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Cellular and molecular mechanisms in the recruitment of immune cells into adipose tissue in obesity

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1. Summary

Obesity is characterized as a state of chronic low-grade inflammation contributing to the pathogenesis of insulin resistance and type 2 diabetes. In the last years, a great deal of evidence indicates an increased infiltration and changed composition of immune cells in the adipose tissue of obese. The infiltration of immune cells and their activation are closely associated with insulin sensitivity. In order to better understand the obesity-associated inflammation it is necessary to gain new insights into the underlying molecular mechanisms and to identify the responsible factors and their cellular source.

(i) There is growing evidence for the involvement of the chemokine IP-10 (CXCL10) in the chronic inflammatory status observed in obesity and type 2 diabetes. The first aim of this thesis was to analyze the role of adipocyte NFκB-signaling in the regulation of the chemokine/adipokine IP-10 and for the adipocyte-mediated T cell migration. Here, the regulation of IP-10 was investigated in adipose tissue of male C57BL/6J mice, primary human and mouse 3T3-L1 preadipocytes/adipocytes. In order to specifically block the NFκB pathway, 3T3-L1 cells stably overexpressing a transdominant mutant of IκBα were generated using retroviral gentransfer. Furthermore, the chemical NFκB-inhibitor Bay117082 was used. Adipocyte-mediated T cell migration was assessed by a migration assay. It could be shown that IP-10 expression was significantly higher in mature adipocytes compared to preadipocytes. IL-1β-induced IP-10 expression and secretion were completely blocked by an NFκB-inhibitor in 3T3-L1 and primary human adipocytes. Stable overexpression of transdominant IκBα in 3T3-L1 adipocytes led to an inhibition of basal and stimulated IP-10 expression and secretion. T cell migration was induced by 3T3-L1 adipocyte-conditioned medium, and both basal and induced T cell migration were strongly inhibited by stable overexpression of transdominant IκBα. In addition, using an anti-IP-10 antibody a significant decrease of adipocyte-induced T cell migration was shown. Altogether, an important role for the NFκB pathway in regulating IP-10 in 3T3-L1 and primary human adipocytes could be demonstrated. Adipocytes rather than preadipocytes contribute to NFκB-dependent IP-10 expression and secretion. Furthermore, NFκB-dependent factors such as IP-10 represent novel signals from adipocytes to induce T cell migration.

(ii) Recent data indicate that obesity and insulin resistance are also associated with activation of immune cells in the periphery. PBMCs might respond to intra- and extracellular signals in various ways like a change of leukocyte subsets by an increased activation and infiltration of

leukocytes into target tissues. The second aim of this thesis was to compare peripheral immune cells from obese and normal-weight women with regard to composition of immune cell subpopulations, surface expression of the chemokine receptors CCR2, CCR3, CCR5 and CXCR3 as well as cell-intrinsic migration capacity. In a case-control study, PBMCs were prepared from fasting blood samples of obese and normal-weight control subjects. Using FACS analysis, the frequencies of immune cell subpopulations as well as chemokine receptor expression of PBMCs were measured. A significant increase of activated $CD4^+CD25^+$ T cells and inflammatory $CD14^+CD16^+$ monocytes was observed in obese as compared to control subjects. Moreover, in contrast to T cells, the chemokine receptor profile on monocytes differed significantly in the obese state. Both receptors, CCR5 and CCR2, showed a significantly increased expression in obese subjects. In addition, a higher chemotactic activity of monocytes from obese subjects was observed in a migration assay, which was associated with both insulin resistance and CCR2 expression. In summary, these results demonstrate that the enhanced migratory capacity of monocytes may result from the here observed increased chemokine receptor expression levels. Therefore, these data further support the close relationship between peripheral immune cell dysfunction and obesity.

2. Zusammenfassung

Adipositas ist gekennzeichnet als ein Zustand chronisch niedrig-gradiger Entzündung, der zur Pathogenese von Insulinresistenz und Typ 2 Diabetes beiträgt. In den letzten Jahren deuteten viele Hinweise auf eine erhöhte Akkumulation, sowie veränderte Zusammensetzung von Immunzellen in dem Fettgewebe von Übergewichtigen. Die Infiltration von Immunzellen und deren Aktivierung sind eng mit der Insulinsensitivität verbunden. Um die mit Adipositas assoziierte Entzündung besser zu verstehen ist es notwendig neue Einblicke in die zugrunde liegenden molekularen Mechanismen zu gewinnen und die verantwortlichen Faktoren, sowie deren zellulären Ursprung zu identifizieren.

(i) Es gibt immer mehr Hinweise für die Beteiligung des Chemokines IP-10 (CXCL10) an dem chronischen entzündlichen Zustand, der bei Adipositas und Typ 2 Diabetes beobachtet wird. Das erste Ziel dieser Arbeit bestand darin, die Rolle des Adipozyten-spezifischen NFκB-Signalweges in der Regulation des Chemokines/Adipokines IP-10 sowie die Adipozyten-vermittelte T Zell Migration zu charakterisieren. Hierfür wurde die Regulation von IP-10 im Fettgewebe von männlichen C57BL-/6J Mäusen, primären humanen, sowie 3T3-L1 Präadipozytes/Adipocytes untersucht. Um den NFκB-Signalweg spezifisch zu blockieren wurde mittels retroviralen Gentransfer eine dominat negative IκBα Mutante in 3T3-L1 Zellen überexprimiert. Darüber hinaus wurde der chemische NFκB-Inhibitor Bay117082 verwendet. Die Adipozyten-vermittelte T Zell-Migration wurde mithilfe eines Migrationassays untersucht. Es konnte gezeigt werden, dass die IP-10 Expression in primären humanen Adipozyten im Vergleich zu Präadipozyten signifikant höher war. Die IL-1β-induzierte IP-10 Expression und Sekretion wurden durch den NFκB-Inhibitor in 3T3-L1 und primären humanen Adipozyten vollständig blockiert. Die Überexpression von transdominanten IκBα in 3T3-L1 Adipozyten führte zu einer Abnahme der basalen, sowie induzierten IP-10 Sekretion bzw. Expression. Die Migration von T Zellen konnte durch konditioniertes Medium von Adipozyten induziert werden und sowohl die basale als auch die induzierte T Zell-Migration wurde durch die Überexpression von transdominanten IκBα stark inhibiert. Darüber hinaus konnte durch die Zugabe eines IP-10-neutralisierenden Antikörpers eine signifikante Abnahme der Adipozyten-induzierten T Zell-Migration gezeigt werden. Zusammengefasst konnte eine wichtige Rolle des NFκB Signalweges in der Regulation von IP-10 in 3T3-L1 sowie primären humanen Adipozyten nachgewiesen werden. Im Vergleich zu Präadipozyten zeigen Adipozyten eine größere Beteiligung an der NFκB-abhängigen IP-10

Expression und Sekretion. Des Weiteren zeigen sich NFκB-abhängigen Faktoren wie IP-10 als neue Signale, die verantwortlich sind für die Adipozyten-induzierte T Zell-Migration.

(ii) Neuere Daten deuten darauf hin, dass Adipositas und Insulinresistenz auch mit der Aktivierung von Immunzellen in der Peripherie assoziiert sind. PBMCs reagieren möglicherweise auf die intra- und extrazellularen Signale mit einer Veränderung der Leukozytenzusammensetzung, einer gesteigerten Leukozytenaktivierung sowie Infiltration von Leukozyten in das Fettgewebe. Das zweite Ziel dieser Arbeit bestand darin, periphere Immunzellen von adipösen und normalgewichtigen Frauen hinsichtlich der Zusammensetzung von Immunzell-Subpupolationen, Oberflächenexpression von den Chemokinrezeptoren CCR2, CCR3, CCR5 und CXCR3 sowie Migrationskapazität zu vergleichen. In einer Fall-Kontroll-Studie wurden PBMCs aus nüchtern Blutproben von Übergewichtigen und normalgewichtigen Kontroll-Probanden isoliert. Mittels FACS-Analysen wurden die prozentualen Anteile der Immunzell-Subpupolationen sowie Chemokinerezepter-Expression an der Oberfläche von PBMCs gemessen. Ein signifikanter Anstieg von aktivierten $CD4^+CD25^+$ T Zellen und inflammatorischen $CD14^+CD16^+$-Monozyten wurde bei Übergewichtigen verglichen mit Kontrollprobanden beobachtet. Darüber hinaus zeigte sich im Gegensatz zu T Zellen ein signifkanter Unterschied des Chemokine Rezeptor Profils auf Monozyten im adipösen Zustand. Die beiden Rezeptoren CCR2 und CCR5 zeigten eine significant gesteigerte Expression in adipösen Probanden. Des Weiteren, konnte eine höhere chemotaktische Aktivität von Monozyten von adipösen Probanden in einem Migrationassay beobachtet werden. Zusammengefasst zeigen diese Ergebnisse, dass die verstärkte Migrationskapazität von Monozyten im adipösen Zustand von den beobachteten erhöhten Chemokinerezeptor-Expressions Leveln resultieren könnte. Diese Daten belegen dadurch weiter den engen Zusammenhang zwischen der Dysfunktion von peripheren Immunzellen und Adipositas.

3. Introduction

3.1 Obesity

Obesity is now recognized as one of the most important public health problems facing the world today. Although its incidence is increasing rapidly, obesity has been known since prehistoric times, as epitomized by the famous Venus of Willendorf (Figure 1). Obesity was once a sign of health and prosperity and demonstrated social respect. Thus, obese people are not a modern occurrence, however, its prevalence has changed dramatically. Today the prevalence of obesity is on continuous rise in all age groups of the developed countries in the world. It has tripled in many European countries since the 1980s. According to the World Health Organization, more than 1.9 billion adults are overweight and at least 600 million of them are clinically obese (WHO, 2015). Obesity is defined as a state of excess adipose tissue mass, which is associated with an increased risk of several major diseases, particularly type 2 diabetes, hypertension, other cardiovascular diseases and cancer (Koehler et al., 2007).

Figure 1: The Venus of Willendorf. Limestone figure from the Late Paleolithic Period, 25000 BC. (Naturhistorisches Museum, Vienna, Austria). Photo: www.nhm-wien.ac.at

The obese state is a complex, multi-factorial disease developing from the interaction of individual genotype and environment. It involves the integration of social, behavioral, cultural, physiological, metabolic, and genetic factors (Barsh et al., 2000, Marti et al., 2008). The fundamental cause of obesity and overweight is the imbalance between energy intake and expenditure. Both adipocyte hypertrophy and hyperplasia contribute to an increase of the fat mass and thus to the development of obesity (Jo et al., 2009). In fact, obesity is one of the key factors for the development of insulin resistance, a condition of reduced glucose utilization by insulin-sensitive tissues due to an impaired insulin action. Numerous factors contribute to this metabolic dysfunction, including increased circulating inflammatory cytokines, decreased protective factors and communication between inflammatory and metabolic cells. Obesity reflects a derangement in cellular and molecular mediators of immunity (Hauner, 2004, Lee and Lee, 2014). The tight connection of metabolism and inflammation, referred to as "immunometabolism" involves many components of the classical inflammatory response to pathogens, such as increased circulating cytokines, recruitment of leukocytes and activation of fibrosis (Mathis and Shoelson, 2011). Moreover, it is also unique in its multi-organ involvement and chronic low-grade activation lacking the cardinal signs of classic inflammation like dolor, rubor, calor and tumor (Mraz and Haluzik, 2014). Further understanding of the biology and mechanistic pathways of obesity and associated disorders is of central importance for development of rational therapy strategies.

3.2 The white adipose tissue

The classical view on the function of adipose tissue has changed dramatically. In the past, the adipose tissue was simply considered to be a passive storage depot with the main function to store excess energy in form of triglycerides. It is now recognized as a multi-functional organ that produces and secretes factors involved in the regulation of many physiologic and pathologic processes. To date, more than one hundred adipose tissue secreted products, called adipokines, have been described. These adipokines influence various metabolic processes in the body as well as immune function and inflammatory processes by autocrine, paracrine and endocrine effects (Hauner, 2005, Vachharajani and Granger, 2009). In the obese state, the expression and secretion of adipokines is disturbed, with elevated levels of pro-inflammatory cytokines like TNFα and IL-6, as well as chemokines, such as MCP-1, IP-10, RANTES and IL-8, and decreased levels of anti-inflammatory factors like adiponectin and IL-10 (Herder et al., 2006a, Herder et al., 2006b, Trayhurn, 2005, Trayhurn and Wood, 2005) (Figure 2).

The source of these adipokines are not only adipocytes but also cells present in the adipose tissue stromal–vascular fraction (SVF), such as preadipocytes, immune cells and endothelial

cells (Hauner, 2004). In obesity, the secretory status is also modified by specific changes in the cellular adipose tissue composition, such as adipocyte hypertrophy, followed by increased angiogenesis, immune cell infiltration and extracellular matrix overproduction. This dynamic change found in the adipose tissue is referred as "adipose tissue remodeling" (Suganami and Ogawa, 2010). Notably, the relative contribution of adipocytes versus SVF cells to the release of adipokines in obesity remains elusive in most cases. The adipocyte size has also been shown to influence adipokine secretion, with increasing adipocyte size resulting in a shift toward pro-inflammatory adipokines (Skurk et al., 2007). Moreover, white adipose tissue can be distinguished as subcutaneous and visceral adipose tissue. Visceral adipose tissue is better vascularized than subcutaneous depots and has a higher metabolic activity (Bjorntorp, 1996, Hauner, 2004).

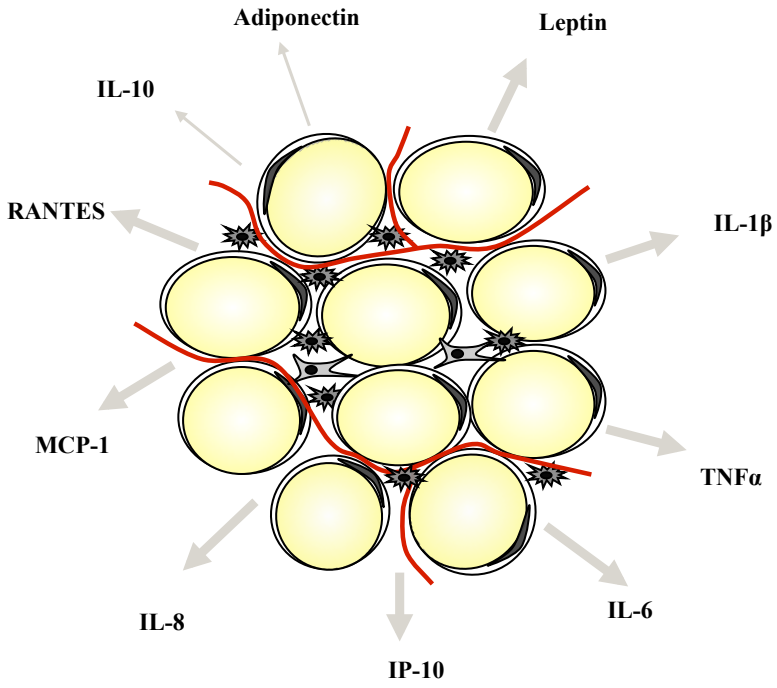

Figure 2: Secretory products of the white adipose tissue. This figure represents secretion of selected adipokines by white adipose tissue. IL, Interleukin; IP-10, Interferon gamma-induced protein10 kDa; MCP-1, Monocyte chemoattractant protein-1; RANTES, Regulated upon activation, normally T-expressed, and presumably secreted; TNFα, Tumor necrosis factor alpha.

In particular, increased visceral adipose tissue is associated with an enhanced secretion of pro-inflammatory adipokines and thus with an increased incidence of insulin resistance and type 2 diabetes (Giorgino et al., 2005, Maury et al., 2007). Furthermore, the adipose tissue communicates with multiple organs or tissues by virtue of a large number of adipokines and thus influences a variety of physiologic and pathophysiologic processes of the whole body.

3.3 Inflammation in obesity and type 2 diabetes

As mentioned above, one of the milestones in obesity research is the finding that obesity is characterized by a subacute chronic inflammatory response (Wellen and Hotamisligil, 2005), which is thought to play a central role in the pathogenesis of insulin resistance and type 2 diabetes. In contrast to acute inflammation, which is stopped by an active termination program (Serhan and Savill, 2005), chronic inflammation is characterized by sustained interaction between parenchymal and stromal cells in response to tissue stress or malfunction (Medzhitov, 2008).

The association of inflammation and diabetes date back to reports from the 1800s, when high-dose salicylates appeared to decrease glycosuria in diabetic patients (Ebstein, 2002). This work suggested remarkable benefits of salicylates for treating diabetes. The effect was rediscovered in 1957 when clinical observations suggested that the use of high-dose aspirin in individuals with diabetes resulted in marked improvements in glycemia (Reid et al., 1957). However, the mechanism behind these effects was not really identified since, the focus on investigation at that time was clearly on insulin secretion.

The first molecular link between inflammation, obesity and insulin resistance results from research findings from the early 1990s. This study in mice demonstrated that adipocytes directly express TNFα and led to the concept of a role for inflammation in obesity (Hotamisligil et al., 1995). With the discovery of TNFα in 1993 (Hotamisligil et al., 1993) and leptin (Zhang et al., 1994) in 1994 from the adipose tissue, the endocrine role of the adipose tissue was recognized for the first time. These observations were paralleled by human studies showing increased TNFα expression in the adipose tissue of obese individuals with a decrease of TNFα expression after weight loss (Kern et al., 1995). Further evidence supporting a key role for TNFα in obesity-related insulin resistance came from studies showing that ob/ob mice (leptin-deficient mice) that were also deficient for TNFα or TNFα receptors had improved insulin sensitivity in diet-induced obesity compared with TNFα- and TNFα receptor-sufficient ob/ob mice (Uysal et al., 1997). Following the discovery of TNFα and leptin in adipocytes, a diverse range of other protein factors have been identified such as IL-6, MCP-1, RANTES, IP-10 and IL-8 (Herder et al., 2006b, Trayhurn, 2005, Trayhurn and Wood, 2005, Wu et al., 2007). It rapidly become clear that obesity is characterized as a chronic low-grade inflammatory state and that diverse inflammatory factors secreted by adipose tissue affect insulin action in a manner similar to that of TNFα.

In searching for the precise mechanisms involved in inflammation-induced insulin resistance, many inflammatory mediators and also nutrients such as lipids or organelle stress have been shown to activate a signaling cascade that triggers inflammatory kinases such as JNK and IKK as well as protein kinase C, S6K, mTOR and ERK (Osborn and Olefsky, 2012). Yuan and co-workers identified the IKK2/NFκB pathway as a mediator of TNFα-induced insulin resistance (Yuan et al., 2001). They could show that overexpression of IKK2 in a human embryonic kidney cell line attenuated insulin signaling and that ob/ob mice expressing only one copy of the gene for IKK2 were protected against the development of insulin resistance. The JNK pathway, also activated by many inflammatory stimuli including TNFα and ligation of Toll-like receptors (TLRs), is a further important regulator of insulin resistance in obesity (Hirosumi et al., 2002). JNK activity is increased in the adipose tissue, liver and muscle, and loss of JNK prevents insulin resistance in both, dietary and genetic mouse models of obesity (Hirosumi et al., 2002, Qatanani and Lazar, 2007). Another important mechanism involved in the development of insulin resistance is the endoplasmic-reticulum (ER) stress (Ozcan et al., 2004). Energy or nutrient excess can trigger ER stress, which is directly linked to activation of inflammatory signaling pathways that inhibit insulin action and transcriptionally regulate production of inflammatory factors. Reactive oxygen species (ROS) that are produced during organelle stress and mitochondrial dysfunction also contribute to this cycle. The consequences are increased ER stress, increased inflammation, inhibition of insulin action and possibly leptin action, culminating in systemic metabolic dysfunction (Hummasti and Hotamisligil, 2010). Together, it became evident in the last two decades that inflammatory pathways are critically involved in the pathogenesis of insulin resistance.

3.4 The NFκB pathway

NFκB was first described as a transcription factor in mature B cells and plasma cells that binds to the intronic enhancer element controlling immunoglobulin kappa light chain expression (Sen and Baltimore, 1986). Ever since its discovery in 1986, the NFκB family of dimeric transcription factors and its role in inflammatory response and immunological reactions has been extensively studied. NFκB is ubiquitously expressed and activated in response to a great variety of extracellular stimuli and translocates to the nucleus, where it controls the expression of numerous components of the innate immune system (Li and Verma, 2002). These include pro-inflammatory cytokines (e.g. TNFα, IL-6), chemokines (e.g. MCP-1, IP-10), adhesion molecules and inducible enzymes, such as cyclooxygenase-2, and inducible nitric oxide synthase. Potent NFκB inducers are cytokines, such as IL-1β and TNFα, bacterial and viral products like LPS, hypoxia, and other stressful conditions like oxygen free radicals and ultraviolet light (Hayden and Ghosh, 2004). Inappropriate activation of the NFκB

signaling pathway is implicated in the pathogenesis of chronic inflammation and autoimmunity, certain hereditary disorders and various cancers (Lawrence, 2009). The mammalian NFκB/Rel family includes five members sharing a highly conserved Rel homology domain: RelA (p65), c-Rel, RelB, NFκB1 (p50; p105) and NFκB2 (p52; p100). The first three contain C-terminal transactivation domains, while the others share a long C-terminal domain with multiple copies of ankyrin repeats, which inhibit their activation. They form homo- or heterodimeric complexes with each other that constitute the NFκB transcription factor complex. Partial proteolysis of p105 and p100 results in the formation of the DNA-binding proteins p50 and p52, respectively. In resting cells, NFκB proteins are predominantly cytoplasmic, associating with members of the inhibitory IκB (inhibitor of NFκB) family, such as IκBα, IκBβ, IκBγ, IκBε, IκBζ, IκBNS and the precursor proteins p105 and p100 as well as Bcl3 (B cell leukemia 3 protein) (Ghosh et al., 1998). All IκB family members contain ankyrin repeats, which are necessary for binding NFκB and blocking its nuclear import. IκB proteins mask the nuclear localization sequence of the NFκB proteins thereby preventing its translocation into the nucleus (Karin and Ben Neriah, 2000). Upon inflammatory activation, IκB is phosphorylated at two serine residues (e.g. serines 32 and 36 in human IκBα) located within the N-terminal domain of the proteins. This phosphorylation of IκB results in ubiquitination on lysine residues (e.g. lysines 21 and 22 in human IκBα) mediated by ubiquitin conjugating enzyme, together with the E3-ubiquitin-protein ligase SCF-bTrCP (Skp1-Cul1-Fbox ligase containing the F-box protein b-transducin repeat-containing protein (bTrCP)). This step represents the signal for degradation by the 26S proteasome (Chen, 2005), this further allows nuclear translocation of NFκB and binding to cognate DNA motifs in the promoter region of target genes, which subsequently initiates transcription. The critical step in NFκB activation is the phosphorylation of IκB by a large multisubunit kinase complex consisting of IKK 1/α and 2/β as well as an additional essential protein, NEMO/IKKγ. NEMO represents the regulatory component of the IKK complex, whereas IKK1 and IKK2 act as catalytic subunits. Each kinase contains in its N-terminus the catalytic domain and in its C-terminus a helix-loop-helix, a leucine zipper structure and the NEMO-binding domain. Both IKKs can phosphorylate IκB proteins to a similar extent. However, from gene knockout experiments it became clear that IKK2 plays the dominant role in signal-induced phosphorylation/degradation of IκB protein. IκB degradation and subsequently NFκB activity can be induced in many cell types by different stimuli. Additionally, individual NFκB response can be characterized as consisting of waves of activation and inactivation of the various NFκB family members (Hayden and Ghosh, 2004). This is due to sustained activation and inactivation of the IKK complex and its selectivity for different IκBs, as well as the differential regulation of IκB expression by NFκB dimers. The

various NFκB dimers exhibit some preferences for distinct κB motifs, which can result in differential regulation of target genes (Menetski, 2000). NFκB activation as described above is referred to as the canonical or classical NFκB pathway. Several parallel signal transduction pathways appear to exist, all of which ultimately result in IKK activation and IκB degradation. Among the best understood are that ones for the inflammatory cytokines TNFα, IL-1ß and LPS (Figure 3). IL-1β is one of the major pro-inflammatory cytokines produced by monocytes and macrophages (Martin and Wesche, 2002) and recognized by its receptor IL-1R. LPS is a well preserved component of the external part of the gram-negative bacterial cell wall. This molecule is recognized by the innate immune system via the TLR4 present, in particular, on monocytes/macrophages. In contrast to their distinct extracellular domains, all members of the TLR/IL-1R family are characterized by an intracellular TIR domain. Due to the presence of the TIR domain, they share a common myeloid differentiation protein 88 (MyD88)-dependent signaling pathway that is involved in NFκB activation. Upon binding of the pro-inflammatory signals IL-1β or LPS to their corresponding receptors, IL-1R and TLR4 engage the TIR-containing cytosolic adaptor molecule MyD88 through homotypic interactions, with subsequent recruitment of IL-1R–associated kinase (IRAK) and IRAK2, IRAK4 and TRAF6. TRAF6 is thought to subsequently activate NFκB either through IKK complex and the kinases TAB-1 and TAK-1 (Andreakos et al., 2004). Beside this MyD88-dependent NFκB activation, MyD88-independent pathway also exist leading to NFκB activation upon LPS or IL-1β stimulation (Verstrepen et al., 2008).

