002). "Peroxisome proliferator-activated receptor-gamma in macrophage lipid homeostasis." Trends Endocrinol Metab **13**(8): 331-5.

Lee, J. S., H. D. Campbell, et al. (1989). "The IL-4 and IL-5 genes are closely linked and are part of a cytokine gene cluster on mouse chromosome 11." Somat Cell Mol Genet **15**(2): 143-52.

Leung, D. Y., M. Boguniewicz, et al. (2004). "New insights into atopic dermatitis." J Clin Invest **113**(5): 651-7.

Matsuzawa, Y. (2006). "Therapy Insight: adipocytokines in metabolic syndrome and related cardiovascular disease." Nat Clin Pract Cardiovasc Med **3**(1): 35-42.

McKenzie, A. N., S. C. Barry, et al. (1991). "Structure-function analysis of interleukin-5 utilizing mouse/human chimeric molecules." Embo J **10**(5): 1193-9.

MedlinePlus. (2007). "Obesity and overweight." Overview - obesity, from http://www.nlm.nih.gov/medlineplus/obesity.html.

Meneki. (2004). "Interleukin-5 receptor signaling." from http://www.ims.u-tokyo.ac.jp/meneki/e-img.f/index-e.html.

Milburn, M. V., A. M. Hassell, et al. (1993). "A novel dimer configuration revealed by the crystal structure at 2.4 A resolution of human interleukin-5." Nature **363**(6425): 172-6.

Minamitake, Y., S. Kodama, et al. (1990). "Structure of recombinant human interleukin 5 produced by Chinese hamster ovary cells." J Biochem (Tokyo) **107**(2): 292-7.

Moller, D. E. and J. P. Berger (2003). "Role of PPARs in the regulation of obesity-related insulin sensitivity and inflammation." Int J Obes Relat Metab Disord **27 Suppl 3**: S17-21.

Moon, B. G., S. Takaki, et al. (2004). "The role of IL-5 for mature B-1 cells in homeostatic proliferation, cell survival, and Ig production." J Immunol **172**(10): 6020-9.

Oliver, P., C. Pico, et al. (2003). "Resistin expression in different adipose tissue depots during rat development." Mol Cell Biochem **252**(1-2): 397-400.

Ouchi, N., S. Kihara, et al. (2000). "Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-kappaB signaling through a cAMP-dependent pathway." Circulation **102**(11): 1296-301.

Panagiotakos, D. B., C. Pitsavos, et al. (2005). "The implication of obesity and central fat on markers of chronic inflammation: The ATTICA study." Atherosclerosis **183**(2): 308-15.

Pazdrak, K., D. Schreiber, et al. (1995). "The intracellular signal transduction mechanism of interleukin 5 in eosinophils: the involvement of lyn tyrosine kinase and the Ras-Raf-1-MEK-microtubule-associated protein kinase pathway." J Exp Med **181**(5): 1827-34.

Peraldi, P. and B. Spiegelman (1998). "TNF-alpha and insulin resistance: summary and future prospects." Mol Cell Biochem **182**(1-2): 169-75.

Perez, C., J. Vandesompele, et al. (2003). "Quantitative real time polymerase chain reaction for measurement of human interleukin-5 receptor alpha spliced isoforms mRNA." BMC Biotechnol **3**: 17.

Powrie, F. and R. L. Coffman (1993). "Cytokine regulation of T-cell function: potential for therapeutic intervention." Immunol Today **14**(6): 270-4.

PP. (2004). "Body Part - Adipocyte." IUPS Physiome Project, from http://www.bioeng.auckland.ac.nz/anatml/anatml/database/cells/cells/parts/part/part_37.html.

Rajala, M. W. and P. E. Scherer (2003). "Minireview: The adipocyte--at the crossroads of energy homeostasis, inflammation, and atherosclerosis." Endocrinology **144**(9): 3765-73.

Rayner, D. V. and P. Trayhurn (2001). "Regulation of leptin production: sympathetic nervous system interactions." J Mol Med **79**(1): 8-20.

Reaven, G. M. (1997). "Banting Lecture 1988. Role of insulin resistance in human disease. 1988." Nutrition **13**(1): 65; discussion 64, 66.