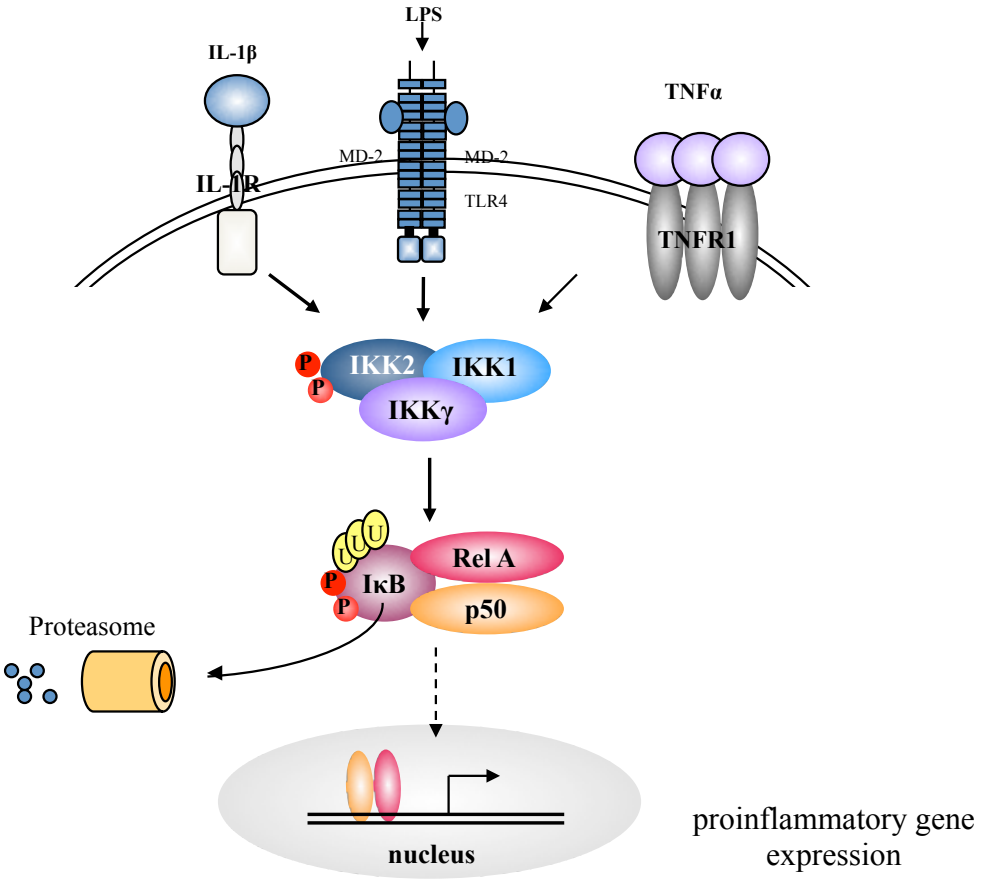

Figure 3: Signaling pathways leading to NFκB activation. IL-1β, interleukin-1 beta; IL-1R, interleukin-1 receptor: LPS, lipopolysaccharide; TLR, toll-like receptor; TNFα, tumor necrosis factor alpha; TNFR, tumor necrosis factor receptor; IKK, inhibitor of NFkappaB kinase; P, phosphorylation; U, ubiquitination; IκB, inhibitor of NFkappaB.

3.5 The NFκB pathway and its role in obesity

NFκB is arguably an essential transcription factor for initiation and progression of numerous human diseases, and to date, the NFκB pathway reveals to be the "master control system" in the development of metabolic inflammation (Cai, 2009). Recent studies have highlighted the role of NFκB in the development of insulin resistance and type 2 diabetes (Arkan et al., 2005, Cai et al., 2005, Yuan et al., 2001). Moreover, the milestone discovery that salicylates mainly target the IKK2/NFκB axis (Arkan et al., 2005, Cai et al., 2005, Kopp and Ghosh, 1994, Yin et al., 1998), brought NFκB in the focus of diabetes research. In fact, treatment of type 2 diabetic patients with salsalate, a modified form of salicylates, improves insulin sensitivity and decreases biomarkers of inflammation along with inhibiting NFκB activity in their PBMCs (Goldfine et al., 2010, Goldfine et al., 2008). Shoelson's group provides the most compelling evidence linking NFκB with obesity-associated insulin resistance by studying heterozygous deletion of IKK2 and insulin resistance (Yuan et al., 2001). Nevertheless, it was

unknown at that time whether IKK2 directly affected the process of insulin resistance, such as serine phosphorylation of IRS, or indirectly via NFκB. In obesity, the NFκB pathway is stimulated through the IKK complex by several pro-inflammatory mediators, like TNFα, IL-1β, saturated free fatty acids and endotoxins, like LPS (Oeckinghaus et al., 2011). So far, IKK2 was an established critical signaling molecule to modulate obesity-associated inflammation and insulin resistance (Baker et al., 2011, Tornatore et al., 2012). In various peripheral metabolic tissues this pathway possesses specific effects. To date, a large body of research has documented metabolic consequences of manipulating NFκB in these tissues.

In obese adipose tissue, increased expression and secretion of many inflammatory cytokines and chemokines has been demonstrated, which are known NFκB target genes (Ajuwon and Spurlock, 2005, Fain and Madan, 2005, Rotter et al., 2003, Shoelson et al., 2006). Moreover, in the mouse adipocytes cell line 3T3-L1, NFκB activity was shown to be upregulated by fatty acid palmitate (Ajuwon and Spurlock, 2005), adipogenesis (Berg et al., 2004) and incubation with macrophage-conditioned medium (Permana et al., 2006). Activation of NFκB by TNFα has been shown to cause dedifferentiation of adipocytes in culture, an effect specifically antagonized by the adipogenic transcription factor PPARγ and mediated by its ability to override the inhibitory effects of NFκB on the expression of key adipocyte genes (Ruan et al., 2002). Moreover, activation of NFκB in primary human adipose tissue of obese has been observed (Ekstrom et al., 2010). However, Jiao and coworkers generated mice overexpressing constitutively active IKK2 in adipose tissue under the control of murine adipocyte fatty acid binding protein (aP2) (Jiao et al., 2012). The activation of IKK2 in adipose tissue of mice results in local and systemic inflammation but protects mice from developing age-related and diet-induced obesity. The reduced adiposity may be at least partially attributable to increased energy expenditure (Jiao et al., 2012).

The NFκB pathway has also been suggested to be involved in the pathogenesis of skeletal muscle insulin resistance (Austin et al., 2008, Radin et al., 2008, Sriwijitkamol et al., 2006). For example, IKK2 silencing prevented TNFα-induced impairments in insulin action on Akt phosphorylation and glucose uptake and metabolism in human skeletal muscle (Austin et al., 2008). Moreover, it has been implicated as an essential transcription factor for the β-cell-specific expression of Glut2 (Norlin et al., 2005). Transgenic mice expressing a nondegradable form of IκBα in β-cells are protected from induction of diabetes (Eldor et al., 2006). Evidence for a systemic effect of this pathway was given by studying mice with deletion of IKK2 in either hepatocytes or myeloid cells. Mice lacking IKK2 in hepatocytes retain insulin responsiveness in liver but develop insulin resistance in muscle and fat in

response to high-fat diet-induced obesity (Arkan et al., 2005). By contrast, mice lacking IKK2 in myeloid cells retain global insulin sensitivity. Thus, this study indicates that myeloid cells, such as monocytes and macrophages, regulate systemic insulin sensitivity and are involved in development of inflammation-associated systemic insulin resistance, whereas hepatic IKK2 expression contributes to insulin resistance in liver. Beside its role in metabolic tissues, the NFκB pathway also affects the hypothalamus in obesity. Both acute and chronic overnutrition result in the activation of IKK2 in the hypothalamus, leading to impaired insulin and leptin signaling (Zhang et al., 2008). Moreover, central inhibition of IKK2/NFκB signaling attenuates high-fat diet-induced obesity and glucose intolerance (Benzler et al., 2015).

Altogether, NFκB signaling in various cell types is involved in regulating obesity-associated disorders, including adipocytes, hepatocytes, muscle cells, β-cell immune cells and neurons. These findings clearly implicate NFκB signaling as a molecular link between inflammation and metabolic dysregulation in obesity and type 2 diabetes. However, the NFκB pathway activates cell-specific programs, which most likely crosstalk to each other. An exact picture of its signaling role in various tissues is still missing.

3.6 Leukocyte infiltration in obesity

Another hallmark of chronic inflammation in obesity is the recruitment and migration of leukocytes into adipose tissue. The process of leukocyte extravasation from the blood into the inflamed tissue comprises several adhesive steps, including the initial selectin-dependent rolling and tethering of the leukocytes, the chemokine-induced leukocyte activation, the integrin-mediated firm adhesion and the trans-endothelial migration of leukocytes. Finally, leukocytes migrate in the extracellular matrix. Chemokines direct the migration across the endothelium and control segregation of cells into specific microenvironments within tissues (Ley et al., 2007). In the last years a great deal of evidence has pointed to the role of immune cell infiltration in adipose tissue in the regulation of glucose homeostasis and inflammation.

Initial studies focused on the role of macrophages, which were shown to accumulate in adipose tissue of obese mice and humans (Curat et al., 2006, Weisberg et al., 2003). To date, numerous types of immune cells have been identified within the obese adipose tissue (Huh et al., 2014). The involvement of multiple immune cell subpopulations further highlights the complexity of obesity-associated inflammation.

3.6.1 Adipose tissue macrophage infiltration

Macrophages, sentinels of innate immunity, fulfill homeostatic functions in numerous tissues (Davies et al., 2013). In adipose tissue, these cells are implicated in angiogenesis, extracellular matrix remodeling, proliferation and differentiation of preadipocytes (Sun et al., 2011). Macrophages surround dead adipocytes in so-called "crown-like structures" (Murano et al., 2008, Strissel et al., 2007), where they fuse to scavenge residual lipid droplets (Cinti et al., 2005, Zaragosi et al., 2010). In obesity, the number of adipose tissue macrophages increases and their function is changed. In obese mice, adipose tissue macrophages represent almost 40% of the total adipose cell content in contrast to only 10% in lean counterparts (Weber et al., 2008). The human visceral adipose tissue comprises 4% macrophages of all cells and rises to 12% in obesity (Harman-Boehm et al., 2007). Adipose tissue macrophages originate from bone-marrow-derived monocytes, which infiltrate the tissue from the circulation (Weisberg et al., 2003). Moreover, a recently published study also demonstrated cell division of macrophages within the obese adipose tissue (Amano et al., 2014). In obese mice, macrophage accumulation temporally precedes an increase in circulating insulin levels and contributes to insulin resistance (Xu et al., 2003). The conditional deletion of CD11c-expressing macrophages results in a marked reduction of both local and systemic inflammation and, importantly, normalization of insulin sensitivity in obesity (Patsouris et al., 2008). Moreover, adipose tissue macrophages have been shown to function as antigen presenting cells, which are able to induce adipose tissue resident T cell proliferation (Morris et al., 2013). Chemokines secreted from diverse adipose tissue cells play a crucial role in attracting circulation monocytes. Although a number of chemokines have been demonstrated to be involved in this process, MCP-1, RANTES and their receptors are the most promising pathways (Ito et al., 2008, Kamei et al., 2006, Kanda et al., 2006, Keophiphath et al., 2009, Weisberg et al., 2006).

3.6.2 Phenotypic diversity of adipose tissue macrophages

Recent studies have also pointed to the heterogeneity of macrophages infiltrated into obese adipose tissue. Macrophages can be divided into at least two subgroups: "classical activate" M1 macrophages and "alternatively activated" M2 macrophages (Mosser, 2003). Macrophages invading the adipose tissue of obese animals exhibit an M1 polarization compared to M2 macrophages identified in the adipose tissue of lean animals. The classical activated M1 macrophages are positive for surface markers F4/80, CD11b and CD11c and produce pro-inflammatory cytokines, like TNFα, IL-6 and IL-1β (Lumeng et al., 2007). In contrast, resident adipose tissue macrophages in lean animals also express F4/80 and CD11b

surface markers but lack CD11c expression almost completely. These cells exhibit an M2/anti-inflammatory profile with enhanced expression of IL-10 and IL-1 receptor antagonist, which protects against insulin resistance (Lumeng et al., 2007). However, mixed M1/M2 phenotypes have also been identified in obese adipose tissue (Bourlier et al., 2008, Shaul et al., 2010). Interestingly, the obesity-induced switch in the adipose tissue macrophage activation state is not dependent on the conversion of resident M2 macrophages to an M1 phenotype but arises rather from the recruitment of inflammatory macrophages out of the circulation (Lumeng et al., 2008). Prior to macrophage infiltration, increased M1 markers are detected in circulating peripheral blood monocytes from both obese and obese type 2 diabetic patients, relative to normal-weight controls (Satoh et al., 2010). This imbalance of M1 and M2 phenotype is known to be associated with enhanced insulin resistance and inflammation and may be an important link to metabolic disorders (Chawla et al., 2011).

3.6.3 Adipose tissue T cell infiltration

In addition to macrophages, various cells of the adaptive immunity have been detected in adipose tissue that may play important roles in obesity-related disease by influencing preadipocyte/adipocyte functions and polarization of macrophages. T cells in human adipose tissue were first described 15 years ago (Bornstein et al., 2000). Five years later, they were identified again along with NK, NKT and B cells in the SVF of mice, where they constituted 10-15% of the SVF of visceral adipose tissue (Caspar-Bauguil et al., 2005). 2007 Wu et al. showed an increased accumulation of T cells in adipose tissue in the obese state (Wu et al., 2007). Diet-induced obese mice contain at least three-fold more resident T cells per gram adipose tissue than lean mice. Additionally, FTY720, a sphingosine-1-phosphate receptor modulator preventing T cells from editing lymphatic tissue, reverses high-fat diet-induced weight gain, insulin resistance and adipose tissue inflammation in mice (Kendall and Hupfeld, 2008). In obese adipose tissue, $CD3^+$ T cells were found dispersed in between adipocytes but also in crown-like structures associated with macrophages and dead adipocytes (Rausch et al., 2008).

3.6.4 Adipose tissue T cell subpopulations

Recent research more extensively characterized subpopulations of adipose tissue lymphocytes of humans and mice and revealed a functional link between specific T cell subpopulations and obesity-associated insulin resistance (Duffaut et al., 2009, Nishimura et al., 2009, Winer et al., 2009). In lean adipose tissue, $CD4^+$ $Foxp3^+$ T (Treg) cells and Th2-polarized $CD4^+$ T cells are predominant. Anti-inflammatory Treg cells regulate immune homeostasis, control self-

tolerance and suppress immune responses. Specifically, Tregs control the function of other T cell subpopulations but also of cells of the innate immune system, like macrophages (Peterson, 2012). Increased Treg cell percentage in visceral adipose tissue ameliorates insulin sensitivity, whereas depletion of Treg cells resulted in an increase in systemic insulin resistance (Feuerer et al., 2009a, Winer et al., 2009). Tregs modulate insulin sensitivity through a number of mechanisms, including reduction of adipose tissue M1 polarization, prevention of Th1 differentiation and increased glucose uptake into adipocytes (Deiuliis et al., 2011, Feuerer et al., 2009a). Treg cells are a heterogeneous population showing a high degree of phenotypic and functional specialization (Burzyn et al., 2013, Chaudhry and Rudensky, 2013). Recent data demonstrated that visceral adipose tissue Treg cells are specialized with high levels of PPARγ expression as compared with Treg cells from other tissues (Cipolletta et al., 2015, Cipolletta et al., 2012). Moreover, accumulation of adipose tissue Tregs depends on antigens presented by MHC class-II molecules and soluble mediators, notably IL-33 (Kolodin et al., 2015). Th2 cells express the transcription factor GATA-3 and secrete a unique pattern of cytokines, including IL-4, IL-5 and IL-13 (Sad et al., 1995, Salgame et al., 1991). IL-4 and IL-13 stimulate PPARγ and PPARδ activation and thereby induce M2 macrophages (Kang et al., 2008, Odegaard et al., 2007).

In the obese adipose tissue, there is a shift towards decreased levels of anti-inflammatory Treg cells and Th2 cells and an increased level of inflammatory $CD8^+$ and Th1-polarized $CD4^+$ T cells (Figure 4) (Lee and Lee, 2014). This shift occurs before the infiltration of inflammatory macrophages and might be a signal for the recruitment of the inflammatory, polarized M1 macrophages. Indeed, it has been demonstrated that Th2 cells, Treg cells, NKT cells, or eosinophils contribute to the M2 activation of macrophages by secreting IL-4 or IL-10. In contrast, obesity causes alteration of the constituent immune cells, in which Th1 cells, B cells, neutrophils, or mast cells induce M1 activation of macrophages by the elevated secretion of TNFα and IFNγ. Moreover, increased secretion of TNFα and free fatty acids from hypertrophic adipocytes also contribute to the M1 activation of macrophages (Tateya et al., 2013).

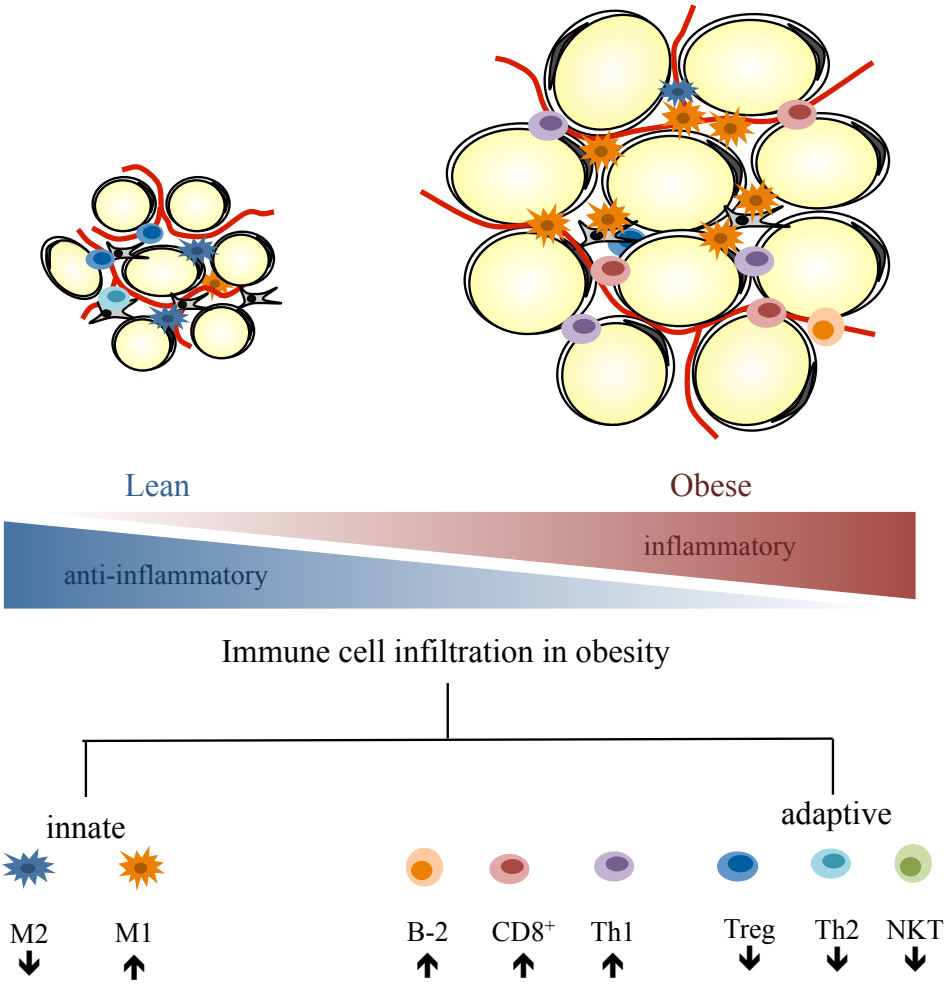

Figure 4: Adipose tissue leukocyte migration in the obese state. Obesity is associated with an increased infiltration of cells from both the innate and adaptive immune systems. In the lean state anti-inflammatory cells like M2-polarized macrophages (M2), type 2 helper T cells (TH2), regulatory T cells (Treg) and natural killer T cells (NKT) predominate. In the obese state a shift towards inflammatory immune cells, like M1-polarized macrophages, type 1 helper T cells (Th1), cytotoxic T cell ($CD8^+$), and B-2 cells is observed.

Nishimura et al. focused on the increase in $CD8^+$ adipose tissue T cells and found that within 2 weeks of high-fat diet, $CD8^+$ T cells infiltration increased in obese epididymal adipose tissue of mice. This infiltration preceded the accumulation of macrophages, and depletion of $CD8^+$ T cells lowered macrophage infiltration, adipose tissue inflammation and ameliorated systemic insulin resistance (Nishimura et al., 2009). $CD8^+$ T cells recognize peptide antigens loaded on MHC class-I molecules on antigen-presenting cells. Moreover, $CD8^+$ cells produce a variety of cytolytic substances but also secrete a number of cytokines that activate other immune cells (Lee and Lee, 2014). Other groups focused on $CD4^+$ T cells within adipose tissue (Kintscher et al., 2008, Winer et al., 2009). These cells recognize peptide antigens loaded on MHC class-II molecules of antigen-presenting cells. The Th1 lineage produces pro-inflammatory cytokines such as IFNγ. In obesity, the percentage and total number of Th1

cells is increased in adipose tissue of mice, and Th1 cell deficiency in mice improved insulin sensitivity (Winer et al., 2009). Similarly, weight gain in humans is also accompanied by an increase in Th1 cell numbers (Winer et al., 2009). Interestingly, the accumulation of Th1 cells in obese adipose tissue appears to be antigen driven, since rearrangements of the T cell receptor in adipose tissue T cells suggest that antigens in fat may communicate with the adaptive immune system (Winer et al., 2009, Yang et al., 2010). In mice, IFNγ production by T cells is significantly increased after six weeks of high-fat diet, indicating early T cell activation (Strissel et al., 2010). IFNγ induces both macrophage expression levels of pro-inflammatory effector cytokines and of MHC class-II molecules (Schroder et al., 2004). This in turn can promote macrophage antigen-presentation to $CD4^+$ T cells and induces Th1 cell polarization and proliferation in adipose tissue (Cho et al., 2014; Morris et al., 2013). Interestingly, it has been suggested that adipocytes can act as antigen presenting cells to T cells in adipose tissue inflammation; i.e. adipocytes expressing MHC class-I could mediate $CD8^+$ T cell responses, whereas those expressing MHC class-II molecules could regulate $CD4^+$ T cell responses (Huh et al., 2014).

However, the initial events leading to infiltration, timing and order of leukocyte infiltration in the obese state have not been firmly established. Two studies showed macrophages as the first cells recruited to adipose tissue, with six-fold increase in adipose tissue by eight weeks of high-fat diet, whereas the absolute T cell numbers ($CD3^+$, $CD4^+$ and $CD8^+$ T cells) did not show any significant changes (Strissel et al., 2010, Winer et al., 2009). In contrast, other studies demonstrated that $CD8^+$ T cells are the first cells recruited to adipose tissue, with a significant increase within two weeks of high-fat diet, while the proportion of total macrophages did not increase until six weeks of high-fat diet (Nishimura et al., 2009). Kintscher et al. also proposed that $CD4^+$ T cell infiltration might be the primary event before macrophage accumulation and can modify macrophage behavior (Kintscher et al., 2008). Finally, T helper 17 cells, γδ T cells, NKT cells and B cells have also been shown to be recruited into adipose tissue (Huh et al., 2014).

Thus, further data are needed to determine the precise order of leukocyte accumulation within obese adipose tissue. Another essential question is to decipher which signals lead to activation and migration of immune cells in this context. The molecular mechanism involved in leukocyte infiltration into adipose tissue is still poorly understood. Obesity provides an extracellular environment that is enriched with lipids, high glucose levels, pro-inflammatory cytokines and chemokines, which can contribute to activation of these processes. To further unravel the precise mechanisms underlying obesity-induced insulin resistance and to identify

potential new entry points for therapy development, it is necessary to identify the main responsible factors, their timing and location in order to understand the obesity-associated inflammation.

3.7 Chemokines and chemokine receptors in obesity

Chemokines are small proteins that have originally been shown to direct the movement of circulating leukocytes to sites of inflammation or injury by chemotaxis. In addition, they activate the production and secretion of inflammatory mediators (Charo and Ransohoff, 2006). Chemokines are a superfamily of about 50 low molecular weight chemotactic cytokines (8– 14 kDa) that bind to and signal through G-protein coupled heptahelical receptors that are differentially expressed on leukocyte subpopulations. Chemokines share homologous sequences and can be subdivided into four main classes (CC, CXC, CX3C and C) depending on the orientation of conserved cysteine residues in their amino acid sequences. Chemokine receptors are classified into different families (CCR, CXCR, CX3CR and CR) that correspond to the four distinct subfamilies of chemokines they bind. *In vitro* the chemokine system is characterized by a considerable redundancy, since most chemokines bind to several receptors and most chemokine receptors bind more than one chemokine (Charo and Ransohoff, 2006). *In vivo*, however, the blockade of single chemokines or receptors has been shown to reduce significantly the disease activity in numerous animal models of inflammation. Chemokine receptor expression on leukocytes is tightly regulated during cell differentiation and cytokine activation. The differentiation of helper T cells into a Th1 phenotype is associated with CXCR3 and CCR5 expression, while Th2 cells predominantly express CCR3, CCR4 and CCR8 (Sallusto et al., 1998). *In vitro* studies have shown a downregulation of CCR2 expression and an upregulation of CCR5 expression during monocyte differentiation into macrophages (Fantuzzi et al., 1999, Kaufmann et al., 2001).

Chemokines and their receptors came into the focus of diabetes research, since studies in mouse models and in humans have shown that obesity is associated with an enhanced infiltration of leukocytes into adipose tissue (Curat et al., 2006, Feuerer et al., 2009b, Kintscher et al., 2008, Weisberg et al., 2003, Wu et al., 2007). Moreover, in humans, an upregulation of CCR1, CCR2, CCR3 and CCR5 expression has been described in adipose tissue of obese patients (Huber et al., 2008). Therefore, chemokines and chemokine receptors responsible for leukocyte infiltration have to be analyzed in a comprehensive manner to better understand the regulation of the inflammatory process in adipose tissue of obese patients.

3.7.1 IP-10/CXCR3

One important chemokine for T cell migration is IP-10 / CXCL10. It was identified as a 10 kDa secreted protein induced by IFNγ and LPS in a variety of cell types, like endothelial cells, keratinocytes, fibroblasts, monocytes, neutrophils and epithelial cells (Luster and Ravetch, 1987, Ohmori and Hamilton, 1990). It is upregulated in a wide range of human inflammatory diseases, including atherosclerosis, multiple sclerosis, inflammatory bowel disease and others (Grimm and Doe, 1996, Mach et al., 1999, Sorensen et al., 1999). Through its chemotactic activity towards subsets of leukocytes preferentially activated Th1 lymphocytes, IP-10 regulates multiple aspects of inflammatory and immune responses primarily. Human serum levels range from 20-400 pg/ml, with higher levels found most commonly among individuals with chronic inflammatory conditions (Romagnani et al., 2004, Rothenbacher et al., 2006). IP-10 also enhances adherence of inflammatory cells to endothelial cells (Neville et al., 1997). Additionally, IP-10 has been demonstrated to inhibit proliferation of human endothelial cells (Luster et al., 1985) and bone marrow-derived haematopoietic progenitors (Sarris et al., 1993).

In contrast to most other chemoattractants that generally act on several receptors, IP-10 only signals via one specific chemokine receptor CXCR3. Among the CXCR3 ligands, IP-10 is unique in that its promoter has two functional NFκB binding sites, whereas the MIG and I-TAC promoters have none (Ohmori and Hamilton, 1993).

However, so far little was known about the role of IP-10 in adipose tissue leukocyte migration and its regulation in preadipocytes and adipocytes. Studies in other cell types showed that the IP-10 promoter is regulated in a complex way with interrelated roles of the transcription factor binding sites for NFκB and the family of interferon regulatory factor (IRF) proteins. Analysis of the IP-10 promoter revealed two κB sites and an additional IRF binding site as critical regulatory sequence elements (Ohmori and Hamilton, 1995, Ohmori et al., 1997). IFNγ-mediated induction of IP-10 is observed in most IP-10-expressing cell types, including macrophages and monocytes (Taub et al., 1993). However, in skin fibroblasts, TNFα and not IFNγ is the main inducer of IP-10 (Villagomez et al., 2004). Apart from this, in human intestinal epithelial cell lines, IL-1ß alone or in synergism with IFNγ regulates IP-10 by activating the NFκB pathway (Yeruva et al., 2008). Furthermore, NFκB is also necessary for the induction of IP-10 expression in LPS- plus IFNγ-induced neutrophiles, as well as in LPS and IFNγ-stimulated monocytes (Tamassia et al., 2007). Interestingly, increased secretion of IP-10 from monocytes under hyperglycemia is activated via the TLR2 and TLR4 pathway (Devaraj and Jialal, 2009). In addition, palmitic acid induces IP-10 expression in human

macrophages via NFκB activation (Laine et al., 2007). Thus, the regulation of IP-10 by immunological stimuli is highly cell-type-specific. Nevertheless, the molecular mechanism that regulates IP-10 in preadipocytes and adipocytes, has not been analyzed yet. A recent study could show that primary human mature adipocytes constitutively express and secrete IP-10, which is strongly upregulated by IFNγ (Herder et al., 2007). Serum concentrations of IP-10 have been found to correlate positively with BMI in adipose tissue and other parameters of obese, in some but not all studies (Herder et al., 2007). In addition, elevated concentrations of IP-10 are associated with incidence of type 2 diabetes (Herder et al., 2006a). Thus, it may be hypothesized that IP-10 is also involved in the chronic inflammatory process observed in obesity and type 2 diabetes. Knowledge of its regulation in preadipocytes/adipocytes and its pathophysiological role in obese adipose tissue may therefore also give insight into the progression of low-grade inflammation in obesity.

The IP-10 receptor CXCR3 is not only important in lymphocyte recruitment to inflammatory sites but is also involved in T cell activation (Taub, 1996). CXCR3 is expressed on several immune cell types, like NK cells, plasmacytoid and myeloid dendritic cells, B cells, and, especially, activated T cells. Sallusto et al. demonstrated that CXCR3 is preferentially expressed by Th1-activated T cells (Sallusto et al., 1998). Likewise, blockade or depletion of CXCR3 severely attenuates recruitment of Th1 cells to the sites of inflammation (Xie et al., 2003). CXCR3 has been implicated in diseases like atherosclerosis (Mach et al., 1999), multiple sclerosis (Sorensen et al., 1999), type 1 diabetes (Frigerio et al., 2002) and others. A prominent role for CXCR3-mediated migration of inflammatory cells in atherosclerosis has been reported. Targeted deletion of CXCR3 in Apolipoprotein E (ApoE)-deficient mice resulted in decreased lesion formation in the abdominal aorta (Veillard et al., 2005). Moreover, recent studies demonstrated that an antagonist of CXCR3 could attenuate atherosclerotic lesion formation by blocking direct migration of $CXCR3^+$ effector cells from the circulation into the atherosclerotic plaques (van Wanrooij et al., 2008).

Thus, the CXCR3/CXCL10 axis is pivotal in dysbalancing T cell responses during atherogenesis. An abnormal expression of CXCR3 may also lead to a change in the lymphocytes recruited to the obese adipose tissue and disturb the balance between Th1 and Th2 cells.

3.7.2 MCP-1/CCR2

MCP-1 and its receptor CCR2 were found to be crucial for monocyte/macrophage recruitment in several inflammatory models (Ajuebor et al., 1998). MCP-1 is even thought to be the main

chemokine responsible for recruiting monocytes/macrophages (Palframan et al., 2001). In addition, it has been shown to be a potential intervention point for the treatment of a variety of diseases, including multiple sclerosis (Sorensen et al., 2004), rheumatoid arthritis (Hayashida et al., 2001) and atherosclerosis (Kusano et al., 2004).

The chemokine receptor CCR2 is expressed abundantly on the so-called "inflammatory" subset of blood monocyte and on other immune cell types, such as dendritic cells and memory Th1 cells (Sozzani et al., 1997). CCR2 binds multiple ligands, including MCP-1, MCP-2, MCP-3 and MCP-4. Of these ligands, MCP-1 is studied most extensively, and CCR2 is considered to be the exclusive receptor for MCP-1 (Gerard and Rollins, 2001).

There is considerable evidence for the pathophysiologic role of MCP-1/CCR2 in the obese adipose tissue. MCP-1 was found to be remarkably increased in adipose tissue and plasma in obesity (Sartipy and Loskutoff, 2003, Takahashi et al., 2003). Moreover, studies in MCP-1-deficient mice and mice overexpressing MCP-1 support a role of this chemokine in attracting macrophages to the adipose tissue and affecting insulin sensitivity in obesity (Kamei et al., 2006, Kanda et al., 2006). Genetic deficiency of the MCP-1 receptor CCR2 and pharmacological antagonisms of CCR2 reduce macrophage accumulation in adipose tissue of high-fat diet-fed obese C57BL⁄ 6J mice and partially protect against the development of obesity-induced inflammation, hepatic steatosis and insulin resistance (Kamei et al., 2006, Kanda et al., 2006, Weisberg et al., 2003). Another study found that recruited adipose tissue macrophages in obese animals show an increase in CCR2 expression compared to lean mice (Lumeng et al., 2007). Furthermore, CCR2 in bone marrow cells plays an important role in the recruitment of macrophages into obese adipose tissue (Ito et al., 2008). In addition, increased expression levels of monocyte CCR2 and MCP-1 can be seen in patients with type 2 diabetes (Mine et al., 2006). However, as not all studies agree on the roles of CCR2 and MCP-1 in macrophage recruitment, this issue remains to be fully resolved (Inouye et al., 2007, Kirk et al., 2008). Moreover, it is still unknown through what cell type CCR2 exerts its effect.

3.7.3 RANTES/CCR5/CCR3

RANTES (CCL5) is a ligand for the receptors CCR1, CCR3 and CCR5. Its expression by fibroblasts, platelets and monocytes/macrophages is a particular feature of inflammatory disorders such as atherosclerosis (Sjoberg et al., 2009). Interestingly, circulating levels of RANTES are associated with impaired glucose tolerance and type 2 diabetes (Herder et al., 2005). RANTES is also increased in white adipose tissue in the setting of murine and human

obesity (Wu et al., 2007). In addition, RANTES mRNA was found to be expressed in both adipocytes and SVF, with higher levels in SVF (Wu et al., 2007).

The increased expression of RANTES in white adipose tissue associated with obesity is thought to play an important role in recruiting monocytes to adipose tissue. In obese subjects, adipose tissue expression of RANTES and its receptors correlated positively with expression of CD68, a macrophage-specific marker (Huber et al., 2008). RANTES mRNA expression also positively correlated with the inflammatory macrophage marker CD11b (Keophiphath et al., 2009). In addition, monoclonal antibodies directed against RANTES reduced T cell chemotaxis induced by media conditioned by adipose tissue isolated from obese male mice (Wu et al., 2007).

The RANTES receptor CCR5 is expressed predominantly on macrophages differentiated from blood monocytes and Th1 cells activated in response to inflammatory stimuli. Multiple ligands beside RANTES, including MIP-1β, MIP-1α, MCP-2 and MIP-1α/LD78β, bind to CCR5. Of these ligands, MIP-1β and MIP-1α/ LD78β are considered to be selective to CCR5, yet RANTES is the most extensively studied chemokine to date (Charo and Ransohoff, 2006). As compared to CCR2, the *in vivo* function of CCR5 is less well defined. CCR5 has been shown to contribute to the survival of macrophages during inflammation and infection (Tyner et al., 2005). It may function to retain tissue macrophages in inflamed tissue and is important for both Th1 cell recruitment and activation in inflammation (Charo and Ransohoff, 2006, Katschke et al., 2001).

Another receptor for RANTES, CCR3, also binds and responds to the eotaxin, eotaxin-3, MCP-3 and MCP-4 chemokines. It is highly expressed in eosinophils and basophils, and is also detected in Th1 and Th2 cells, as well as in airway epithelial cells. CCR3 is also widely expressed in other pro-inflammatory cells like basophils, mast cells and Th2 lymphocytes (Charo and Ransohoff, 2006).

Interestingly, both RANTES receptors CCR5 and CCR3 have been reported to be induced in adipose tissue of obese mice by cells of the SVF as well as by adipocytes. The most striking increase was observed for CCR5. CCR3 was modestly increased, whereas expression levels of the third RANTES receptor CCR1 was not changed (Wu et al., 2007). More recent data suggest a critical role of CCR5 in obesity-induced adipose tissue inflammation and insulin resistance by regulating both macrophage recruitment and M1/M2 status (Kitade et al., 2012).

Overall, current data suggest an important role of the here-introduced chemokines and their receptors for the enhanced leukocyte infiltration into obese adipose tissue. However, the exact

mechanisms are still not well understood. It is under current discussion how the low-grade adipose tissue inflammation is initiated and maintained. One challenge in understanding this process will be to identify the pathways and mediators that initiate and maintain the communication between metabolic and immune cells. The regulated expression of chemokines and their receptors is a critical determinant for migration within specific tissues and thereby contributes to the control of both tissue and inflammation-specific immune processes in many pro-inflammatory diseases.

3.8 PBMCs

PBMCs are a critical component of the immune system, playing an integral role in the body's defense mechanisms. Lymphocytes are the largest subpopulation within PBMCs, playing an important role in the adaptive immune response, and monocytes are important cells of the innate immune response. The adaptive immune system recognizes and remembers specific pathogens, which results in stronger attacks each time the pathogen is encountered. The innate immune system provides immediate defense against infections and functions in a non-specific manner (Murphy, 2007).

3.8.1 PBMCs, inflammation and insulin resistance

PBMCs, monocytes and T cells, in particular, are not directly involved in insulin-regulated glucose metabolism but are pivotal players in inflammatory responses. Adipose tissue macrophages and adipose tissue T lymphocytes are derived from bone marrow precursors that migrate from the peripheral circulation into adipose tissue (Weisberg et al., 2003). Therefore, the composition of PBMC populations and their specific activation state may also contribute to regulation of the obesity-induced pro-inflammatory activity of adipose tissue itself.

In fact, numerous evidence indicates that obesity and insulin resistance are associated with activation of these cells. An increased NFκB activity in mononuclear cells has been shown in patients with type 2 diabetes (Hofmann et al., 1998) as well as in obese individuals (Ghanim et al., 2004). These cells are also characterized by increased secretion of pro-inflammatory cytokines. This pro-inflammatory state of PBMCs in obesity is also in part due to excessive macronutrient intake (Mohanty et al., 2000). Additionally, a weight loss intervention in individuals with the metabolic syndrome was shown to result in a decreased expression of inflammatory genes in PBMCs. This altered expression was associated with an increase in systemic insulin sensitivity (Crujeiras, 2008). Remarkably, the expression of RANTES and ICAM-1, both involved in leukocyte migration into inflamed tissue, were decreased. Furthermore, insulin infusions, which have an anti-inflammatory effect on PBMCs, suppress

the NFκB-binding activity and increase IκB protein levels in PBMCs from obese subjects (Dandona et al., 2001). PBMCs are also target cells for leptin (Fernandez-Riejos et al., 2010), adiponectin (Lovren et al., 2010) and PPARγ (Macias-Gonzalez et al., 2008), all relevant factors in adipose tissue biology. These findings support the idea that the paracrine/endocrine regulatory mechanisms observed in adipose tissue may work in a similar manner in PBMCs and raise the possibility that PBMCs may contribute to the inflammatory state in obesity. Indeed, leukocyte count is positively related to BMI, fat mass percentage and abdominal adiposity (Kim and Park, 2008).

Arkan et al. found that mice lacking IKK2 in hepatocytes retain liver insulin responsiveness but develop insulin resistance in muscle and fat in response to high-fat diet. In contrast, IKK2 deletion in myeloid cells retains global insulin sensitivity and protects against systemic insulin resistance in mice under high-fat diet. Thus, IKK2 acts locally in liver and systemically in myeloid cells, where NFκB activation induces inflammatory mediators that cause insulin resistance (Arkan et al., 2005). Besides the NFκB/IKK2 signaling pathway, the JNK pathway in immune cells has also been proposed to link inflammation and insulin resistance (Hirosumi et al., 2002). In haematopoietically derived cells, a JNK deletion confers protection against high-fat diet-induced insulin resistance by decreasing obesity-associated inflammation and production of cytokines that induce insulin resistance in insulin target cells in mice (Solinas et al., 2007). These findings were supplemented by the results of a human trial with healthy, non-diabetic individuals. JNK activity in adipose tissue and elevated NFκB activity in PBMCs were discovered to be important determinants of insulin resistance. Moreover, JNK activity in contrast to NFκB activity in PBMCs was shown to be an independent determinant of insulin resistance indicating that these pathways are co-dependent (Sourris et al., 2009). Together, these findings suggested that PBMCs crucially participate in the regulation of systemic, metabolic homeostasis. In conclusion, PBMCs might reflect metabolic and immune responses of adipocytes or hepatocytes and probably also their communication with other sites, such as pancreatic islets and skeletal muscle. These findings suggested that PBMCs crucially participate in the regulation of systemic, metabolic homeostasis. Therefore, PBMCs may provide an easy and representative view of the overall inflammatory status of the body in terms of activation of pro-inflammatory transcription factors, migratory ability and correlation with systemic markers of inflammation in circulation.

4. Aim of the work

Obesity is characterized as a state of chronic low-grade inflammation with elevated plasma levels of pro-inflammatory adipokines. Chemokines came into the focus of diabetes research, since studies in mouse models and in humans have shown that obesity is associated with an enhanced infiltration of macrophages and T cells, in particular Th1-polarized T cells, into adipose tissue. Recent research revealed a functional link between leukocyte infiltration and obesity-associated insulin resistance. However, the molecular mechanisms involved in this process are still poorly understood. NFκB is a major regulator of inflammatory processes with an important function in the pathogenesis of obesity-related insulin resistance; its specific function for the accumulation of immune cells in adipose tissue remains elusive. Moreover, there is growing evidence for the involvement of the chemokine IP-10 in the chronic inflammatory status observed in obesity and type 2 diabetes. Nevertheless, its regulation in preadipocytes/adipocytes and chemoattractant properties for T cells in the adipose tissue remain to be answered. Therefore, the first aim of this work was to investigate whether NFκB is involved in the regulation of IP-10 in preadipocytes and adipocytes and to characterize the potential role of NFκB-activity and IP-10 for T cell infiltration into adipose tissue.

Moreover, in humans, an upregulation of CCR1, CCR2, CCR3 and CCR5 expression has been described in adipose tissue of obese subjects, indicating that the enhanced migration of leukocytes into adipose tissue of obese may not be exclusively triggered by the elevated production of adipose tissue chemokines. Notably, it remains unclear whether chemokine receptor upregulation is restricted to adipose tissue or can be observed also in peripheral blood leukocytes. Recent data indicate that obesity and insulin resistance are also associated with activation of immune cells in the periphery. PBMCs might respond to intra- and extracellular signals in various ways, including a change of leukocyte subsets as well as an increased activation and infiltration of leukocytes into target tissues. Therefore, the second aim of this study was to compare PBMCs from obese and normal-weight women with regard to composition of immune cell subpopulations, surface expression of the chemokine receptors CCR2, CCR3, CCR5 and CXCR3 and cell-intrinsic migration capacity.

5. Material and Methods

5.1 Regulation of IP-10 in preadipocytes and adipocytes and its pathophysiological role in the obese adipose tissue

5.1.1 Subjects

For the isolation of preadipocytes, adipocytes and SVF, human subcutaneous adipose tissue was obtained from healthy women and men undergoing elective abdominal surgery; no selection was made for BMI, age or gender. Patients with diabetes, cancer, serious diseases, acute infection, malignancies or any other consuming disease were excluded. Informed consent was obtained from all subjects. The study was approved by the ethical committee of the Technische Universität München, Germany.

5.1.2 Mice

For all experiments, male C57BL/6J mice were housed in a temperature controlled (25°C) facility with a 12h light/dark cycle. Mice were fed a standard chow D12450B (% kcal: protein 20%, carbohydrate 70%, fat 10%) or a high-fat diet D12492 (% kcal: protein 20%, carbohydrate 20%, fat 60%) from Sniff (Soest, Germany). Experimental feeding was started at the age of 10 weeks for then 16 weeks (Figure 5) or for 10 weeks (Figure 6a). Beside these experiments, cDNA was obtained from mouse experiments published earlier (Kintscher et al., 2008) for additional analysis of gene expression. Male C57BL/6J mice were fed a high-fat diet for 10 weeks vs. a control diet, and adipose tissue samples were collected after 0, 5 and 10 weeks. All animal procedures were in accordance with institutional guidelines, with national law and were approved.

5.1.3 Cell culture

Freshly isolated primary mature adipocytes and preadipocytes/SVF (stromal vascular fraction) were isolated and cultured as previously described (Herder et al., 2006b). Isolation of SVF and adipocytes from murine gonadal fat tissue was performed according to the protocol published by Weisberg et al. (Weisberg et al., 2006). 3T3-L1 cells (Xantos Biomedicine, Munich, Germany) and the retroviral packaging cell line Phoenix-Eco provided by B. Baumann (University of Ulm, Germany) were cultured in basal medium, containing DMEM (Invitrogen, Karlsruhe, Germany), 10% FCS (Invitrogen) and 50 µg/ml gentamycin (Roth, Karlsruhe, Germany) at 37°C, 5% CO_2. Differentiation of 3T3-L1 cells was performed as described (Laumen et al., 2009). Cells were incubated in the presence or absence of the

following agents: IL-1β (R&D, Wiesbaden, Germany), LPS (Sigma-Aldrich, Munich, Germany), Bay117082 (NFκB-inhibitor, Biomol, Hamburg, Germany), goat anti-IP-10 antibody (R&D) or the respective control goat IgG (R&D). All agents were not cytotoxic at the concentrations used and as demonstrated in a cell viability assay (data not shown).

5.1.4 Transfection of cells

3T3-L1 cells were transfected on various days of differentiation using Lipofectamine 2000 transfection reagent (Invitrogen). 1.5μg of DNA and 3μl of reagent were mixed and added to the cells for 4h. 24h after transfection, luciferase activity was measured using the dual-luciferase assay (Promega, Mannheim, Germany). In all transfections, 0.3 μg of ubiquitin-promoter renilla-luciferase vector was co-transfected to normalize for transfection efficiency. Ubiquitin-promoter renilla-luciferase vector and 6xNFκB-luciferase vector were provided by B. Baumann (University of Ulm, Germany) and IP-10 promoter-luciferase vector by D. Haller (Technische Universität München, Germany).

5.1.5 Retroviral Infection of 3T3-L1

To inhibit NFκB in 3T3-L1 cells a dominant interfering IκBα mutant protein (IκBα-mut, serine 32/36 mutated to alanine, resulting in a nondegradable repressor) or an empty vector as control (mock) were stably integrated into 3T3-L1 cells using retroviral gene transfer. For virus production, the pCFG5-IκBα-mut retroviral vector (provided by B. Baumann, University of Ulm) was transfected using calcium-phosphate transfection into ecotrophic Phoenix virus-producer cell line. Supernatant containing the retrovirus was collected 48h and 72h after transfection and used to infect 3T3-L1 preadipocytes in the presence of 8 μg/ml dietylaminoethyl. 24h after a second infection, the selection with zeocin was started for five days until all cells were 100% positive for green fluorescent protein.

5.1.6 ELISA

3T3-L1 cells were stimulated with IL-1β or LPS for 20h. The supernatant was immediately frozen at -80°C. IP-10 protein level was determined by a mouse-specific DuoSetELISA assay, according to the manufacturer's instructions (R&D).

5.1.7 Quantitative RT-PCR

Total RNA was isolated using NucleoSpinRNAII-kit (Macherey-Nagel, Düren, Germany) and reverse transcribed using cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). PCR for detection of human/mouse IP-10, CXCR3 (chemokine CXC motif

receptor 3), GAPDH (glyceraldehyde-3-phosphate-dehydrogenase) and mouse adiponectin were performed using qPCR-SYBR-Green (ABgene, Epsom, GB) or for primary mouse adipose tissue using the universal probe library (Roche, Germany). Primers ordered from MWG (Ebersberg, Germany) were designed using PrimerExpress-Software (Applied Biosystems); the sequences are given in Table 1. Quantification of gene regulation was performed by the ΔΔCt method; results were corrected for GAPDH expression (Gorzelniak et al., 2001).

Primer sequences for SYBR-Green PCR			
mIP-10	forward	5´GCCCAGTGACCTTGAGGAG	
	reverse	5'ACGTGACTGCTGAGGAAGC	
mAdiponectin	forward	5'CTACGACCAGTATCAGGA	
	reverse	5'GAAAGCCAGTAAATGTAGAG	
mGAPDH	forward	5'CCTGGAGAAACCTGCCAAGTATG	
	reverse	5'GAGTGGGAGTTGCTGTTGAGTC	
hIP-10	forward	5'AGAGGAACCTCCAGTCTCAGC	
	reverse	5'CCTCTGTGTGGTCCA TCCTT	
hGAPDH	forward	5´ACCACAGTCCA TGCCA TCAC	
	reverse	5´TCCAC-CACCCTGTTGCTGTA	
Primer sequences for universal-probe library PCR			UPL-probe
mIP-10	forward	5´GCTGCCGTCATTTTCTGC	probe 3
	reverse	5´TCTCACTGGCCCGTCATC	
mCXCR3	forward	5'GCAGCACGAGACCTGACC	probe 5
	reverse	5'GGCATCTAGCACTTGACGTTC	
mGAPDH	forward	5'TCCACTCATGGCAAATTCAA	probe 8
	reverse	5'TTTGATGTTAGTGGGGTCTCG	

Table 1: Primer Sequences

5.1.8 Western blot and EMSA

For western blot analysis, 50 µg of total protein extracts were separated on 12.5% polyacrylamid gels and transferred onto polyvinylidenfluorid-membranes (Millipore, Schwalbach Ts., Germany). Membranes were blocked (PBS, 0.1% Tween, 2% Amersham blocking reagent), stained with anti-IκBα (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) followed by incubation with a horseradish-secondary antibody (Dianova, Hamburg, Germany) and visualized by enhanced chemiluminescence ECL (Amersham Bioscience, Freiburg, Germany). As loading control, stripped membrane was incubated with anti-β-actin (Santa Cruz Biotechnology). EMSA was performed as previously described (Brunner et al., 2007).

5.1.9 Migration assay

For the analysis of the ability of splenocytes to migrate towards a chemokine gradient established by adipocytes, 3T3-L1 cells were differentiated as described. Adipocytes were stimulated as indicated in DMEM medium containing 2% heat-inactivated FCS. After 18h the supernatant was taken and filled into the bottom chambers of Costar Transwells (Costar, Cambridge, MA, USA). Lympholyte-M (Cedarlane Labs, Hornby, ON, Canada) purified splenocytes (106 per sample in DMEM medium containing 2% FCS) were assayed for transmigration with 5-µm-pore-size Costar Transwell culture inserts. The migration occurred at 37°C in a humidified atmosphere with 5% CO2 over 3h. After incubation, cells migrated to the lower chamber were harvested, stained with anti-CD8-FITC (eBioscience, Frankfurt, Germany) and anti-CD4-PerCP (Peridinin Chlorophyll Complex Protein) (BD Biosciences, Heidelberg, Germany), counted by Becton Dickinson FACS-LSRII (inhibitory antibody experiments) or Beckton Dickinson FACS-CantoII (all other experiments) and analyzed by FACS-Diva software 6.1.

5.1.10 Oil red O staining

On day 10 of differentiation, cells were washed twice with PBS and fixed with 3.7% formaldehyde incubated for 1h. Formaldehyde was removed and Oil Red O solution (Sigma-Aldrich) (0.3% Oil Red O, 60% Isopropanol) was added to the plate and incubated for 1h. The solution was discarded, and stained cells were washed twice with PBS. Cells were visualized with an inverted phase contrast fluorescence microscope (Zeiss, Göttingen, Germany), and pictures were taken with a digital camera (Leica, Bensheim, Germany).

5.1.11 GPDH enzyme activity

Measurement of GPDH enzyme activity as a marker of adipogenic differentiation was performed as described in a previous work (Van Harmelen et al., 2004). For the measurement of the protein content, RC DC protein assay was used according to the manufacturer's instructions (Biorad, Hercules, Canada).