Requena, L. (2007). "Normal subcutaneous fat, necrosis of adipocytes and classification of the panniculitides." Semin Cutan Med Surg **26**(2): 66-70.

Robinson, D. S., Q. Hamid, et al. (1992). "Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma." N Engl J Med **326**(5): 298-304.

Rolink, A. G., P. Thalmann, et al. (1990). "Characterization of the interleukin 5-reactive splenic B cell population." Eur J Immunol **20**(9): 1949-56.

Ross, R. (1986). "The pathogenesis of atherosclerosis--an update." N Engl J Med **314**(8): 488-500.

Ross, R. (1993). "The pathogenesis of atherosclerosis: a perspective for the 1990s." Nature **362**(6423): 801-9.

Ross, R. (1993). "Rous-Whipple Award Lecture. Atherosclerosis: a defense mechanism gone awry." Am J Pathol **143**(4): 987-1002.

Ross, R. (1999). "Atherosclerosis--an inflammatory disease." N Engl J Med **340**(2): 115-26.

Sanderson, C. J. (1992). "Interleukin-5, eosinophils, and disease." Blood **79**(12): 3101-9.

Sato, S., T. Katagiri, et al. (1994). "IL-5 receptor-mediated tyrosine phosphorylation of SH2/SH3-containing proteins and activation of Bruton's tyrosine and Janus 2 kinases." J Exp Med **180**(6): 2101-11.

Schaffler, A., J. Scholmerich, et al. (2005). "Mechanisms of disease: adipocytokines and visceral adipose tissue--emerging role in intestinal and mesenteric diseases." Nat Clin Pract Gastroenterol Hepatol **2**(2): 103-11.

Schindler, C. (1999). "Cytokines and JAK-STAT signaling." Exp Cell Res **253**(1): 7-14.

Schindler, C., D. E. Levy, et al. (2007). "JAK-STAT signaling: from interferons to cytokines." J Biol Chem **282**(28): 20059-63.

Schonbeck, U., G. K. Sukhova, et al. (2002). "T(H)2 predominant immune responses prevail in human abdominal aortic aneurysm." Am J Pathol **161**(2): 499-506.

ScienceDaily. (2004). "Adipose tissue." Science Reference, from http://www.sciencedaily.com/articles/a/adipose_tissue.htm.

Sferruzzi-Perri, A. N., S. A. Robertson, et al. (2003). "Interleukin-5 transgene expression and eosinophilia are associated with retarded mammary gland development in mice." Biol Reprod **69**(1): 224-33.

Shen, Y., E. Baker, et al. (1992). "Localization of the human GM-CSF receptor beta chain gene (CSF2RB) to chromosome 22q12.2-->q13.1." Cytogenet Cell Genet **61**(3): 175-7.

Steinberg, D. (2002). "Atherogenesis in perspective: hypercholesterolemia and inflammation as partners in crime." Nat Med **8**(11): 1211-7.

Steppan, C. M. and M. A. Lazar (2004). "The current biology of resistin." J Intern Med **255**(4): 439-47.

Stevens, R. J., K. M. Douglas, et al. (2005). "Inflammation and atherosclerosis in rheumatoid arthritis." Expert Rev Mol Med **7**(7): 1-24.

Stienstra, R., C. Duval, et al. (2006). "PPARs, Obesity, and Inflammation." PPAR Res **2007**: 95974.

Takaki, S., H. Kanazawa, et al. (1994). "A critical cytoplasmic domain of the interleukin-5 (IL-5) receptor alpha chain and its function in IL-5-mediated growth signal transduction." Mol Cell Biol **14**(11): 7404-13.

Takatsu, K., S. Takaki, et al. (1994). "Interleukin-5 and its receptor system: implications in the immune system and inflammation." Adv Immunol **57**: 145-90.

Tavernier, J., J. Van der Heyden, et al. (2000). "Interleukin 5 regulates the isoform expression of its own receptor alpha-subunit." Blood **95**(5): 1600-7.

Tominaga, A., T. Takahashi, et al. (1990). "Role of carbohydrate moiety of IL-5. Effect of tunicamycin on the glycosylation of IL-5 and the biologic activity of deglycosylated IL-5." J Immunol **144**(4): 1345-52.