5.1.12 LDH enzyme activity

LDH enzyme activity in cell culture supernatants was measured for evaluation of cell viability. It was performed as described in a previous work (Vassault, 2005).

5.1.13 Statistical analysis

Values for all experiments are expressed as means ± SD or ± SE of minimum three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey's test, students t-test or one-sample t-test, *$p<0.05$, **$p<0.01$.

5.2 Cell-intrinsic migration capacity and surface chemokine receptor expression in PBMCs from lean versus obese women: results from the AdipoRiSc (Adiposity Risk Screening) study

5.2.1 Participants

Female subjects were recruited between October 2008 and July 2009 as part of the AdipoRiSc case-control study. Written informed consent was obtained prior to the start of the study. Recruitment, clinical phenotyping and biosample collection were performed at the study unit of the Else Kroener-Fresenius Centre for Nutritional Medicine (Technische Universität München). Two groups of women were classified by body mass index (BMI) as lean (≥ 18.5 to ≤ 24.9 kg/m^2) and obese (≥ 30.0 kg/m^2). Blood samples of 29 obese and 19 lean women were investigated by migration assay analysis. For a sub-cohort of 14 obese and 16 lean women, we additionally performed FACS (fluorescence-activated cell sorting) analysis to determine chemokine receptor levels on PBMCs. Statistical analysis was performed for both sub-cohorts separately. Please note that for two obese subjects in the FACS cohort no migration data were available and that the RANTES-induced migration assay could only be performed for 8 lean and 12 obese subjects. From these, 4 lean and 8 obese women had enough PBMCs to also allow for FACS analyses.

The weight of the included subjects had been stable for at least 3 months (variation of less than ± 3 kg). Subjects with any evidence of inflammatory or metabolic disease including type 2 diabetes mellitus (assessed by oral glucose tolerance test, data not shown) or other chronic diseases and cancer were excluded. Acute infectious and inflammatory diseases were ruled out by analyzing the leukocyte counts (< 4 and > 10 x 10^3/µl) and by history and thorough clinical examination. Further exclusion criteria were smoking, medication with metabolic adverse effects, pregnancy, lactation and chronic alcohol consumption. This study was approved by the Ethics Committee of the Technische Universität München.

5.2.2 Anthropometric, clinical and plasma chemokine measurements

Weight was determined using an electronic scale (BC 418 segmental body composition analyzer, Tanita, München, Germany). Body circumference assessments were carried out according to WHO criteria. Total body fat mass was determined by dual-energy X-ray absorptiometry (DXA) (Hologic Explorer S/N 90417, QDR-Series, Hologic Inc.; software version 12.4). Venous blood samples were collected in the fasting state (overnight fast of at least 12 hours) for determination of routine biochemical parameters (Laboratory Becker, Olgemöller & Colleagues, Munich, Germany). All subjects underwent a standardized two

hour oral glucose tolerance test (OGTT) after an overnight fast to test for insulin resistance and diabetes mellitus (Krug et al., 2012). Insulin resistance was estimated using the homeostatic model assessment of insulin resistance (HOMA-IR) calculated as insulin (mg/dl) x glucose (mg/dl) / 405 (Matthews, 1985).

5.2.3 Flow cytometric analysis

PBMCs were isolated from whole venous blood using BD Vacutainer CPTs (Becton Dickinson, Heidelberg, Germany) containing sodium heparin according to the manufacturer's instructions and suspended in RPMI 1640 (Gibco, Darmstadt, Germany), 0.5% heat-inactivated FCS and 1000 mg/l glucose. FACS analysis was performed similar to a previously described protocol (Harner et al., 2011). The percentages of chemokine-receptor positive cells were determined within total lymphocytes or monocytes after defining a cut-off value according to the isotype control by using anti-CD3-fluorescein isothiocyanate (FITC) mouse IgG1, κ isotype, anti-CD14-APC-H7 mouse IgG2b, κ isotype, anti-CD14-Peridinin-chlorophyll-protein (PerCP) mouse IgG2b, κ isotype, anti-CD16-phycoerythrine (PE) mouse IgG2b, κ isotype, anti-CD16-PE-Cy7 mouse IgG1, κ isotype, anti-CCR2- Alexa Fluor 647 (A647) mouse IgG2b, κ isotype, anti-CCR3-A647 mouse IgG2b, κ isotype, anti-CCR5-PE mouse IgG2a, κ isotype and anti-CXCR3-PE mouse IgG1, κ isotype (all antibodies were from BD Bioscience, Heidelberg, Germany). Cells were incubated with antibodies for 25 minutes at 4°C, washed two times, and analyzed by flow cytometry (FACS Canto with FACS DIVA software, Version 5.0.3, BD Bioscience, Heidelberg, Germany). Results are expressed as percentages of stained cells and median fluorescence intensity (MFI) representing the receptor surface density.

5.2.4 Chemotactic assay

Monocyte chemotaxis was evaluated using 24-well transwell plates (Costar, Bodenheim, Germany) with polycarbonate membranes of 6.5 mm diameter and a pore size of 5 µm. Chemoattractants (100 ng/ml MCP-1, 100 ng/ml IP-10, 1 ng/ml RANTES and a "chemokine-mix" consisting of 50 ng/ml MCP-1, 50 ng/ml IP-10, 0.5 ng/ml RANTES, Pepro Tech, Hamburg, Germany; the chemokine concentrations sufficient to induce a maximal migration response were determined by assessing the dose-response for each chemokine) were diluted in 600 µl assay medium (RPMI 1640, 0.5% heat-inactivated FCS, 1000 mg/l glucose) and were placed in the lower wells of the chemotaxis chamber. 100 µl aliquots of PBMC suspension containing 4×10^6 monocytes/ml were placed in the upper wells. Migration was quantified by counting monocytes in the lower chamber after 150 min at 37 °C and 5% CO_2. Results are

expressed as chemotactic index (fold increase of monocyte response to stimulants over the response to medium alone, which was set as one).

5.2.5 Statistical analysis

Statistical analyses were performed using SPSS version 20.0 (IBM, Ehningen, Germany). Comparisons between groups were performed using t-test or Wilcoxon signed-rank test as indicated. Correlations between variables were expressed as Pearson's correlation coefficients. A p-value < 0.05 was considered as statistically significant.

6. Results

6.1 Regulation of IP-10 expression in preadipocytes and adipocytes and its pathophysiological role in the obese adipose tissue

The results of this investigation and therewith parts of this thesis were published by Krinninger et al. (Krinninger et al., 2011).

6.1.1 IP-10 mRNA expression levels in white adipose tissue of C57BL/6J mice

In order to investigate IP-10 expression in obesity, a first set of experiments with male mice fed a high-fat diet for 16 weeks was performed.

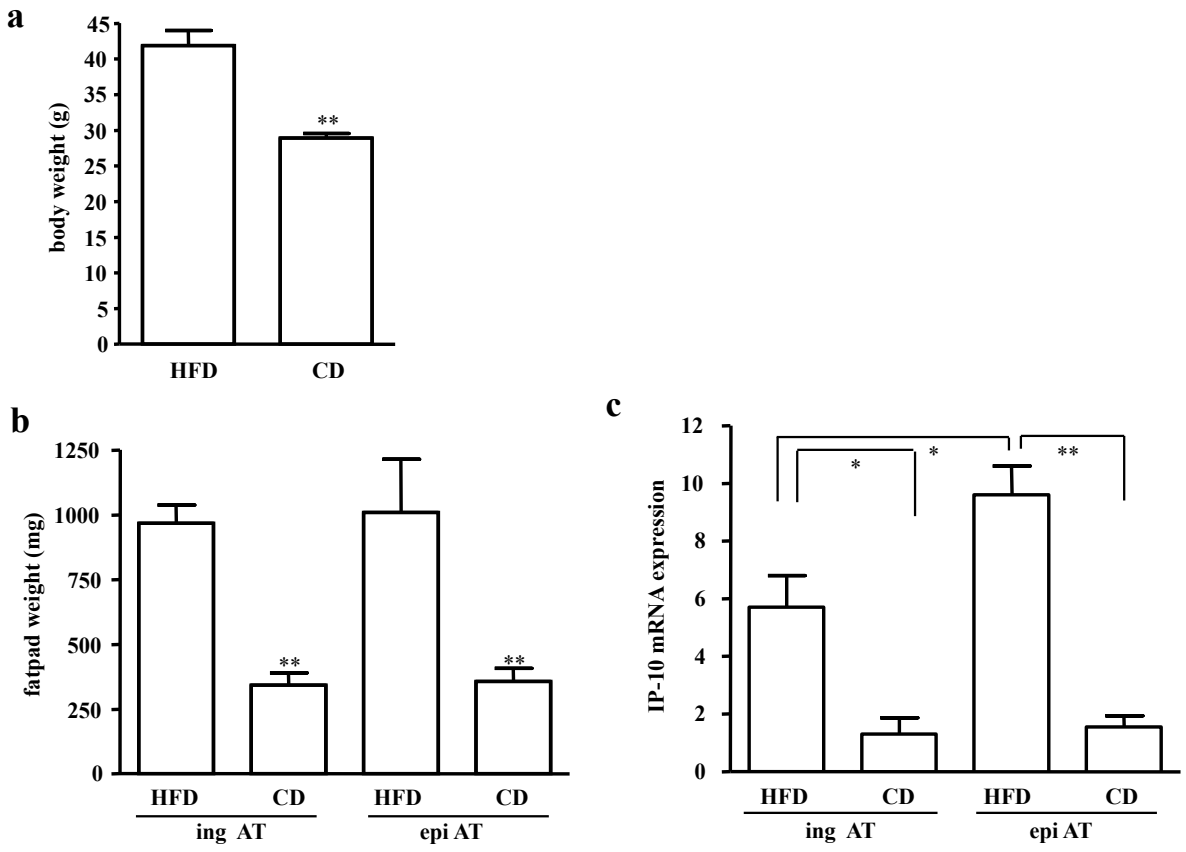

Figure 5: Total weight gain (a) and fat pad weight (b) of HFD-fed mice in epididymal and inguinal adipose tissue and IP-10 expression in mouse adipose tissue (c). Four C75BL/6 mice were fed a standard chow (CD) (sniff D12450B) or a HFD (sniff D12492). Experimental feeding was started at age of 10 weeks for 16 weeks. Total body weight (a) and inguinal (ing) / epididymal (epi) fat pad weight (b) were analyzed. (c) Total mRNA was isolated from inguinal (ing) and epididymal (epi) adipose tissue of male C75BL/6 mice fed a standard chow (CD) compared with mice fed a HFD. Total mRNA was reverse transcribed; IP-10 and GAPDH mRNA expression was determined by qRT-PCR. Results are means (a,b) ±SD (n = 4) and (c) ±SE (n ≥ 3). *P<0.05, **p<0.01. Figures from Krinninger et al. (Krinninger et al., 2011).

These mice showed a significant increase in body weight (Figure 5a) and inguinal/epididymal fat pad weight (Figure 5b). Real-time PCR analysis revealed a 5.7- and 9.6-fold increase in IP-10 mRNA expression in inguinal (subcutaneous) and epididymal (visceral) adipose tissue, respectively, in mice fed the high-fat diet, compared with control mice fed a standard chow (Figure 5c). Moreover, the increase of IP-10 expression levels was significantly higher in epididymal as compared to inguinal adipose tissue.

6.1.2 IP-10 mRNA expression levels in different mouse and human adipose tissue cell types

Adipose tissue is composed of adipocytes and the SVF, including preadipocytes but also macrophages and T cells. To further elucidate the origin of adipose tissue IP-10, both SVF and adipocytes from lean mice as well as from high-fat diet-fed mice were isolated. In lean mice, SVF expressed 2.5-fold higher levels of IP-10 mRNA ($p<0.05$) than adipocytes, whereas in overweight mice (HFD-fed) no difference was detectable due to the increase of IP-10 expression in adipocytes (Figure 6a). Hence, upon weight gain, adipocytes are the cell type that contributes to the higher IP-10 mRNA expression in adipose tissue. Similar to overweight mice, in samples derived from overweight human subjects, a comparable expression level of IP-10 in isolated SVF and adipocytes was observed (Figure 6b).

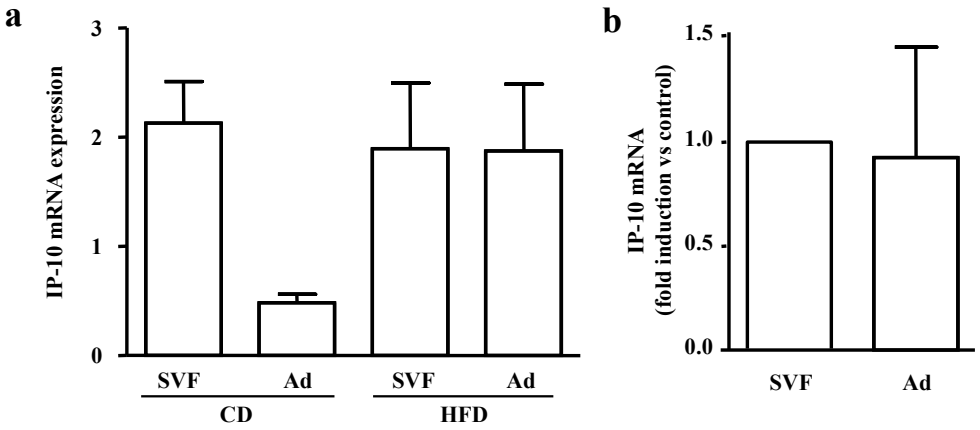

Figure 6: IP-10 expression in mouse and human SVF cells and adipocytes. Total mRNA was isolated from primary SVF (stromal vascular fraction) and adipocytes (Ad), freshly isolated from adipose tissue of six male mice per group either fed a HFD or control mice fed a standard chow diet (CD) for 10 weeks (a) and from adipose tissue of four overweight or obese persons (BMI ranging from 27-36) (b); mRNA was reverse transcribed; IP-10 and GAPDH mRNA expression was determined by qRT-PCR. (a) Expression level of one sample CD/ingAT (a) and of one sample CD/SVF (b) was set to one. Results are means ±SE (a) or ±SD (b). Figures from Krinninger et al. (Krinninger et al., 2011).

6.1.3 IP-10 mRNA expression in primary human and 3T3-L1 adipocytes versus pre-adipocytes

Next, the cellular origin of IP-10 synthesis in adipose tissue was investigated in more detail. Human subcutaneous adipose tissue samples were used for the isolation of preadipocytes and adipocytes. mRNA expression of IP-10 was considerably higher in adipocytes as compared with undifferentiated preadipocytes (Figure 7a). The same results were obtained using mouse 3T3-L1 adipocytes compared with undifferentiated preadipocytes (Figure 7b). On average, adipocytes expressed 8-fold more IP-10 mRNA than preadipocytes in both models.

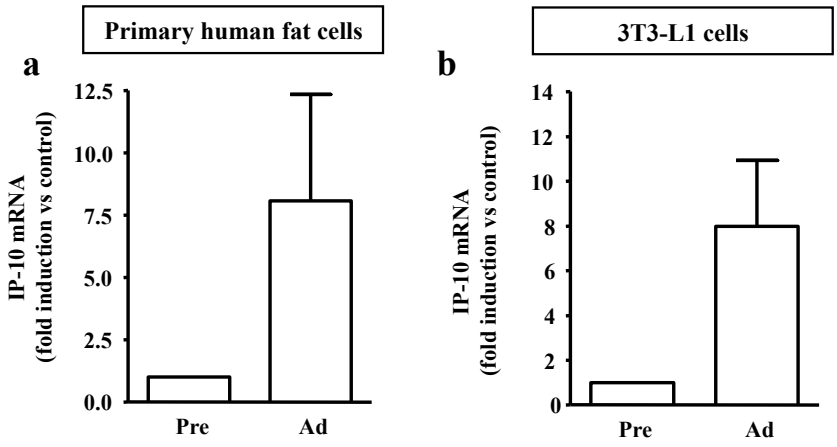

Figure 7: Total mRNA was isolated from freshly isolated primary human preadipocytes (Pre) and mature human adipocytes (Ad) of adipose tissue from subjects (a) and from 3T3-L1 preadipocytes and adipocytes (n=3) (b). mRNA was reverse transcribed; IP-10 and GAPDH mRNA expression was determined by qRT-PCR. Results are means ±SE. Figures from Krinninger et al. (Krinninger et al., 2011).

6.1.4 IL-1β and LPS induce IP-10 protein secretion in 3T3-L1 cells

Next, it was asked whether IP-10 secretion by adipocytes is further enhanced by stimuli that mimic an inflammatory status. 3T3-L1 adipocytes were exposed to IL-1β or LPS. Both stimuli are known to mediate their inflammatory response through NFκB. An increase in IP-10 release was observed at concentrations of 10 ng/ml IL-1β (Figure 8a) or 1 µg/ml LPS (Figure 8b).

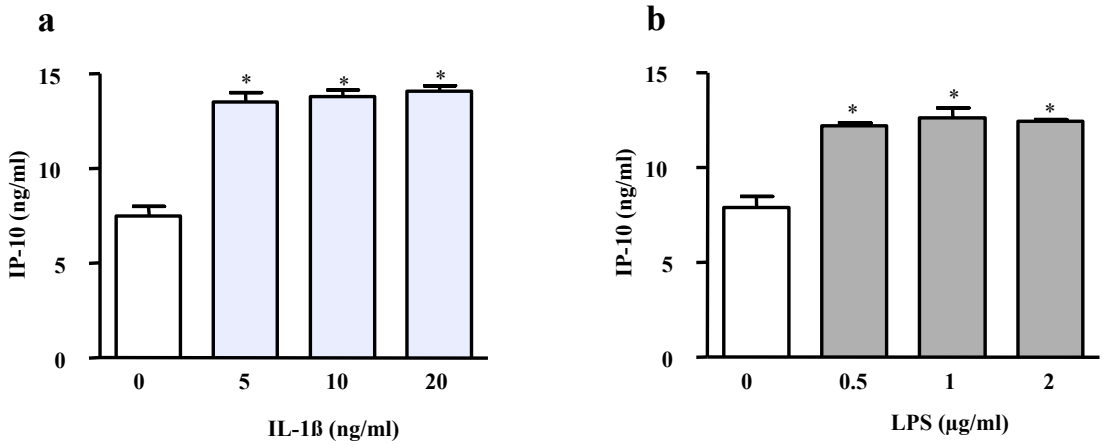

Figure 8: IL-1β- or LPS-stimulated protein secretion of IP-10 in 3T3-L1 adipocytes. Supernatants of 3T3-L1 adipocytes were harvested after 20h of adding fresh media with or without 10 ng/ml IL-1β (a) or 1 µg/ml LPS (b). IP-10 protein concentration was measured using ELISA. Results are means ±SD (n = 6). *P<0.05. Figures from Krinninger et al. (Krinninger et al., 2011).

6.1.5 IP-10 expression and promoter-activity increase during adipogenesis

To further explore the mode of IP-10 regulation in adipocytes IP-10 mRNA expression, IP-10 protein secretion and IP-10 promoter-activity were analyzed during 3T3-L1 adipogenesis. IP-10 mRNA expression was initially low in 3T3-L1 cells (on day 1 to 6 after induction of adipogenesis) but increased continuously during adipogenesis and reached a maximal 8-fold increase in freshly isolated mature adipocytes on day 14 of adipocyte differentiation compared to day 0 preadipocytes (Figure 9a). To verify whether the increase of IP-10 mRNA expression during differentiation is accompanied by an enhanced protein secretion, IP-10 protein release was analyzed during differentiation. IP-10 protein secretion was detectable exclusively in fully differentiated adipocytes, whereas in preadipocytes no protein release could be observed (Figure 9b).

To study the molecular mechanisms involved in the expression of IP-10 in adipocytes, a reporter-construct containing 673 bp of the proximal murine IP-10 promoter (containing two κB-sites) in front of a luciferase gene was transfected into 3T3-L1 cells. In parallel, an NFκB-responsive luciferase-reporter construct containing six κB-consensus-sites was transfected to determine NFκB-activity. The activities of both the IP-10 promoter (Figure 9c) as well as the synthetic NFκB-responsive reporter (Figure 9d) behave exactly the same, with a slight decrease after induction of differentiation and an overall increase in luciferase-activity over the 14-day differentiation period.

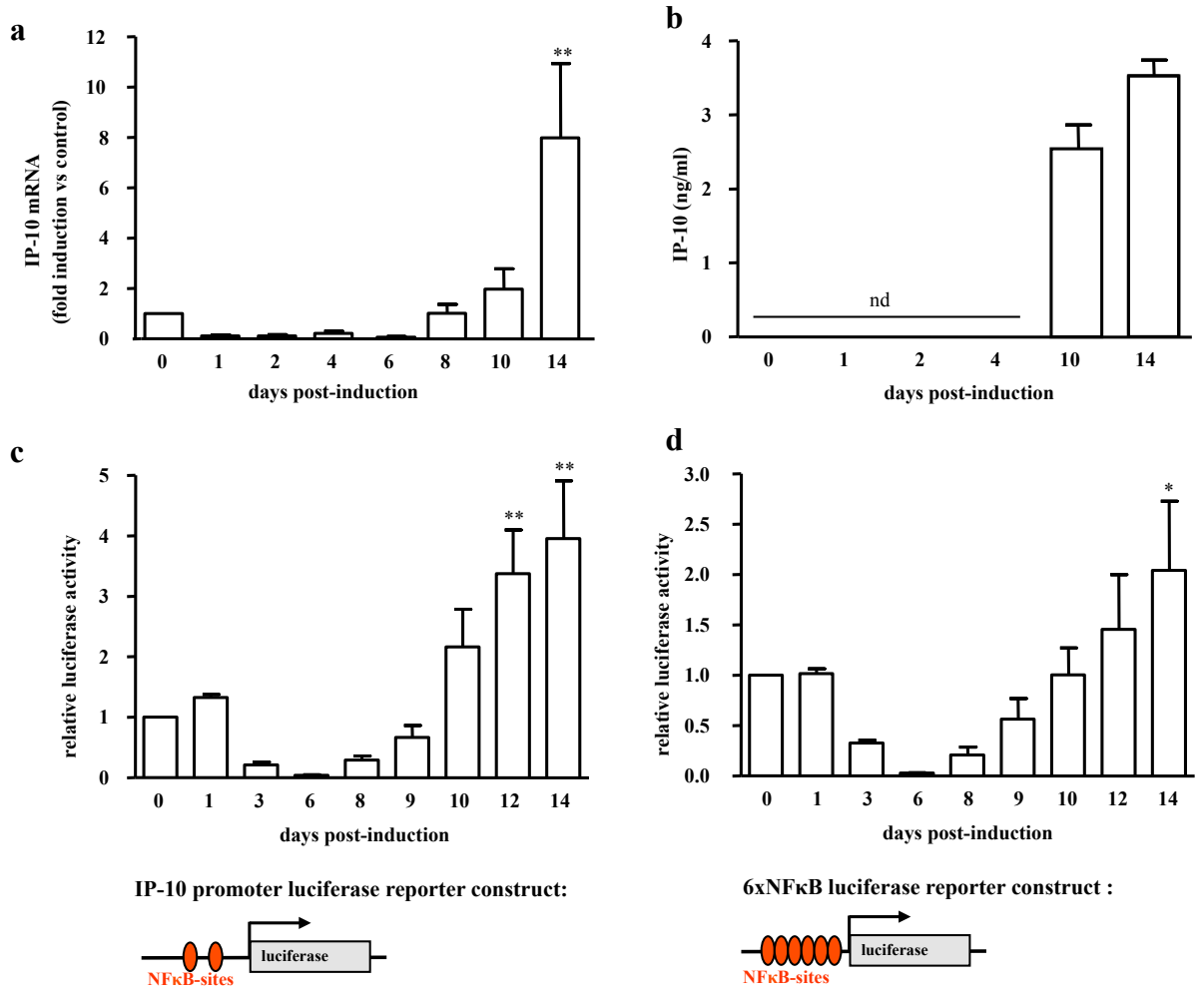

Figure 9: Evolution of IP-10 gene mRNA expression/protein secretion, IP-10 promoter- and 6xNFκB-reporter-activity during adipogenesis. 3T3-L1 preadipocytes were harvested on several days after induction of differentiation. mRNA was reverse transcribed; IP-10 and GAPDH mRNA expression was determined by qRT-PCR (a). IP-10 protein secretion into supernatant was measured by ELISA (b). 3T3-L1 cells were transiently transfected with an IP-10 promoter luciferase-reporter (c) or a 6xNFκB-luciferase-reporter (d). Luciferase activities of extracts were determined 24h after transfection on the indicated days of differentiation and normalized based on Renilla-luciferase-activity. Results are means ±SE ($n \geq 3$). *$P<0.05$, **$p<0.01$. Figures from Krinninger et al. (Krinninger et al., 2011).

6.1.6 NFκB binding to IP-10 promoter κB-sites and inhibition of NFκB

As IP-10 is known to be regulated by two κB-sites in its promoter, NFκB DNA-binding-activity to these sites was analyzed. Electrophoretic mobility shift assays revealed an increased NFκB binding-activity to the IP-10 promoter κB-sites upon IL-1β stimulation in 3T3-L1 adipocytes (Figure 10).

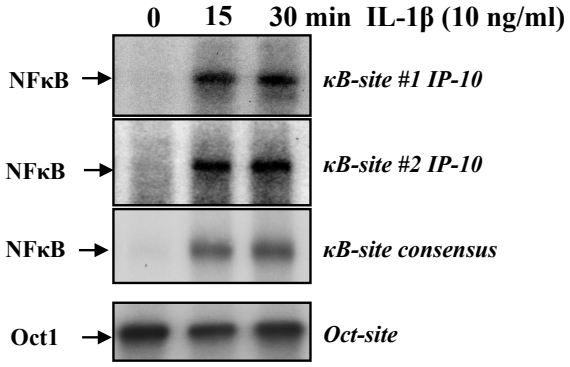

Figure 10: IL-1β induces NFκB-DNA binding to IP-10 promoter κB-sites. Protein of 3T3-L1 cells induced with IL-1β for the indicated times was harvested and NFκB binding activity was analyzed by EMSA. Used radioactive labeled DNA-probes: κB-site#1 IP-10 = first binding site for NFκB in the IP-10 promoter; κB-site#2 IP-10 = second binding site for NFκB in the IP-10 promoter; κB-site consensus = consensus κB-site and Oct-site = octamer binding site here showing Oct1 binding as loading control. Figure from Krinninger et al. (Krinninger et al., 2011).