Trayhurn, P. and J. H. Beattie (2001). "Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ." Proc Nutr Soc **60**(3): 329-39.

Trayhurn, P. and I. S. Wood (2004). "Adipokines: inflammation and the pleiotropic role of white adipose tissue." Br J Nutr **92**(3): 347-55.

Trayhurn, P. and I. S. Wood (2005). "Signalling role of adipose tissue: adipokines and inflammation in obesity." Biochem Soc Trans **33**(Pt 5): 1078-81.

Tuypens, T., G. Plaetinck, et al. (1992). "Organization and chromosomal localization of the human interleukin 5 receptor alpha-chain gene." Eur Cytokine Netw **3**(5): 451-9.

UT-Southwestern-Medical-Center. (2006). "Metabolic Syndrome." from http://www8.utsouthwestern.edu/utsw/cda/dept27717/files/148948.html.

van Leeuwen, B. H., M. E. Martinson, et al. (1989). "Molecular organization of the cytokine gene cluster, involving the human IL-3, IL-4, IL-5, and GM-CSF genes, on human chromosome 5." Blood **73**(5): 1142-8.

Villa, A., C. Sobacchi, et al. (2001). "Recombination activating gene and its defects." Curr Opin Allergy Clin Immunol **1**(6): 491-5.

von der Thusen, J. H., J. Kuiper, et al. (2003). "Interleukins in atherosclerosis: molecular pathways and therapeutic potential." Pharmacol Rev **55**(1): 133-66.

Wakioka, T., A. Sasaki, et al. (1999). "APS, an adaptor protein containing Pleckstrin homology (PH) and Src homology-2 (SH2) domains inhibits the JAK-STAT pathway in collaboration with c-Cbl." Leukemia **13**(5): 760-7.

Wang, Y., H. D. Campbell, et al. (1993). "Sex hormones and dexamethasone modulate interleukin-5 gene expression in T lymphocytes." J Steroid Biochem Mol Biol **44**(3): 203-10.

Weisberg, S. P., D. McCann, et al. (2003). "Obesity is associated with macrophage accumulation in adipose tissue." J Clin Invest **112**(12): 1796-808.

Wellen, K. E. and G. S. Hotamisligil (2003). "Obesity-induced inflammatory changes in adipose tissue." J Clin Invest **112**(12): 1785-8.

Weltman, J. K. and A. S. Karim (2000). "IL-5: biology and potential therapeutic applications." Expert Opin Investig Drugs **9**(3): 491-6.

WHO. (2006). "Obesity." Health topics from http://www.who.int/topics/obesity/en/.

WHO. (2006). "Obesity and overweight." Fact sheet N°311, from http://www.who.int/mediacentre/factsheets/fs311/en/index.html.

Xing, J. and J. D. Chen (2004). "Alterations of gastrointestinal motility in obesity." Obes Res **12**(11): 1723-32.

Xu, H., G. T. Barnes, et al. (2003). "Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance." J Clin Invest **112**(12): 1821-30.

Yamagata, T., J. Nishida, et al. (1995). "Of the GATA-binding proteins, only GATA-4 selectively regulates the human interleukin-5 gene promoter in interleukin-5-producing cells which express multiple GATA-binding proteins." Mol Cell Biol **15**(7): 3830-9.

Yamaguchi, Y., Y. Hayashi, et al. (1988). "Highly purified murine interleukin 5 (IL-5) stimulates eosinophil function and prolongs in vitro survival. IL-5 as an eosinophil chemotactic factor." J Exp Med **167**(5): 1737-42.

Yamaguchi, Y., T. Suda, et al. (1991). "Analysis of the survival of mature human eosinophils: interleukin-5 prevents apoptosis in mature human eosinophils." Blood **78**(10): 2542-7.

Yudkin, J. S. (2003). "Adipose tissue, insulin action and vascular disease: inflammatory signals." Int J Obes Relat Metab Disord **27 Suppl 3**: S25-8.

Zheng, W. and R. A. Flavell (1997). "The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells." Cell **89**(4): 587-96.