To further investigate the role of NFκB for the expression of IP-10 in adipose tissue, an NFκB-inhibitor (Bay117082) was used. Mouse 3T3-L1 adipocytes and isolated mature human adipocytes were preincubated for 1h with or without Bay117082 (1 and 5 μM), followed by incubation with IL-1β (10 ng/ml) for 20h. Bay117082 (1 and 5 μM) significantly prevented IL-1β stimulation of IP-10 protein release from 3T3-L1 adipocytes (Figure 11a) and of IP-10 mRNA expression in mature human adipocytes (Figure 11b) in a dose-dependent manner.

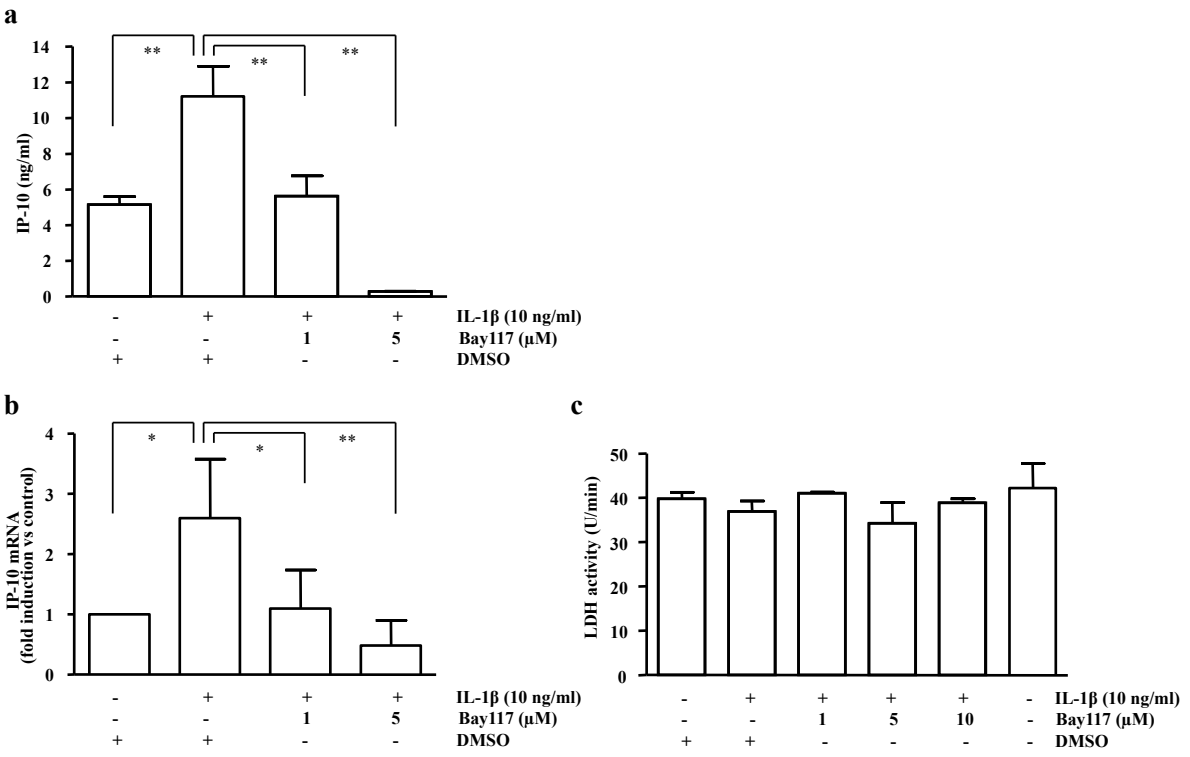

Figure 11: An NFκB-inhibitor decreases IP-10 expression in primary human adipocytes and protein secretion in 3T3-L1 adipocytes. 3T3-L1 adipocytes on day 14 of differentiation (a) and primary human mature adipocytes (b) were preincubated for 1h with or without the NFκB-inhibitor Bay117082 as indicated, followed by incubation with IL-1β (10 ng/ml) for 20h. Medium with vehicle (DMSO) was used as control. IP-10 protein concentration was measured using ELISA (a); mRNA was reverse transcribed; IP-10 and GAPDH mRNA expression was determined by qRT-PCR (b). (c) LDH activity in medium was measured for evaluation of cell viability in media from freshly isolated human adipocytes from adipose tissue preincubated with or without Bay117082 followed by incubation with IL-1β (10 ng/ml). Results are means ±SD ($n \geq 4$). *$P<0.05$, **$p<0.01$. Figures from Krinninger et al. (Krinninger et al., 2011).

6.1.7 Stable overexpression of a dominant-negative IκBα mutant in 3T3-L1 cells

In order to block the NFκB pathway more specifically and to further elucidate the contribution of NFκB signaling to IP-10 regulation in 3T3-L1 preadipocytes and adipocytes, retroviral gene transfer was used in order to express a dominant negative interfering IκBα mutant protein (IκBα-mut) or an empty vector as control (mock) (Huber et al., 2002).

IκBα-mut overexpression was confirmed by western blot (Figure 12a). In addition, EMSA experiments revealed that the mutant protein is functional active, since it was able to almost completely block the NFκB-DNA binding (Figure 12b). Adipogenic differentiation capacity was not affected, monitored morphologically and by Oil red O staining versus control cells (Figure 12c). (Note: Figure 12a/b are also part of my Diploma thesis (Dietl, 2007) and only

shown in this thesis in order to demonstrate the overexpression and functional activity of the mutant protein).

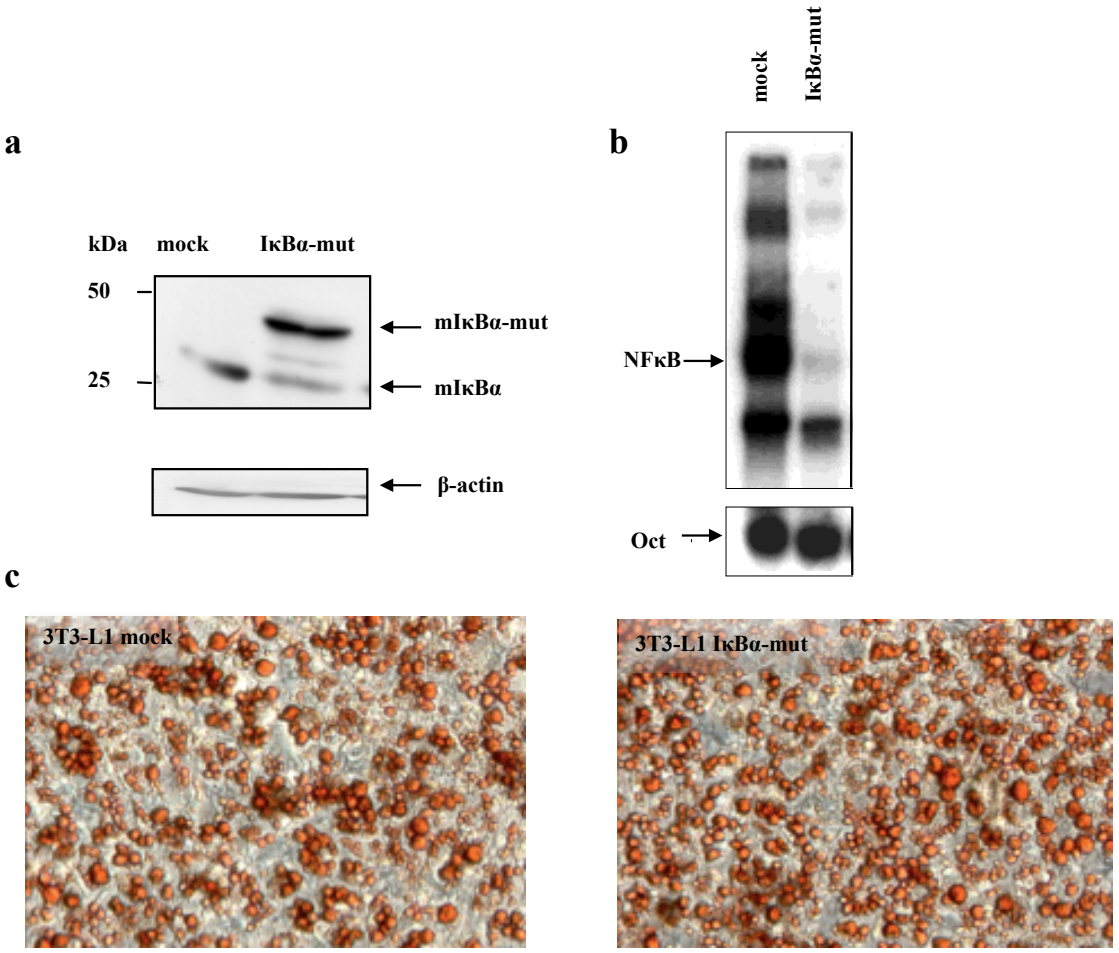

Figure 12: Stable overexpression of IκBα-mut in 3T3-L1 cells inhibits NFκB-activity and IP-10 expression/secretion. Protein from 3T3-L1-mock and 3T3-L1-IκBα-mut preadipocytes was harvested and 50 µg protein were used for western blot with anti-IκBα antibody. β-actin was assayed as an internal loading control (a). Basal NFκB DNA binding activity in 3T3-L1-mock and 3T3-L1-IκBα-mut preadipoyctes (b). Nuclear extracts from stable 3T3-L1 preadipocytes were incubated with ^{32}P-labeled oligonucleotides containing a NFκB binding motif and subjected to EMSA. Oct1 protein DNA-binding was assayed as an internal control for equal loading. (c) Effect of IκBα-mut expression on adipocyte differentiation. 3T3-L1-mock and 3T3-L1-IκBα-mut cells were differentiated for 12 days and intracellular lipid was stained with Oil Red O. Figures from Krinninger et al. (Krinninger et al., 2011) and (Dietl, 2007).

6.1.8 IP-10 expression and secretion in IκBα-mut overexpression 3T3-L1 pre- and adipocytes depending on inhibition of NFκB signaling in pre- and adipocytes

IP-10 expression and secretion by preadipocytes and adipocytes were measured depending on the overexpression of IκBα-mut at basal levels and upon stimulation. Basal and stimulated IP-10 mRNA expression (Figure 13a) and protein secretion (Figure 13b) were higher in mock

adipocytes compared to preadipocytes with no measurable amounts of IP-10 in preadipocyte-conditioned media. In adipocytes, the overexpression of IκBα-mut significantly reduced basal and stimulated IP-10 mRNA expression as well as protein release compared to control cells. These results demonstrate the central role of NFκB in the regulation of IP-10 in 3T3-L1 adipocytes rather than in preadipocytes, in which the reduction of IP-10 expression and secretion in cells overexpressing IκBα-mut was less evident.

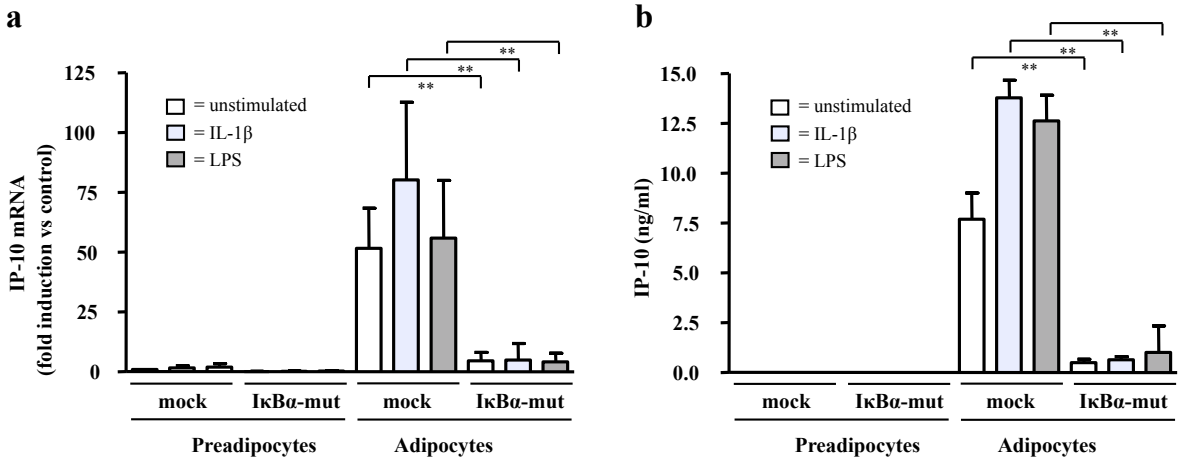

Figure 13: IP-10 expression and secretion by preadipocytes and adipocytes depending on the overexpression of IκBα-mut. RNA and supernatant from stably infected 3T3-L1 preadipocytes and adipocytes were harvested 20h after adding fresh media with or without IL-1β (10 ng/ml) or LPS (1 µg/ml) stimulation. mRNA was reverse transcribed; IP-10 and GAPDH mRNA expression was determined by qRT-PCR (a). IP-10 protein concentration was measured from supernatants using ELISA (b). Results are means ±SD (n ≥ 5). **P<0.01. Figures from Krinninger et al. (Krinninger et al., 2011).

6.1.9 IL-1β- and LPS-induced T cell migration dependent on the overexpression of IκBα-mut

NFκB regulates the expression of several chemokines in many cell types. To elucidate the relevance of the NFκB pathway for adipocyte-induced T cell migration, conditioned medium from the here-established cell lines were used for migration experiments. In a migration assay using isolated mouse spleen leukocytes, minimal induction of $CD4^+$ T cell migration was observed in response to supernatants from preadipocytes stimulated with either IL-1β or LPS (Figure 14a). However, in response to supernatants from IL-1β- or LPS-stimulated adipocytes, $CD4^+$ T cells exhibited a significant increased migratory activity compared to supernatants from unstimulated adipocytes (Figure 14b). This activation of migration was significantly abrogated using media from 3T3-L1 cells overexpressing IκBα-mut. Similar results were obtained analyzing the migration of $CD8^+$ T cells (Figure 14c/d).

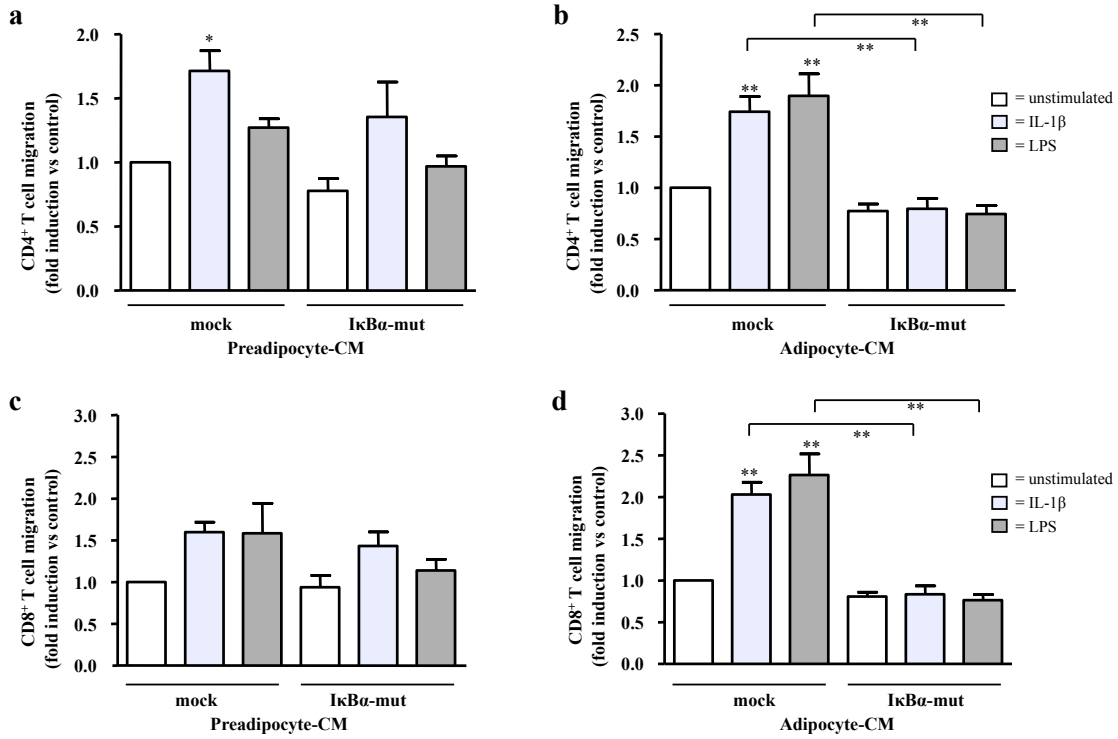

Figure 14: Migration of T cells in response to 3T3-L1 adipocyte-conditioned medium is inhibited by overexpression of IκBα-mut Stable 3T3-L1 preadipocytes (a/c) and adipocytes (b/d) were induced for 20h by adding fresh media with or without IL-1β (10 ng/ml, striped bar) or LPS (1 µg/ml, black bar). Conditioned media (CM) were harvested and transferred to the lower chamber of Costar Transwells. Freshly isolated mouse splenocytes were placed in Costar Transwell permeable supports. Migration of $CD4^+$ T cell (a/b) and $CD8^+$ T cells (c/d) towards conditioned media was assayed by FACS. Results are means ±SE ($n \geq 9$). *P<0.05, **p<0.01. Figures from Krinninger et al. (Krinninger et al., 2011).

6.1.10 Adipocyte-induced T cell migration is inhibited by neutralization of secreted IP-10 protein.

To address the question whether the ability of 3T3-L1 adipocyte supernatants to induce T cell migration was mediated by the chemokine IP-10, a specific neutralizing antibody was added to the supernatants. Addition of this antibody to conditioned 3T3-L1 adipocyte media inhibited basal $CD4^+$ T cell migration (Figure 15a) and $CD8^+$ T cell migration (Figure 15b) in a dose-dependent manner, whereas addition of an isotype control did not alter migration of T cells.

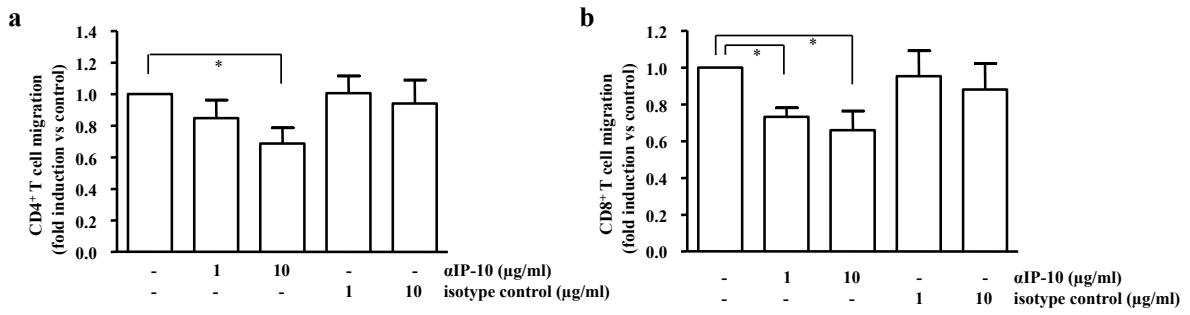

Figure 15: Migration of T cells in response to 3T3-L1 adipocyte-conditioned medium is inhibited by a neutralizing IP-10 antibody. 3T3-L1 adipocytes were cultivated for 20h in fresh medium and conditioned medium was transferred to the lower chamber of Costar Transwells. A neutralizing anti-IP-10 antibody (αIP-10) or as isotype control an anti-IgG antibody was added 30 minutes before addition of splenocytes. For all experiments freshly isolated mouse splenocytes were placed in Costar Transwell permeable supports. Migration of $CD4^+$ T cell (a) and $CD8^+$ T cells (b) towards conditioned media was assayed by FACS. Results are means ±SE ($n \geq 4$). *P<0.05, **p<0.01. Figures from Krinninger et al. (Krinninger et al., 2011).

6.1.11 Expression of IP-10 and its receptor CXCR3 is upregulated in adipose tissue during progression of weight gain.

The progression of leukocyte migration into adipose tissue in obese humans or mice raises the question of whether the expression of responsible chemokines in adipose tissue is upregulated during the time course of weight gain. Samples from a recently published study (Kintscher et al., 2008) showing T lymphocyte infiltration in visceral adipose tissue as a primary event in adipose tissue inflammation were reanalyzed. In adipose tissue from mice fed a high-fat diet for 0, 5 and 10 weeks (w0, w5, w10) or a chow control diet for 10 weeks (CD) a slight upregulation of IP-10 mRNA level after 5 weeks and a significant 3-fold upregulation after 10 weeks of a high-fat diet compared to initial levels (w0) was observed (Figure 16a). In the same tissues, expression of the IP-10 receptor CXCR3 was significantly upregulated at week 5 (1.7-fold) and week 10 (2.5-fold) (Figure 16b). Both IP-10 as well as CXCR3 expression increased in parallel to T cell infiltration as shown recently (Kintscher et al., 2008). These data further support that IP-10 secreted from adipose tissue contributes to the recruitment of T cells into adipose tissue of obese.

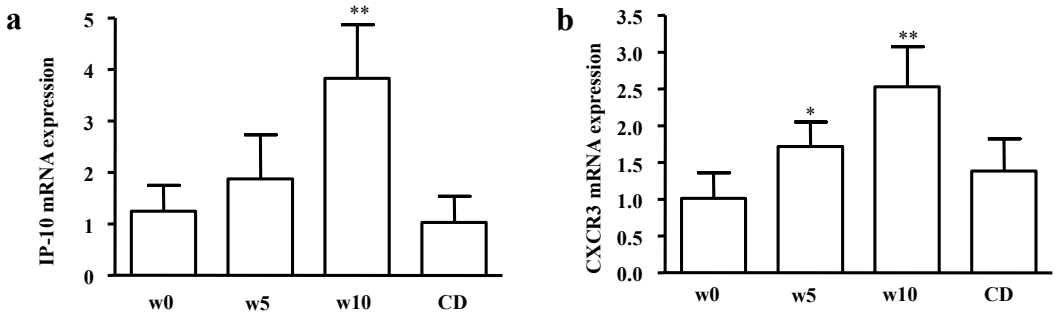

Figure 16: Expression of adipose tissue IP-10 and CXCR3 mRNA increases during progression of weight gain. Total mRNA was isolated from visceral/epididymal adipose tissue of male C57BL/6J mice at baseline (w0), compared with mice fed a high-fat diet for 5 (w5) or 10 weeks (w10) and with mice fed a chow diet for 10 weeks (CD). mRNA was reverse transcribed and IP-10 (a) and CXCR3 (b) expression was determined by qRT-PCR; GAPDH mRNA was measured as control. Results are means ±SD ($n \geq 5$). *$P<0.05$, **$p<0.01$ compared to w0. Figures from Krinninger et al. (Krinninger et al., 2011).

6.2 Cell-intrinsic migration capacity and surface chemokine receptor expression in PBMCs from lean versus obese women: results from the AdipoRiSc (Adiposity Risk Screening) study

The here-presented study is a sub-analysis of the AdipoRiSc case control study, which includes 60 obese (BMI ≥ 30.0 to ≤ 46.0 kg/m^2) versus 60 non-obese women (BMI ≥ 18.5 to ≤ 24.9 kg/m^2). The AdipoRiSc study was set up to (i) investigate biomarkers for obesity prediction and to (ii) characterize functional differences between peripheral immune cells in a subset of obese and normal-weight subjects. The here-presented work focused on the second part of the study, the analyses of composition of immune cell subpopulations, activation/inflammation status, surface expression of chemokine receptors CCR2, CCR3, CCR5 and CXCR3 as well as cell-intrinsic migration capacity. Results of this investigation and therewith parts of this thesis were published by Krinninger et al. (Krinninger et al., 2014).

6.2.1 Anthropometric and metabolic characteristics of the various study populations

Primary PBMCs from recruited obese and lean women were prepared and immediately subjected to surface protein analysis by FACS and migration analysis. For migration assay, 29 obese and 19 lean women were included and the FACS analysis was performed with a subgroup thereof encompassing 14 obese and 12 lean women (for two additional obese subjects solely FACS data are available). As the isolation efficiency of PBMCs varied for different donors, there was not sufficient material available to perform both FACS analysis and migration assay for each donor. Thus for several donors, solely migration assay data are available. However, both subpopulations show similar anthropometric and metabolic phenotypes of a healthy population without indication of chronic or acute diseases but with all expected obesity-related characteristics, summarized in Table 2.

In detail, no differences were detected in age and height between obese and lean women in both subgroups. However, as expected, significant differences were documented according to anthropometry, metabolism and inflammation. Compared with controls, obese women had significantly higher waist, hip circumference and upper arm circumference. Similarly, waist-to-hip ratio and percentage of fat mass were significantly higher in obese compared to controls.

However, both obese subgroups had significantly elevated levels of low-density lipoprotein cholesterol, C-reactive protein (hsCRP), uric acid, γ-glutamyltransferase (γ-GT), insulin HOMA-IR as well as decreased concentrations of high-density lipoprotein (HDL) cholesterol.

	Migration assay population			**FACS analysis population**		
	Lean (n=19)	**Obese (n=29)**	**P-value***	**Lean (n=16)**	**Obese (n=14)**	**P-value***
Anthropometric parameters	mean ± SD	mean ± SD		mean ± SD	mean ± SD	
Age (years)	35.2 ± 6.9	35.5 ± 6.5	0.862	34.9 ± 6.4	35.7 ± 5.0	0.716
Height (cm)	166.0 ± 7.5	167.6 ± 6.32	0.429	164.4 ± 7.4	167.7 ± 6.7	0.214
Weight (kg)	60.1 ± 7.8	99.9 ± 12.3	**<0.001**	58.2 ± 7.3	101.1 ± 12.8	**<0.001**
BMI (kg/m^2)	21.8 ± 1.9	35.6 ± 4.1	**<0.001**	21.5 ± 2.0	36.0 ± 4.7	**<0.001**
Waist circumference (cm)	74.2 ± 6.3	105.8 ± 9.1	**<0.001**	73.3 ± 6.4	105.9 ± 10.5	**<0.001**
Hip circumference (cm)	95.8 ± 5.6	124.1± 8.7	**<0.001**	94.62 ± 5.36	125.6 ± 9.5	**<0.001**
WHR	0.78 ± 0.04	0.85 ± 0.05	**<0.001**	0.77 ± 0.13	0.84 ± 0.09	**<0.001**
Upper arm circumference (cm)	27.4 ± 1.8	36.2 ± 3.0	**<0.001**	27.2 ± 2.0	36.6 ± 3.3	**<0.001**
Fat mass DEXA (%)	30.2 ± 5.3	45.3 ± 3.9	**<0.001**	30.3 ± 4.6	45.9 ± 4.1	**<0.001**
OGTT capillary (mg/dl)						
Blood glucose 0h	81.9 ± 6.5	87.3 ± 8.7	**0.026**	83.3 ± 7.4	86.7 ± 8.0	0.234
Blood glucose 1h	142.9 ± 47.7	158.9 ± 28.9	0.156	148.0 ± 48.9	159.0 ± 31.8	0.481
Blood glucose 2h	115.7 ± 33.6	123.5 ± 26.6	0.374	124.0 ± 35.4	129.7 ± 28.5	0.63
Clinical chemical parameters						
Erythrocytes (10^6/µl)	4.3 ± 0.65	4.5 ± 0.30	0.066	4.2 ± 0.7	4.6 ± 0.3	0.098
Leukocytes (10^3/µl)	6.3 ± 1.4	6.8 ± 1.3	0.203	6.0 ± 1.4	6.9 ± 1.3	0.072
Thrombocytes (10^3/µl)	274.2 ± 54.3	275.3 ± 58.3	0.948	253.9 ± 58.8	260.8 ± 52.5	0.738
Fasting blood glucose (mg/dl)	78.6 ± 5.5	83.8 ± 8.2	**0.02**	79.1 ± 6.7	83.9 ± 6.8	0.065
Creatinin (mg/dl)	0.94 ± 0.90	0.92 ± 0.09	0.556	0.93 ± 0.09	0.91 ± 0.07	0.568
Uric acid mg/dl	4.0 ± 0.6	5.0 ± 0.8	**<0.001**	4.1 ± 0.6	4.9 ± 0.7	**0.002**
Cholesterol (mg/dl)	193.8 ± 41.1	209.9 ± 32.7	0.138	193.8 ± 39.6	215 ± 26.6	0.108
HDL cholesterol (mg/dl)	70.6 ± 10.4	60.0 ± 13.0	**0.004**	70.4 ± 9.9	57.5 ± 12.2	**0.003**
LDL cholesterol (mg/dl)	116.6 ± 31.4	135.3 ± 25.9	**0.03**	115.1 ± 29.3	140.6 ± 23.3	**0.014**
Triglycerides (mg/dl)	90.5 ± 43.5	119.2 ± 54.2	0.059	90.8 ± 39.0	117.8 ± 54.7	0.127
Free fatty acids (mmol/l)	0.40 ± 0.14	0.55 ± 0.19	**0.006**	0.45 ± 0.14	0.57 ± 0.22	0.092
GOT (U/l)	22.4 ± 8.7	23.3 ± 8.5	0.711	21.9 ± 6.7	24.4 ± 10.4	0.427
GPT (U/l)	20.2 ± 14.9	28.4 ± 32.5	0.311	17.7 ± 9.8	33.9 ± 45.6	0.175
γ-GT (U/l)	16.5 ± 9.9	22.8 ± 9.6	**0.034**	14.4 ± 7.3	24.8 ± 11.9	**0.007**
hsCRP (mg/l)	0.89 ± 1.37	6.90 ± 6.12	**<0.001**	0.81 ± 1.1	8.83 ± 6.53	**0.001**
Insulin (mU/l)	4.9 ± 1.4	9.7 ± 3.2	**<0.001**	5.2 ± 2.4	9.8 ± 3.5	**<0.001**
TSH basal (mU/l)	2.2 ± 1.2	2.0 ± 1.1	0.556	2.3 ± 1.2	2.2 ± 1.1	0.737
HOMA-IR	0.95 ± 0.29	2.01 ± 0.71	**<0.001**	0.93 ± 0.19	1.98 ± 0.82	**<0.001**

Table 2: Anthropometric and metabolic parameters of study participants included into migration assay and FACS analysis. BMI, body mass index; WHR, waist-to-hip ratio; DXA, dual-energy X-ray absorptiometry; OGTT, oral glucose tolerance test; HDL, high-density lipoprotein; LDL, low-density lipoprotein; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvate transaminase; γ-GT, γ-glutamyl transpeptidase; hsCRP, high sensitive C-reactive protein; HOMA, homeostatic model assessment of insulin resistance. Table from Krinninger et al. (Krinninger et al., 2014).

Moreover, fasting plasma glucose and free fatty acid levels were increased in the migration assay subgroup compared to lean controls. For leukocyte counts, creatinine, total cholesterol, triglycerides (TG), glutamic oxaloacetic transaminase (GOT), glutamic pyruvate transaminase (GPT) and thyroid-stimulating hormone (TSH) no significant differences were observed.

Overall, the analyzed study populations represent homogenous and healthy study groups without chronic or acute diseases but with all expected obesity-related alterations including impaired insulin sensitivity in the obese. Thus, the recruited individuals match the requirements necessary to analyze the effect of obesity on functional response in peripheral immune cells.

6.2.2 Composition of PBMC from obese and lean women

To gain insight into the proportions of immune cell subpopulations in peripheral blood from normal-weight and obese women, FACS analyses were performed (Figure 17).

Comparing the lean versus the obese group, no significant differences were found for total T cell and T helper cell percentages (Table 3). However, the percentage of activated $CD4^+CD25^+$ T cells was significantly increased in obese versus non-obese women (Figure 18a).

With regard to total monocyte content, no differences were detected between both groups (Table 3). Monocytes can be further subdivided into classical monocytes, characterized by high expression level of the LPS co-receptor $CD14^+$, and non-classical monocytes with expression of CD14 and co-expression of the FcγIII receptor CD16 receptor, the $CD14^+CD16^+$ monocytes (Figure 17). Non-classical monocytes secrete pro-inflammatory cytokines upon activation (Ziegler-Heitbrock, 2006). The percentage of $CD14^+CD16^+$ cells in obese subjects was significantly increased as compared to lean subjects (Figure 18b).

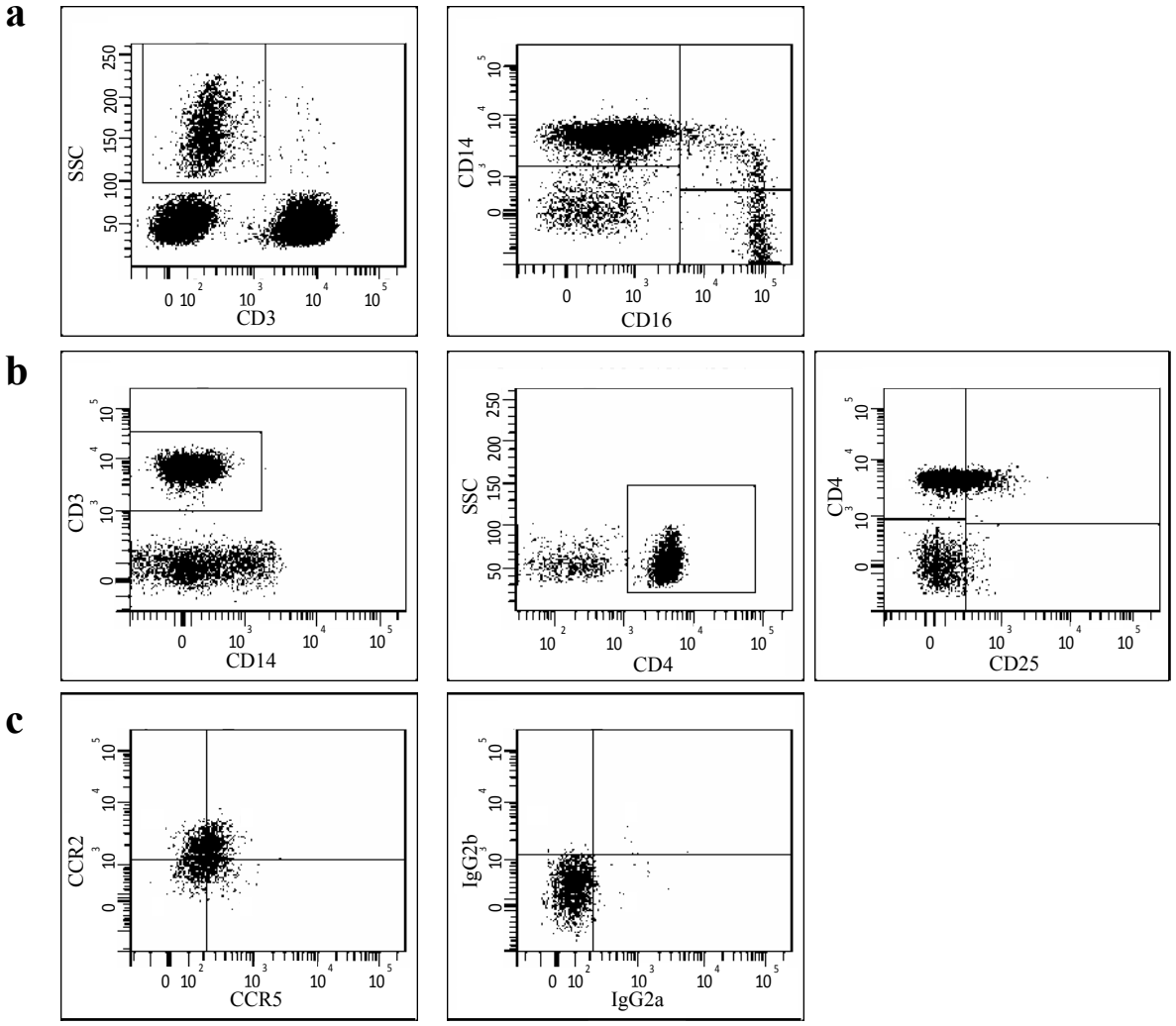

Figure 17: Representative FACS dot plots of a representative normal-weight subject. Monocytes were identified by high granularity and absence of CD3 expression (a, left panel), T lymphocytes by CD3 expression (b, left panel). Monocyte subsets were further characterized by the expression patterns of the LPS co-receptors CD14 and FcγIII receptor CD16 gated as $CD14^{+}CD16^{-}$ and/or $CD14^{+}CD16^{+}$ cells (a, right panel), T lymphocytes according to the expression of CD4 (B, middle panel) and to expression of the co-receptor CD25 in the $CD4^{+}$ population (b, right panel). Representative plot showing the CCR2 and CCR5 expression levels on $CD14^{+}CD16^{+}$ monocytes (c, left panel) and corresponding isotype controls (c, right panel). Figures from Krinninger et al. (Krinninger et al., 2014).

	Lean (n=16)	Obese (n=14)	P-value*
% postive cells	mean ± SD	mean ± SD	
T cells	66.40 ± 9.78	67.24 ± 8.90	0.811
Th cells	61.06 ± 9.39	65.48 ± 9.91	0.232
$CD25^+$ Th cells	9.45 ± 4.17	17.05 ± 6.25	**0.001**
Monocytes	81.28 ± 5.81	82.83 ± 4.61	0.43
$CD14^+$ monocytes	87.30 ± 4.56	80.72 ± 7.94	**0.009**
$CD14^+CD16^+$ monocytes	12.70 ± 4.56	19.3 ± 7.94	**0.009**

* Independent-sample t-test.

Table 3: Percentages of immune cell subpopulations in peripheral blood of obese subjects (n=14) compared with normal-weight subjects (n=16). Table from Krinninger et al. (Krinninger et al., 2014).

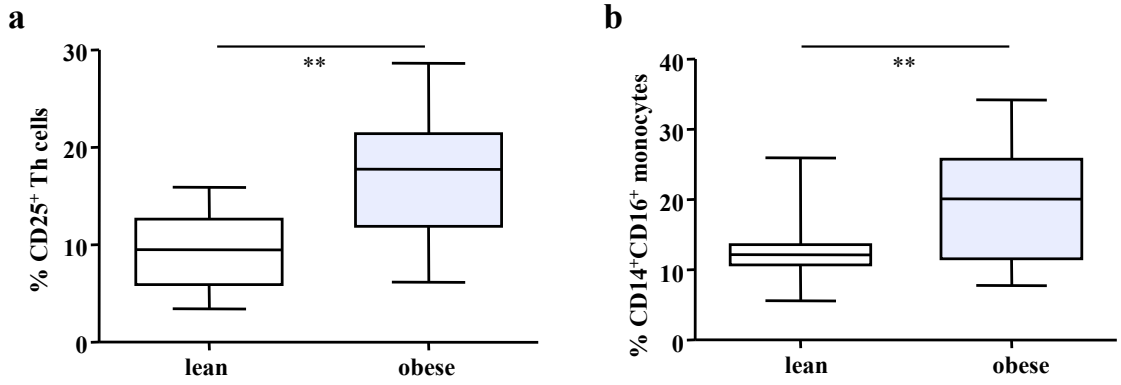

Figure 18: Percentages of $CD4^+CD25^+$ T cells (a) and $CD14^+CD16^+$ monocytes (b) in peripheral blood of obese subjects (n=16) compared with lean controls (n=14). Data are presented as mean (horizontal bar) with 25^{th} and 75^{th} percentiles (boxes) and extreme values (whiskers). Analysis was performed using independent-sample t-test with *$p < 0.05$, **$p < 0.01$. Figures from Krinninger et al. (Krinninger et al., 2014).

6.2.3 Chemokine receptor expression on peripheral T cells and monocytes

To compare cell surface protein expression of the chemokine receptors CCR2, CCR3, CCR5 and CXCR3 on distinct monocyte and T cell subsets in lean versus obese, both the percentage of receptor positive cells and the receptor protein density as median fluorescence intensity (MFI) were assessed. No obvious differences could be detected on various T cell populations in relation to obesity (Table 4/5).

% positive cells	**Lean (n=16)** mean ± SD	**Obese (n=14)** mean ± SD	P-value*
Th cells			
CCR2	0.09 ± 0.35	0.00 ± 0.00	0.333
CCR5	1.09 ± 1.13	1.36 ± 1.09	0.512
CCR3	1.12 ± 1.132	1.16 ± 0.86	0.925
CXCR3	21.49 ± 5.12	22.01 ± 5.45	0.792
$CD25^+$ Th cells			
CCR2	0.39 ± 0.81	0.40 ± 1.20	0.974
CCR5	4.44 ± 3.24	4.45 ± 4.91	0.994
CCR3	3.50 ± 3.03	2.42 ± 2.14	0.262
CXCR3	29.00 ± 5.60	28.62 ± 7.24	0.884

* Independent-sample t-test

Table 4: Surface expression of the chemokine receptors CCR2, CCR5, CCR3 and CXCR3 on Th cells and $CD25^+$ T cells in the peripheral blood of obese subjects (n=14) compared with lean controls (n=16). PBMCs were stained with antibodies against the chemokine receptors, and surface expression was analyzed by FACS. For the expressed chemokine receptors, the mean percentages of positive cells are given. Table from Krinninger et al. (Krinninger et al., 2014).

MFI	**Lean (n=16)** mean ± SD	**Obese (n=14)** mean ± SD	P-value*
Th cells			
CCR2	4.0 ± 9.95	5.21 ± 9.82	0.739
CCR5	3.25 ± 3.23	3.93 ± 2.50	0.523
CCR3	7.75 ± 11.92	5.43 ± 4.34	0.477
CXCR3	30.00 ± 13.04	31.14 ± 9.54	0.785
$CD25^+$ Th cells			
CCR2	9.20 ± 20.04	8.43 ± 25.50	0.929
CCR5	7.56 ± 6.00	7.39 ± 5.38	0.895
CCR3	18.81 ± 24.65	11.50 ± 10.12	0.31
CXCR3	47.63 ± 16.25	43.64 ± 17.54	0.526

* Independent-sample t-test

Table 5. Surface expression of the chemokine receptors CCR2, CCR5, CCR3 and CXCR3 on Th cells and $CD25^+$ T cells in the peripheral blood of obese subjects (n=14) compared with lean controls (n=16). PBMCs were stained with antibodies against the chemokine receptors, and surface expression was analyzed by FACS. For the expressed chemokine receptors, the median fluorescence intensity (MFI) is given. Table from Krinninger et al. (Krinninger et al., 2014).

In contrast to T cells, monocytes and monocyte subsets show increased expression of CCR2 and CCR5 in obesity (Table 6/7). In detail, both the percentage of CCR5-expressing monocytes as well as the MFI for CCR5 was significantly increased in the obese group (Figure 19 a/b). In addition, a significant increase of the receptor surface density of $CCR2^+$

total monocytes was observed (Figure 20b), but no significant change in the percentage of $CCR2^+$ monocytes (Figure 20a). Next it was investigated whether the monocyte subtypes show a differential surface expression of the chemokine receptors in obesity. A significantly increased percentage of $CCR5^+$ cells was solely found for the $CD14^+CD16^-$ monocyte population (Figure 19c), whereas the percentage of CCR2 positive cells was specifically increased in the $CD14^+CD16^+$ monocytes (Figure 20e). A significant increase of surface density for CCR5 (Figure 19b/d/f) and CCR2 (Figure 20b/d/f) was observed in all monocyte subsets. A representative plot of CCR2 and CCR5 staining on $CD14^+CD16^+$ monocytes is shown in Figure 17c. Differences in CCR3 and CXCR3 expression on monocytes as well as on the monocytes subsets $CD14^+$ and $CD14^+CD16^+$ (neither expressed as percentage of positive cells nor as MFI) did not reach statistical significance (Table 6/7).

	Lean (n=16)	**Obese (n=14)**	
% positive cells	mean ± SD	mean ± SD	P-value*
Total monocytes			
CCR2	57.18 ± 18.15	67.92 ± 22.46	0.166
CCR5	10.91 ± 6.30	21.66 ± 13.83	**0.009**
CCR3	3.56 ± 3.32	6.60 ± 5.60	0.091
CXCR3	2.75 ± 2.27	4.96 ± 3.79	0.072
$CD14^+CD16^-$ monocytes			
CCR2	63.31 ± 17.80	72.63 ± 21.52	0.211
CCR5	12.66 ± 6.44	23.91 ± 14.02	**0.007**
CCR3	2.39 ± 2.51	3.76 ± 2.92	0.183
CXCR3	2.69 ± 2.16	4.09 ± 2.76	0.137
$CD14^+CD16^+$ monocytes			
CCR2	21.98 ± 11.97	39.94 ± 24.54	**0.015**
CCR5	7.95 ± 8.69	15.04 ± 17.31	0.182
CCR3	11.23 ± 8.64	14.86 ± 16.08	0.460
CXCR3	6.01 ± 6.65	8.23 ± 6.70	0.372

* Independent-sample t-test.

Table 6: Surface expression of the chemokine receptors CCR2, CCR5, CCR3 and CXCR3 on monocyte subsets in the peripheral blood of obese subjects (n=14) compared with lean controls (n=16). PBMCs were stained with antibodies against the chemokine receptors, and surface expression were analyzed by FACS. For the expressed chemokine receptors, the mean percentages of positive cells are given. Table from Krinninger et al. (Krinninger et al., 2014).

MFI	**Lean (n=16)** mean ± SD	**Obese (n=14)** mean ± SD	P-value*
Total monocytes			
CCR2	1285.13 ± 767.23	2373.71 ± 1795.77	**0.050**
CCR5	49.94 ± 18.50	81.00 ± 37.33	**0.006**
CCR3	43.00 ± 32.42	84.71 ± 80.91	0.068
CXCR3	11.94 ± 8.68	27.57 ± 38.30	0.123
$CD14^+CD16^-$ monocytes			
CCR2	1397.06 ± 809.87	2525.00 ± 1787.77	**0.031**
CCR5	50.38 ± 19.12	78.86 ± 37.10	**0.012**
CCR3	35.63 ± 24.61	72.00 ± 71.50	0.066
CXCR3	10.81 ± 7.27	25.43 ± 35.16	0.115
$CD14^+CD16^+$ monocytes			
CCR2	248.69 ± 238.66	1149.71 ± 1693.60	**0.044**
CCR5	46.19 ± 34.47	97.43 ± 53.94	**0.006**
CCR3	123.63 ± 75.36	183.36 ± 215.27	0.307
CXCR3	29.88 ± 31.49	61.71 ± 84.99	0.169

* Independent-sample t-test.

Table 7. Surface expression of the chemokine receptors CCR2, CCR5, CCR3 and CXCR3 on monocyte subsets in the peripheral blood of obese subjects (n=14) compared with lean controls (n=16). PBMCs were stained with antibodies against the chemokine receptors, and surface expression were analyzed by FACS. For the expressed chemokine receptors, the median fluorescence intensity (MFI) is given. Table from Krinninger et al. (Krinninger et al., 2014).

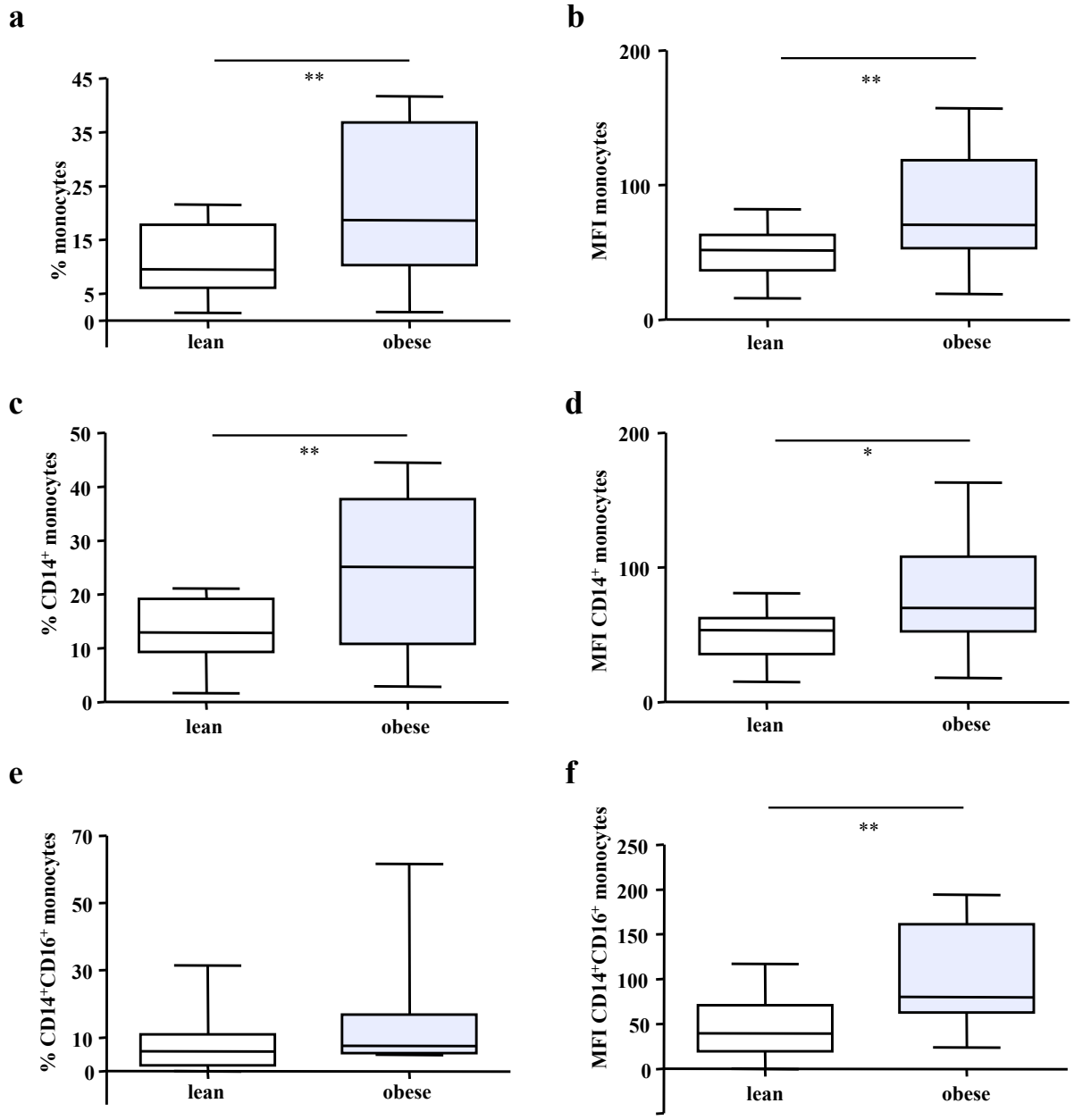

Figure 19: Surface expression and density of chemokine receptor CCR5 on monocytes. Peripheral blood total monocytes (A, B), $CD14^+$ monocytes (C, D) and $CD14^+CD16^+$ monocytes (E, F) of obese subjects (n=14) were compared to lean control subjects (n = 16). Percentages of positive cells (A, C, E) and the receptor surface density (MFI) (B, D, F) are given. Data are presented as median (horizontal bar) with 25^{th} and 75^{th} percentiles (boxes) and extreme values (whiskers). Analysis was performed using independent-sample t-test with *$p < 0.05$, **$p < 0.01$. Figures from Krinninger et al. (Krinninger et al., 2014).

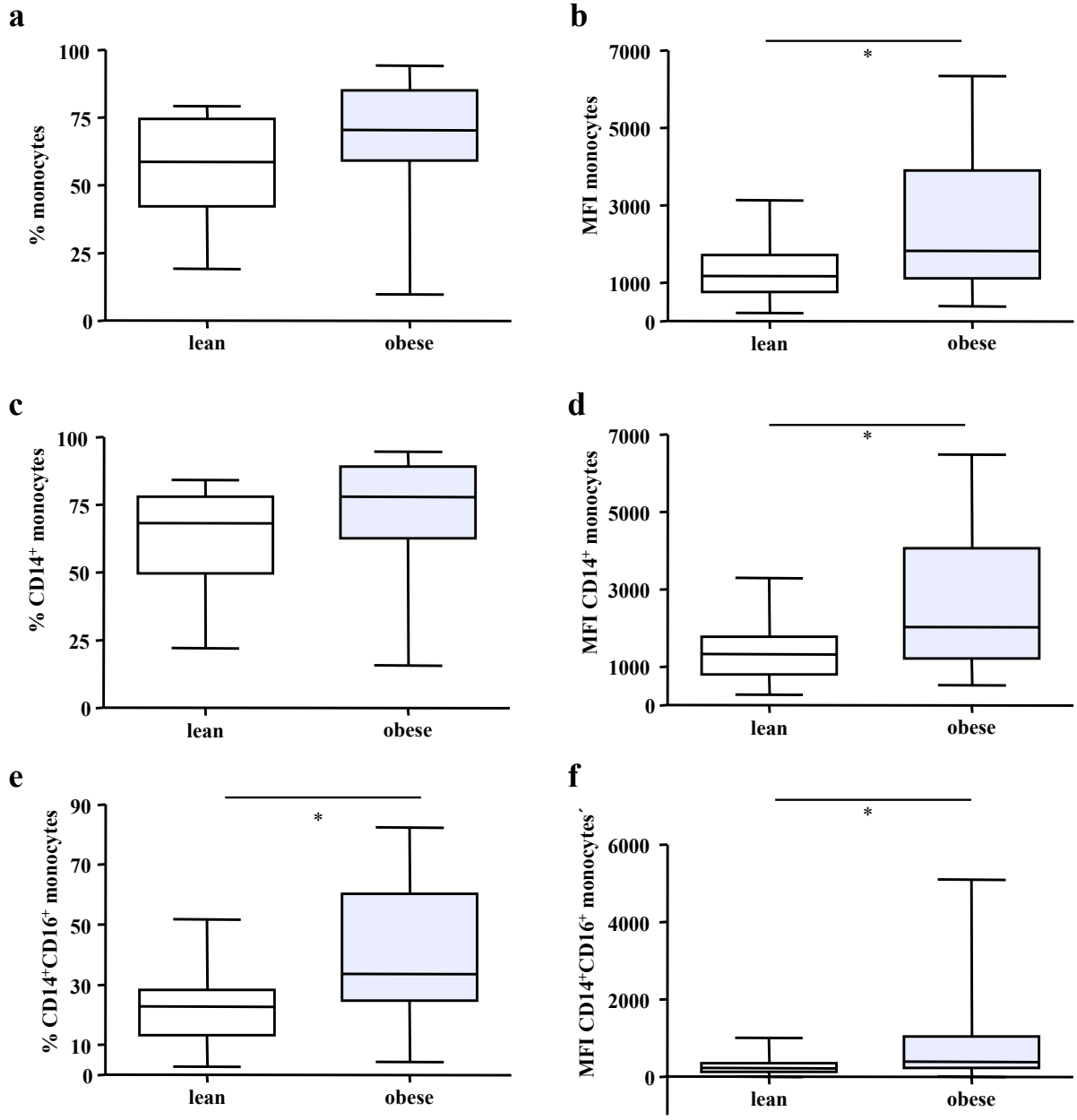

Figure 20: Surface expression and density of chemokine receptor CCR2 on monocytes. Peripheral blood total monocytes (A, B), $CD14^+$ monocytes (C, D) and $CD14^+CD16^+$ monocytes (E, F) of obese subjects (n=14) were compared to lean control subjects (n = 16). Percentages of positive cells (A, C, E) and the receptor surface density (MFI) (B, D, F) are given. Data are presented as median (horizontal bar) with 25^{th} and 75^{th} percentiles (boxes) and extreme values (whiskers). Analysis was performed using independent-sample t-test with $*p < 0.05$, $**p < 0.01$. Figures from Krinninger et al. (Krinninger et al., 2014).

6.2.4 Migration of monocytes

The here-observed increase of chemokine receptor surface expression on monocytes from obese subjects suggests an enhanced responsiveness of these cells to chemokine signaling. Using a migration assay, the migratory response of primary monocytes towards various chemokines was investigated.

The chemokines MCP-1, RANTES and IP-10 have been reported to be upregulated in adipose tissue of obese humans and are thought to play a role in adipose tissue leukocyte infiltration (Herder et al., 2006b, Kamei et al., 2006, Kanda et al., 2006, Wu et al., 2007). Besides induction of leukocytes with these chemokines, additionally a combination of all three chemokines, termed "chemokine-mix", was included to mimic a more physiological situation. A migration medium RPMI-1640 supplemented with a physiologic concentration of glucose (5.5 mM) was used in order to prevent a pro-inflammatory effect of hyperglycemia (Mohanty et al., 2000).

PBMCs from the peripheral blood of 19 normal-weight and 29 obese subjects were isolated and used for migration assays. The assay with the chemokine RANTES was performed with a lower number of participants (8 lean and 12 obese). After two and a half hours migration at 37°C towards the chemokines, the number of migrated monocytes in the lower well was counted under the microscope. The number of cells collected in the lower well containing solely RPMI-1640 was used as control and their migration was set as one to calculate the migration-index.

Monocytes efficiently migrated in response to all chemokines (Figure 21a). The chemokine-mix showed the strongest chemoattractant effect followed by MCP-1, RANTES and IP-10. Comparing the migration-indices of lean versus obese (Table 8), a significantly enhanced migration of monocytes from obese towards the chemokine-mix by 1.5-fold, towards MCP-1 by 1.5-fold and towards RANTES by 2-fold with a trend for increased migration for IP-10 (Figure 21b-e). Furthermore, the migratory activity of the obese group could show a higher inter-individual variability.

Overall, it could be shown that monocytes from obese subjects exhibit an increased migratory ability to diverse chemokines compared to monocytes from normal-weight subjects. Notably, the here-presented data demonstrate that obesity results in a cell-intrinsic increase of monocyte migration-capacity to chemokine stimuli. This activation of monocytes may contribute to the increased infiltration of monocytes to adipose tissue observed in obesity.

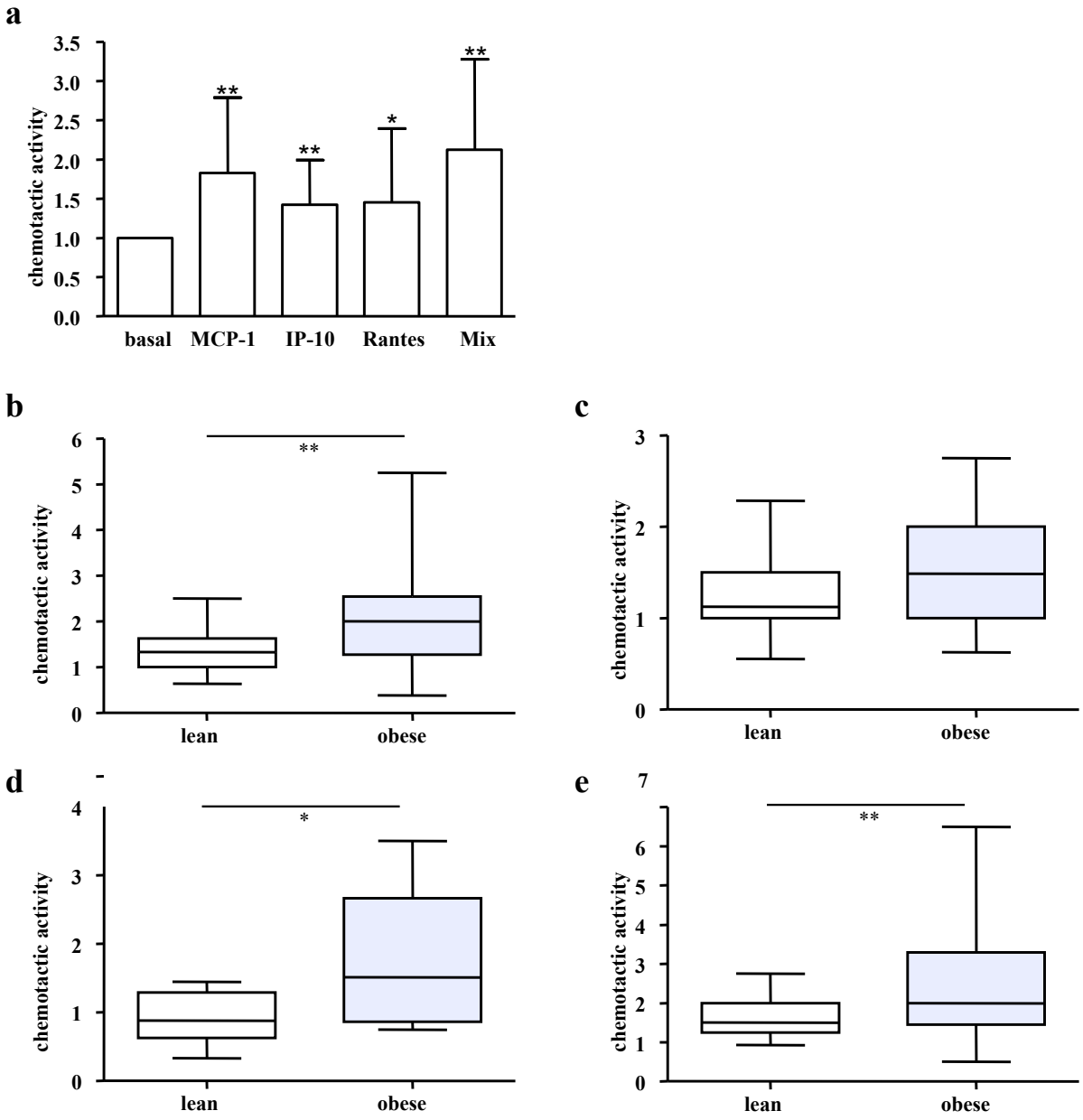

Figure 21: Chemokine-induced monocyte migration. (a) The chemoattractants MCP-1, IP-10, RANTES and a chemokine-mix (MCP-1, IP-10, RANTES) efficiently induced monocyte migration (n=48, n= 20 for RANTES). Data are presented as mean ± SD. Analysis was performed using sample t-test with *p < 0.05, **p < 0.001 (b-e). Migration indices of monocytes from lean and obese women according to the chemokines MCP-1 (B), IP-10 (C), RANTES (D) and the chemokine-mix (E). Lean/obese groups in (B-E) with n=19/29, 19/29, 8/12 and 19/29 subjects, respectively; results are expressed as chemotactic activity (fold increase of monocyte response to stimulants over the basal response to medium alone). Data are presented as median (horizontal bar) with 25th and 75th percentiles (boxes) and extreme values (whiskers). Analysis was performed using independent-sample t-test with *p < 0.05, **p < 0.001. Figures from Krinninger et al. (Krinninger et al., 2014).

Migration index	Lean mean ± SD	n	Obese mean ± SD	n	P-value*
MCP-1	1.4 ± 0.5	19	2.1 ± 1.1	29	**0.005**
IP-10	1.2 ± 0.5	19	1.6 ± 0.6	26	0.059
RANTES	0.9 ± 0.4	8	1.8 ± 1.0	12	**0.017**
Chemokine-mix	1.6 ± 0.5	19	2.4 ± 1.3	29	**0.006**

* Independent-sample t-test.

Table 8: Migrated monocytes from lean women versus from obese women. Migration of monocytes was quantified by counting monocytes in the lower chamber. The results are expressed as migration index (fold increase of monocyte response to stimulants over the response to medium alone). Table from Krinninger et al. (Krinninger et al., 2014).

6.2.5 Correlation of monocytes migration capacity with obesity related phenotypes and chemokine receptor level

Correlation analyses were applied to identify whether the increased migratory ability of monocytes from obese subjects is explained by the variability in anthropometry, metabolic parameters and chemokine receptor expression (Table 9).

The migration responsiveness towards MCP-1, RANTES and the chemokine-mix were significantly positive correlated with BMI and fat mass. No significant correlations were found for the inflammatory marker CRP with any of the investigated chemotactic indices. The chemotactic indices of MCP-1, RANTES and the chemokine-mix did not correlate with the glucose levels. Notably, the chemotactic indices of MCP-1, RANTES and the chemokine-mix revealed a significant correlation with fasting insulin levels and the insulin sensitivity measure HOMA-IR.

Finally, it was investigated whether there is a relationship between the increased migratory capacity of monocytes upon the induction with MCP-1, IP-10 and chemokine-mix and the enhanced chemokine receptor expression in obesity (Table 10/11). For RANTES-induced migration, such correlation analysis was not possible due to the small overlap of the FACS and migration assay study population.

Interestingly, a positive association was found for CCR2 expression on monocytes and monocyte subtypes with migration towards MCP-1 for both the percentages of $CCR2^+$ cells (Table 10) and MFI (Table 11). Correspondingly, migration towards the chemokine mix correlates with the percentages of $CCR2^+$ cells (Figure 22). No correlation was observed with CCR5 expression on monocytes and the migratory ability of monocytes. In conclusion, the cell-intrinsic migratory capacity of monocytes towards the chemokines MCP-1, RANTES and

the chemokine mix seems to be associated with anthropometry markers and insulin resistance as well as the expression of CCR2 on monocytes.

Migration index	**MCP-1**		**RANTES**		**Chemokine-mix**	
	r	P-value	r	P-value	r	P-value
BMI (kg/m^2)	0.339	**0.018**	0.588	**0.006**	0.374	**0.009**
WHR	0.194	0.188	0.228	0.333	0.242	0.097
Fat mass (%)	0.354	**0.015**	0.517	**0.024**	0.324	**0.026**
Fasting blood glucose (mg/dl)	0.140	0.341	0.187	0.431	0.126	0.395
Blood glucose OGTT 1h (mg/dl)	0.004	0.979	0.146	0.539	0.024	0.870
Insulin (mU/l)	0.343	**0.017**	0.479	**0.033**	0.337	**0.019**
HOMA-IR	0.336	**0.020**	0.447	**0.048**	0.329	**0.022**
hsCRP (mg/l)	-0.002	0.990	0.104	0.663	0.137	0.354

P-value and r from Pearson's correlation.

Table 9. Correlation coefficients between migration indices and obesity markers from obese (n=14) and lean subjects (n=14). BMI, body mass index; HOMA-IR, homeostatic model assessment of insulin resistance; hsCRP, high-sensitive CRP; OGTT, oral glucose tolerance test; WHR, waist-to-hip ratio. Table from Krinninger et al. (Krinninger et al., 2014).

Migration index	**MCP-1**		**IP-10**		**Chemokine-mix**	
	r	P-value	r	P-value	r	P-value
Total monocytes						
CCR2	0.406	**0.032**	0.288	0.137	0.484	**0.009**
CCR5	-0.047	0.812	0.145	0.461	0.147	0.455
CCR3	-0.166	0.398	-0.067	0.736	-0.273	0.160
CXCR3	-0.050	0.801	0.094	0.633	0.001	0.996
CD14$^+$ monocytes						
CCR2	0.393	**0.039**	0.322	0.094	0.465	**0.013**
CCR5	-0.048	0.808	0.143	0.468	0.084	0.673
CCR3	-0.135	0.494	0.009	0.963	-0.293	0.131
CXCR3	-0.012	0.952	0.330	0.087	0.108	0.586
CD14$^+$CD16$^+$ monocytes						
CCR2	0.499	**0.017**	0.389	**0.041**	0.627	**<0.001**
CCR5	0.148	0.452	0.027	0.890	0.069	0.726
CCR3	-0.290	0.135	-0.186	0.345	-0.398	**0.036**
CXCR3	-0.082	0.677	-0.023	0.909	-0.293	0.130

P-value and r from Pearson's correlation.

Table 10. Correlation coefficients between migration indices and chemokine receptor expression levels on monocytes and subtypes (percentages of receptor-positive cells). Table from Krinninger et al. (Krinninger et al., 2014).

Migration index	**MCP-1**		**IP-10**		**Chemokine-mix**	
	r	P-value	r	P-value	r	P-value
Total monocytes						
CCR2	0.541	**0.003**	0.328	0.088	0.505	**0.006**
CCR5	-0.082	0.678	0.033	0.868	0.086	0.662
CCR3	-0.083	0.673	-0.077	0.696	-0.248	0.203
CXCR3	-0.024	0.902	0.040	0.839	-0.099	0.615
$CD14^+$monocytes						
CCR2	0.543	**0.003**	0,33	0.087	0.493	**0.008**
CCR5	-0.079	0.688	0.046	0.816	0.111	0.573
CCR3	-0.042	0.830	-0.057	0.774	-0.218	0.265
CXCR3	-0.021	0.917	0.028	0.877	-0.091	0.645
$CD14^+CD16^+$ monocytes						
CCR2	0.332	0.085	0.422	**0.025**	0.555	**0.002**
CCR5	0.004	0.984	-0.041	0.837	-0.019	0.922
CCR3	-0.222	0.257	-0.225	0.250	-0.303	0.117
CXCR3	-0.188	0.338	-0.180	0.360	-0.308	0.111

P-value and r from Pearson's correlation.

Table 11. Correlation coefficients between migration indices and chemokine receptor expression levels on monocytes and subtypes (receptor MFI). Table from Krinninger et al. (Krinninger et al., 2014).

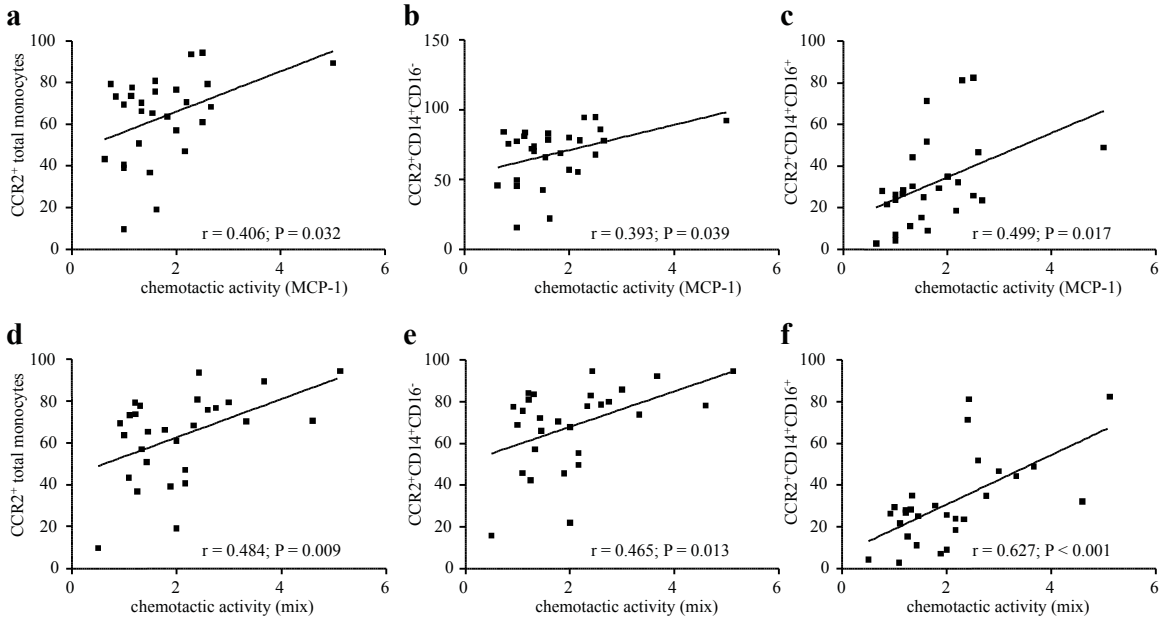

Figure 22: Correlation of CCR2 expression levels with monocyte migration capacity. The correlation of CCR2 chemokine receptor expression (percentage of $CCR2^+$ cells) with migration capacity (shown as migration index) in response to both MCP-1 (a-c) and to chemokine-mix (d-f) is shown for total monocytes (a/d), $CD14^+$ monocytes (b/e), and $CD14^+CD16^+$ monocytes (c/f). Data from 16 lean and 14 obese women; the regression coefficient was obtained from linear regression analysis and correlations between variables are expressed as Pearson's correlation coefficients. Figures from Krinninger et al. (Krinninger et al., 2014).

7. Discussion

7.1 Regulation of IP-10 in preadipocytes and adipocytes and its patho-physiological role in the obese adipose tissue

IP-10 is a CXC chemokine well established as chemoattractant for activated T cells, monocytes, natural killer cells, dendritic cells and eosinophiles via its receptor CXCR3 (Taub et al., 1993). Serum concentrations of IP-10 have been found to correlate positively with BMI and other parameters of obesity (Herder et al., 2006b, Kitahara et al., 2014). Furthermore, elevated concentrations of IP-10 are associated with the incidence of type 2 diabetes (Herder et al., 2006a). Hence, there is growing evidence for the involvement of IP-10 in the chronic inflammatory status observed in type 2 diabetes. Interestingly, Herder et al. could show that IP-10 is produced by mature human adipocytes from various depots (Herder et al., 2006b). However, no characterization on the mechanism of IP-10 regulation in preadipocytes/adipocytes has been reported so far. Furthermore, many questions on the contribution of IP-10 to adipose tissue inflammation and infiltration remain to be answered; the molecular mechanisms underlying adipocyte-mediated lymphocyte recruitment are also largely unclear.

In a first set of experiments IP-10 expression in various depots of white adipose tissue of mice under a high-fat diet was investigated. An enhanced expression of IP-10 in white adipose tissue of obese mice compared with their lean counterparts was demonstrated, suggesting that the adipose tissue may be a major source of the increased plasma levels of IP-10 observed in obesity. This is in line with a study showing increased IP-10 expression levels in the intra-abdominal adipose tissue of type 2 diabetic/obese KKAγ mice (Lee et al., 2009). Upon feeding a high-fat diet, IP-10 expression was significantly stronger upregulated in epididymal (visceral) adipose tissue compared to subcutaneous (inguinal) adipose tissue. Moreover, in humans, a correlation with visceral but not subcutaneous adipose tissue mass and circulating IP-10 levels have been reported (Faber et al., 2013), sustaining the importance of visceral adipose tissue in the pathophysiology of obesity (Ibrahim, 2010).

Adipokines are expressed by several different cell types in adipose tissue (Hauner, 2005). It has been postulated that adipose tissue SV cells, especially preadipocytes, are the main source of pro-inflammatory adipokines. For example MCP-1 and IL-6 secretion have been shown to be upregulated in preadipocytes compared to adipocytes (Bruun et al., 2005, Gerard and Rollins, 2001, Harkins et al., 2004). However, microarray gene profiling of adipocytes or SV

cells from obese versus nonobese Pima Indians also pointed out an active role of mature adipocytes in obesity-related inflammation (Lee et al., 2005, Nair et al., 2005). The here-presented data provide further evidence that adipokines released from mature adipocytes contribute to the local inflammation in obesity. Compared to preadipocytes, IP-10 was predominantly expressed and secreted in fully differentiated adipocytes. Furthermore, IP-10 was also expressed in the SVF in lean mice with increased levels compared to adipocytes. However, under high-fat diet, the IP-10 expression in adipose tissue is turned to equal expression levels when comparing SVF versus adipocytes in obese mice. This illustrates the strong contribution of adipocytes to the obesity-dependent increased expression of IP-10. The here-observed predominant expression and secretion of IP-10 in adipocytes and its upregulation in obesity provides further evidence that adipokines released from freshly isolated mature adipocytes contribute to local inflammation in obesity.

The molecular mechanisms regulating IP-10 expression as well as its contribution to the mild inflammation observed in obese adipose tissue is still a topic that requires further investigations.

Very little has been reported on the upstream signaling mechanism of the IP-10 regulation in adipocytes so far. From studies in other cell types, it is known that the promoter is regulated in a complex way with interrelated roles of the transcription factor binding sites for NFκB and the family of IRF proteins. The IP-10 promoter contains two κB sites and an additional IRF binding site as critical regulatory sequence elements (Ohmori and Hamilton, 1995, Ohmori et al., 1997). IFNγ-mediated induction of IP-10 is observed in most IP-10-expressing cell types, including macrophages and monocytes (Taub et al., 1993). In colonic epithelial cells, IL-1β alone and/or in synergism with IFNγ may play a major role in the induction of IP-10 (Yeruva et al., 2008). However, in skin fibroblasts, TNFα and not IFNγ is the main inducer of IP-10 (Villagomez et al., 2004). These observations show that the regulation of IP-10 by immunological stimuli is highly cell-type-specific.

NFκB is arguably the most important transcription factor for the initiation or progress of numerous human diseases (Verma, 2004). It has been shown to be highly activated at sites of inflammation in several diseases like type 2 diabetes (Arkan et al., 2005). In addition, increasing evidence suggests that adipokine-induced NFκB activation contributes to the development of obesity related insulin resistance and impaired insulin secretion. In 1876 Ebstein demonstrated that sodium salicylate could completely abrogate the symptoms of diabetes (Ebstein, 2002). This anti-hyperglycemic effect of salicylates focused attention on NFκB (Kopp and Ghosh, 1994), since salicylates mainly target NFκB and its upstream kinase

IKK2. Further evidence linking NFκB with insulin resistance is provided by Shoelson et al. who studied a heterozygous deletion of IKK2 gene in mice. In this study reduced IKK2 gene expression was able to correct insulin resistance in obese animals (Yuan et al., 2001). In light of these findings, it was assessed whether NFκB is involved in the regulation of IP-10 in preadipocytes and adipocytes.

LPS and IL-1β are key cytokines for the initiation of pro-inflammatory responses and mediate their inflammatory activities for example through NFκB (Flower et al., 2003, Juge-Aubry et al., 2004, Lin et al., 2000). A number of findings suggest that IL-1β is involved in the development of insulin resistance and type 2 diabetes (Tack et al., 2012). Circulating levels of IL-1β together with IL-6 has been shown to increase the risk of type 2 diabetes (Spranger et al., 2003). Moreover, IL-1β release is enhanced in mouse and human obese adipose tissue (Fain, 2006, Juge-Aubry et al., 2004). IL-1 receptor I knockout mice are protected against high-fat diet-induced adipose tissue inflammation (McGillicuddy et al., 2011). Moreover, antagonizing or neutralizing IL-1β reduces hyperglycemia and tissue inflammation (Larsen et al., 2007).

LPS is an endotoxin commonly found in the outer membrane of gram-negative bacteria. An increase in LPS levels has been observed in subjects with increased fat intake (Amar et al., 2008), suggesting a change in the proportion of gram-negative bacteria in the gut or a change in the gut permeability (Cani et al., 2007, Cani et al., 2008). In turn, LPS can cause a condition of "metabolic endotoxemia" characterized by low-grade inflammation and insulin resistance (Moreno-Indias et al., 2014). In adipocytes LPS can promote activation of the NFκB pathway and expression of several target genes (Berg et al., 2004, Chung et al., 2006). Therefore, IL-1β and LPS are interesting cytokines to study NFκB-dependent IP-10 gene regulation. The responsible signaling mechanisms have not been evaluated in preadipocytes and adipocytes so far. Our experiments revealed that both pro-inflammatory stimuli are able to induce IP-10 secretion by adipocytes. These results are in agreement with recent data demonstrating IL-1β-induced IP-10 expression in a variety of cell types, including epithelial cells (Yeruva et al., 2008), while LPS is a potent inducer for IP-10 in monocytes (Tamassia et al., 2007).

In addition, it was shown that the NFκB-binding site of the IP-10 promoter specifically recruits p65 homodimers or heterodimers in 3T3 fibroblast cell lines (Hoffmann et al., 2003). Here, a supershift assay of 3T3-L1 cells, basal or treated with IL-1β, confirmed the presence of p65 in the complex bound to the consensus κB-2 oligonucleotides. Taken together, these

data support a role of NFκB in IP-10 gene expression, and this is likely to be mainly dependent on the κB-2 site present in its promoter (Ohmori and Hamilton, 1993).

Based on these data and on the growing evidence implicating NFκB as an important factor for IP-10 regulation, NFκB-dependent IP-10 regulation during adipocyte differentiation was further characterized. The increased luciferase-activity of both an IP-10-promoter-driven reporter and a synthetic NFκB-dependent reporter, is in parallel with an increased IP-10 expression/secretion during adipogenesis. This is in line with the known upregulation of p65 during fat cell differentiation. p65 protein levels, RNA expression and DNA-binding activity increased from low levels in preadipocytes to a continuously and an overall increased expression during adipogenesis with a downregulation on day 2 (Berg et al., 2004). This slight downregulation has also been observed for NFκB- and IP-10-promoter activity as well as IP-10 expression and secretion and is most likely due to the effects of dexamethasone and/or insulin in the differentiation cocktail added to the cells at day 0 (Almawi and Melemedjian, 2002, Dandona et al., 2001). Together, the increased IP-10 expression in adipocytes is in agreement with the increase in constitutive activity of the pro-inflammatory transcription factor NFκB and thus supports a NFκB-dependent regulation for IP-10 in adipocytes.

To further investigate the role of NFκB in the regulation of IP-10 in adipose tissue, a synthetic NFκB-inhibitor (Bay117082) was used. Bay117082 is an irreversible inhibitor of IκBα phosphorylation, which results in the downregulation of the cytokine-induced NFκB activation (Pierce et al., 1997). It significantly prevented IL-1β stimulation of IP-10 protein release from 3T3-L1 adipocytes and of IP-10 mRNA expression in mature human adipocytes in a dose-dependent manner, further supporting a central role for NFκB in the regulation of IP-10 in 3T3-L1 adipocytes. Since the specificity of inhibitors is limited, a 3T3-L1 cell line stably expressing a nondegradable mutant of NFκB inhibitor IκBα was established. A retroviral gene transfer was used in order to express a dominant negative interfering IκBα mutant protein (IκBα-mut) or an empty vector as control (mock). Expression of the dominant negative IκBα protein was controlled by western immunoblotting, revealing a strong overexpression of the mutant as compared with the endogenous protein. In the presence of high levels of exogenous dominant negative IκBα protein, expression of endogenous IκBα was barely detectable. This is most likely due to reduction in NFκB activity, resulting in a decreased expression of the NFκB target gene IκBα. In addition, EMSA experiments revealed that the mutant protein is functional active, since it was able to almost completely block the NFκB-DNA binding. In these stably infected cells, IL-1β- or LPS-induced IP-10

expression/secretion was higher in mock adipocytes compared to preadipocytes with no measurable amounts of IP-10 in preadipocyte-conditioned media, indicating that the responsiveness to cytokines might be dependent on the state of differentiation. These data are in accordance with the predominant expression and secretion of IP-10 in naïve (not infected) 3T3-L1 adipocytes mentioned above. However, the preadipocyte/adipocytes difference in IP-10 mRNA expression was more pronounced in the stably infected versus naïve 3T3-L1 cells, indicating an influence of infection on IP-10 expression. However, the amount of secreted IP-10 protein was similar comparing infected versus naïve 3T3-L1. Therefore, it can be assumed that infection and selection of cells influence the expression levels of IP-10, but only marginal its protein secretion.

In adipocytes, the overexpression of IκBα-mut significantly reduced both basal and IL-1β or LPS-stimulated IP-10 mRNA expression, as well as protein release, compared to control cells. However, no significant changes in the induced IP-10 expression/secretion could be observed in preadipocytes, as they produce almost no detectable amounts of IP-10 at all. Thus, NFκB-dependent regulation of IP-10 secretion and expression appears to be a mechanism more important in adipocytes than in preadipocytes. This finding is also consistent with the overall increase in constitutive NFκB signaling as well as changes in NFκB subunit expression and activity during differentiation (Berg et al., 2004).

Thus, the here-presented data further support an important role of adipocytes in the obesity-associated inflammatory process. Although SVF/preadipocytes undoubtedly contribute to the circulating levels of inflammatory factors to some extent (Harkins et al., 2004, Nair et al., 2005, Wu et al., 2007), the here presented results indicate that adipocytes are a dominant fraction in adipose tissue contributing to the increased circulating levels of IP-10 in obesity.

In light of an increased constitutive NFκB activity and IP-10 expression in obesity, the physiological relevance of IP-10 for obesity-associated chronic inflammation was further analyzed. It is now generally accepted that cells of the innate and adaptive immune system are crucially involved in adipose inflammation and systemic metabolic abnormalities (Lee and Lee, 2014). Initial studies focused on the role of macrophages, which were shown to accumulate in adipose tissue of obese mice and humans (Curat et al., 2006, Weisberg et al., 2003). However, lymphocyte infiltration occurs during the early phases of insulin resistance development in high-fat diet-induced obesity and precedes adipose tissue macrophage accumulation (Kintscher et al., 2008). Therefore, pro-inflammatory T lymphocytes may contribute to the local inflammatory cell activation even before appearance of macrophages Recent research more extensively characterized the different subsets of adipose tissue T cells

and their alterations in obesity (Duffaut et al., 2009, Nishimura et al., 2009, Winer et al., 2009).

In obese adipose tissue, a shift towards an inflammatory phenotype of T cells was observed, with an increase in $CD8^+$ and Th1-polarized T cells and a decrease in regulatory T cells (Huh et al., 2014). Furthermore, the accumulation of Th1 cells in obese adipose tissue seems to be antigen driven. Winer et al. and Feuerer et al. both described restricted TCR repertoires in adipose $CD4^+$ T and Treg cells, suggesting that antigens in fat may communicate with the adaptive immune system (Feuerer et al., 2009a, Winer et al., 2009). Indeed, MHC II-mediated antigen presentation by macrophages and adipocytes stimulates polarization of $CD4^+$ T cells towards a Th1 phenotype (Cho et al., 2014, Deng et al., 2013, Morris et al., 2013). In regard to $CD4^+$ and $CD8^+$ T lymphocytes, the data are not yet reconciled. In patients with type 2 diabetes, Kintscher et al. detected only moderate expression of CD8 in white adipose tissue but demonstrated the presence of $CD4^+$ T lymphocytes and a positive correlation between CD4 expression and body weight (Kintscher et al., 2008). In contrast, other studies demonstrate an increase in $CD8^+$ T cells in obese mice, while the $CD4^+$ T cells were diminished (Kwon et al., 2014, Nishimura et al., 2009). These variances may be due to species differences and the dynamic of the interaction of inflammatory cells within white adipose tissue.

Despite the increasing recognition of the participation of T lymphocytes in the pathophysiology of obesity, the molecular mechanisms leading to the trafficking of T cells into adipose tissue are so far poorly understood. For instance, we still do not know how T cell infiltration is initially activated. A functional alteration in Th2 and Treg cells might contribute to it. Moreover, T cell recruitment to sites of inflammation is usually mediated by chemokines released from endothelial cells, stromal cells or macrophages. Thus, chemokines produced by adipocytes could also be important. Therefore, the potential role of NFκB activity and the chemokine IP-10 for T cell infiltration into adipose tissue was determined. Since, in regard to $CD4^+$ and $CD8^+$ T lymphocytes, the data are not yet reconciled, migration of both $CD4^+$ and $CD8^+$ T cell was studied.

Adipose tissue inflammation assessed by the expression of CD68, RANTES and TNFα was shown to correlate with various T cell markers in adipose tissue from obese patients (Zeyda et al., 2011). Therefore, it was interesting to examine to what extent adipokines secreted by 3T3-L1 preadipocytes and adipocytes can activate T cell migration in an NFκB-dependent manner. Here, conditioned media from LPS- or IL-1β-stimulated 3T3-L1 adipocytes could induce both $CD4^+$ and $CD8^+$ T cell migration. This finding was substantiated by the observation that T

cell migration was reduced to basal level by overexpression of dominant-negative IκBα in 3T3-L1 adipocytes, clearly indicating the importance of the pathway for the migration process. However, only a minimal induction of T cell migration was observed in response to supernatants from preadipocytes stimulated with either IL-1β or LPS, and no difference could be observed in the $CD4^+$ and $CD8^+$ T cell migration induced by conditioned media of preadipocytes overexpressing dominant-negative IκBα. Hence, NFκB-dependent activation of T cell migration by adipocytes seems to play a more prominent role compared to preadipocytes. These data are in line with the known upregualtion of NFκB during adipogenesis (Berg et al., 2004). In conclusion, NFκB-regulated adipokines secreted by adipocytes are of central importance for the migration of T cells and are thereby possibly involved in the recruitment into adipose tissue. Indeed, for most of the chemokines secreted by adipose tissue (CCL20, RANTES and IP-10), a contribution of NFκB to their promoter-activity was reported (Bonizzi and Karin, 2004). Moreover, PPARγ activation reduces high-fat diet-induced T lymphocyte infiltration into adipose tissue and development of insulin resistance in mice (Foryst-Ludwig et al., 2010). Since PPARγ is known to inhibit NFκB-dependent transcriptional activation by inhibition of IκBα degradation, reduction of p65 nuclear translocation, and diminished binding of RelA to the DNA these results further sustain our data. Even more, a recently published study demonstrated that fish-oil-derived omega-3 poly unsaturated fatty acids reduce inflammatory- and chemokine-mediated crosstalk between $CD8^+$ T cell and adipocytes together with decreased NFκB p65 activation (Monk et al., 2015).

Together, the here-presented data further support the strong molecular link of adipocyte-specific NFκB activity to inflammation, metabolic dysregulation in adipose tissue and finally diabetes.

Since IP-10 has a potent chemotactic effect on stimulated T cells (Taub et al., 1993), it could be one of the relevant NFκB target genes responsible for the enhanced T cell migration inhibited by overexpression of IκBα-mut in adipocytes. A growing body of evidence suggests that IP-10 plays a role in chronic inflammatory diseases, including atherosclerosis by its chemotactic effect on Th1-primed T cells. IP-10 promotes atherogenesis by modulating the local balance of effector and regulatory T cells. Deletion of IP-10 decreased the $CD4^+$ T cell accumulation in the plaques of ApoE –/– mice, whereas numbers and activity of regulatory T cells were enhanced (Heller et al., 2006). Since atherosclerosis and obesity share common pathophysiological features, a similar functional role for IP-10 in the recruitment of T cells can be postulated. IP-10 primarily acts via its receptor CXCR3 on activated T cells (Farber,

1997, Qin et al., 1998). The here-shown increased expression of CXCR3 in epidydimal and inguinal adipose tissue of obese mice further points out the involvement of the chemokine IP-10 in the enhanced T cell infiltration in obese adipose tissue.

In this study it could be demonstrated that the increased migration of $CD4^+$ and $CD8^+$ T cells towards media from stimulated 3T3-L1 adipocytes is inhibited by the use of an anti-IP-10 antibody in a dose-dependent manner. Therefore, IP-10 seems to be an important factor for recruitment of T cells into adipose tissue and might be a new potent candidate responsible for the development of low-grade inflammation in obesity. The observation of this study might represent an initial step towards understanding the role IP-10 plays in adipose tissue. Beside the role of IP-10 for T cell attraction, it is likely that the basic regulatory principles elucidated here might be shared also by a number of other chemokines genes like RANTES or SDF. These chemokines are reported to be involved in T cell recruitment into adipose tissue (Duffaut et al., 2009, Kintscher et al., 2008, Wu et al., 2007). Both are NFκB target genes and are regulated in obesity. However, there are no data concerning the biological activity of these chemokines secreted by adipocytes.

Moreover, it could be shown here that expression of both IP-10 and its receptor CXCR3 increases during weight gain in mouse adipose tissue in parallel to T cell infiltration shown recently (Kintscher et al., 2008). Notably, CXCR3 is highly expressed on activated T cells (Rocha et al., 2008), mainly on Th1 cells and recent publications indicate that adipose tissue infiltrating T cells represent Th1 cells (Duffaut et al., 2009, Feuerer et al., 2009a, Rocha et al., 2008, Winer et al., 2009). Indeed, in a recently published study, obese CXCR3-deficient mice accumulate fewer T cells in periepididymal adipose tissue compared to obese wild-type mice (Rocha et al., 2014), further sustaining our data.

In conclusion, the present study could demonstrate that IP-10 is upregulated in adipose tissue of a diet-induced obese mouse model. In addition, adipocytes, rather than SVF and preadipocytes significantly contribute to elevated IP-10 levels in obesity. These data clearly show that the NFκB pathway plays a central role for the regulation of IP-10 in 3T3-L1 and primary human adipocytes. These results also provide evidence that NFκB target genes such as IP-10 are major activators of adipose tissue $CD4^+$ and $CD8^+$ T cell migration.

It has not been possible to study intercellular IP-10 protein levels, since commercial antibodies were very unspecific in western blot analysis. Therefore, IP-10 protein release has been measured by ELISA. Moreover, the signal transduction experiments with stable 3T3-L1 cells overexpressing a transdominant IκBα as well as T cell migration experiments could not

be performed in primary human adipocytes. However, the use of the 3T3-L1 line prevents the bias of macrophage contamination that is concomitant to cell purification of human or animal fat tissue samples, and allows the differential analysis of both preadipocytes and fully differentiated adipocytes. To further prove the physiological relevance of IP-10 and NFκB for adipose tissue infiltration, future experiments with cell-type-specific knockout models will be necessary.

7.2 Conclusion and perspective

Taken together, the presented data demonstrate that the NFκB pathway plays a central role for the regulation of IP-10 expression in mouse 3T3-L1 and primary human adipocytes. Of note, adipocytes, rather than preadipocytes, contribute to the obesity-associated elevated IP-10 levels. This work also provides evidence that NFκB target genes and in particular IP-10 are major activators of adipose tissue lymphocyte migration. Thus, these observations highlight the role of IP-10 secreted from adipocytes as a new candidate for the recruitment of T cells into adipose tissue.

Altogether, this study contributes to a better understanding of the molecular and cellular crosstalk and underlying mechanisms in adipose tissue regulating the leukocyte infiltration in adipose tissue in the etiology of obesity, the metabolic syndrome and diabetes. To date, it is generally accepted that activation of IKK2/NFκB signaling pathway in adipose tissue contributes to the pathogenesis of systemic insulin resistance. However, new data suggests that inflammation is also required for energy balance, which is called “beneficial activity” of inflammation in obesity (Ye and McGuinness, 2013). The maintenance of low-grade inflammation may also serve to create a state of persistent insulin resistance to limit the amount of obesity. Indeed, a very recent study could demonstrate that adipocyte-specific IKK2 deletion suppresses adipose tissue inflammation through an IL-13-dependent paracrine feedback pathway (Kwon et al., 2014). A further study even supports different activities of the NFκB pathway in the lean versus obese condition. NFκB is required for expression of pro-inflammatory genes in the lean state. However, in the obese state NFκB exhibits an anti-inflammatory effect though the inhibition of adipocyte apoptosis (Gao et al., 2015). Together, these data support the concept that an impaired local pro-inflammatory response in the adipocyte may also lead to increased ectopic lipid accumulation, glucose intolerance and systemic inflammation. The IKK2/NFκB signaling pathway in adipocytes, in contrast to most other tissues (Arkan et al., 2005, Cai et al., 2005, Yuan et al., 2001), plays an essential anti-inflammatory modulatory role in this context (Kwon et al., 2014, Yuan et al., 2001). In light of these new findings, it would be further necessary to study the NFκB-dependent regulation

of IP-10 in adipocytes and its role for T cell migration under this new point of view. It would be interesting to test whether adipocyte-specific IKK2 or p65 deficiency exert an influence on IP-10 expression and secretion in adipocytes. Another open question is whether a possible modulation of IP-10 regulation can affect distinct T cell subpopulations in these knockout mice. Here it could be demonstrated that expression of both IP-10 and its receptor CXCR3 increases during weight gain in mouse adipose tissue in parallel to T cell infiltration shown recently (Kintscher et al., 2008). Notably, in a very recently published study obese CXCR3-deficient mice accumulate fewer CD4+ and CD8+ T cell in periepididymal adipose tissue than obese wild-type mice. Moreover, these obese CXCR3-deficient mice also had decreased expression of IL-10 (an anti-inflammatory cytokine) and Foxp3 (a Treg cell marker) (Rocha et al., 2014). These results indicate that CXCR3 participates in the accumulation of various T cell subsets into obese adipose tissue, including inflammatory and also anti-inflammatory T cells, like Treg cells. Thus it would also be interesting to examine a possible anti-inflammatory effect of IP-10, via activation of Treg cell infiltration. An adipocyte-specific IP-10 knockout model could provide additional insight into the physiological relevance of IP-10 in the context of adipose tissue inflammatory and anti-inflammatory effects.

7.3 Cell-intrinsic migration capacity and surface chemokine receptor expression in PBMCs from lean versus obese women: results from the AdipoRiSc (Adiposity Risk Screening) study

Obesity is known to represent a state of chronic low-grade inflammation with increased accumulation of leukocytes within adipose tissue. Numerous lines of evidence associate both obesity and insulin resistance, with the activation of adipose tissue-resident immune cells (Winer and Winer, 2012), which have been shown to be derived from bone marrow precursors that migrate via the peripheral circulation into adipose tissue (Weisberg et al., 2003). Excessive energy intake induces both oxidative stress and an increase of NF-κB activity in PBMCs (Mohanty et al., 2000). NF-κB activation and secretion of pro-inflammatory cytokines was also observed in PBMCs from obese individuals (Ghanim et al., 2004), whereas weight loss in individuals with metabolic syndrome was shown to result in a decreased expression of inflammatory genes in PBMCs together with an increase of systemic insulin sensitivity (Crujeiras, 2008). Therefore, in obesity, PBMCs might respond to intra- and extracellular signals in various ways, including a change of leukocyte subsets as well as an increased activation and infiltration of leukocytes into target tissues, such as adipose tissue or arterial intima media.

The here-presented study, a sub-analysis of the AdipoRiSc case-control study, included 60 obese women and 60 normal-weight women. Since FACS analyses of PBMCs and migration assay could only be completed with PBMCs from a limited number of individuals, subgroups had to be defined. In the first part of the work, a comprehensive phenotyping of the study population and subgroups was performed in order to prove the recruitment of homogenous, healthy, representable study populations. There were no differences in age and height. Furthermore, on the basis of the BMI as the only distinguishing factor, a number of obesity-related changes were identified. As expected, all anthropometric and body-composition measurements were significantly elevated in the obese group compared to the control group in both subpopulations. The BMI has been recommended to evaluate overweight and obesity; however, it does not account for variations in body fat distribution. Since abdominal fat correlates with metabolic abnormalities (Demerath et al., 2008, Gastaldelli et al., 2002) WHR was quantified in addition to BMI. In order to assess body fat percentage, two types of devices (BIA and DXA) were applied. Since DXA is the standard technique in the measurement of body composition (Prior et al., 1997), it was used for all further correlations. Furthermore, obesity is associated with impaired glucose metabolism, dyslipidemia and inflammation (Jung and Choi, 2014).

All markers of insulin resistance (insulin levels, fasting glucose levels and HOMA) were significantly higher in the obese group compared to the lean group, indicating impaired insulin sensitivity. However, fasting glucose levels were significantly lower in the lean group compared to the obese group, indicating impaired fasting glucose tolerance. This phenotype has not been observed in the FACS analysis subpopuplation, which might be due to the limited number of subjects. Glucose tolerance was assessed by a standardized two-hour OGTT. There were no differences in 1h and 2h postload glucose levels between both groups, excluding type 2 diabetes. However, no type 2 diabetes was manifested yet. Hypertriglyceridemia, reduced HDL, elevated LDL as well as FFA levels characterize the dyslipidemia associated with obesity (Mooradian et al., 2008). In this study, the obese group also exhibited a tendency towards dyslipidemia by showing significantly increased TG, FFA, total cholesterol and LDL levels, whereas HDL levels were significantly decreased compared to the control group. As mentioned above, obesity can be regarded as a state of low-grade inflammation. CRP is an acute phase protein that is widely used to predict chronic inflammatory conditions in clinical settings. In line with literature, the CRP level was about seven times higher in the obese group compared to the control group, demonstrating an inflammatory process (Aronson et al., 2004). Furthermore, a recent study performed in female obese adolescents showed that leukocyte count is positively related to BMI, WC and fat mass (Kim and Park, 2008). However, Desai et al. failed to demonstrate a correlation between BMI and leukocyte count (Desai et al., 2006). These results are in line with the here-presented data; leukocyte count did not show a statistically significant change in obesity.

In summary, both subpopulations had similar values in respect to anthropometric and metabolic characteristics. The population analyzed here represents homogenous and healthy study groups without indication of chronic or acute diseases, but with nonetheless all expected obesity-related alterations, including impaired insulin sensitivity in the obese. Thus, the recruited individuals match the requirements necessary to analyze the effects of obesity on functional response in peripheral immune cells. The study is restricted to one gender in order to avoid an influence of sex-specific differences in the small cohort.

To determine the relative contribution of obesity to systemic inflammation, this study comprehensively characterized circulating immune cell phenotypes. No differences were observed regarding T cells, T helper cells and monocytes percentages in PBMCs of obese compared to normal-weight women. However, there was a significant upregulation of activated $CD4^+CD25^+$ T cells and inflammatory $CD14^+CD16^+$ monocytes in obese subjects compared to lean subjects, indicating that PBMCs from obese are already in an activated

state. Low-grade inflammation in obesity is considered to be accompanied by an activated innate immune system, with activated circulating (van Oostrom et al., 2004) and adipose tissue-resident innate immune cells (Wellen and Hotamisligil, 2005). However, the contribution of the adaptive immune system, especially T cells, has not been sufficiently investigated.

CD25, the alpha chain of the trimeric IL-2 receptor, is expressed by T lymphocytes after triggering of the T cell receptor (Poulton et al., 1988). The expression of CD25 on T cells occurs within 2–24 h after stimulation and persists for only a few days after decline of stimulating antigens (Poulton et al., 1988). The presence of CD25 on T lymphocytes, therefore, serves as a maker for recent T cell activation. Variations in CD25 expression on T cells have been reported in diseases in which immunological activation plays an important role in the pathogenesis, such as acute coronary syndrome (Neri Serneri et al., 1997), atheriosclerosis, rheumatoid arthritis (Wood et al., 1988) and Crohn's disease (Crabtree et al., 1990). Here, the effect of obesity on the expression of the leukocyte activation marker CD25 has been investigated. The presented results demonstrate that circulating $CD4^+$ T cells from obese subjects have a significant enhanced percentage of $CD25^+$ T cells compared with lean subjects, indicating a recent stimulation of these cells in a cell-mediated immune response. This is in accordance with studies published during progression of this work, demonstrating an increase in peripheral $CD25^+$ T cells in obesity and type 2 diabetes (van Beek et al., 2014, Viardot et al., 2012). Together these results provide evidence that the circulating adaptive immune cells are also activated in obesity. Similarly, energy restriction with weight loss significantly reduced the surface expression of activation markers on circulating T lymphocytes (CD25 and CD69) (Viardot et al., 2010). Moreover, gastric bypass decreased T lymphocyte CD69 and CD95 expression, indicative of reduced T lymphocyte activation (Cottam et al., 2003). The increased T cell activation in obesity could suggest that clonal expansion had taken place. However, detailed analysis of T cell receptor recombination should be performed to proof this assumption. Furthermore, identification of antigens responsible for triggering this process in the circulation will be necessary. Given the fact that T cell receptors can recognize lipid antigens, it is conceivable that lipid antigens may direct this immune response (Cao et al., 2008).

In addition, other candidate antigens have to be taken into account and also non-antigen related T cell activation, for example by the adipocyte-secreted protein leptin. Costimulated by PHA (Polyhydroxyalkanoate) or Con A (Concanavalin A), leptin has been shown to enhance activation and proliferation of human circulating T lymphocytes (Martin-Romero et

al., 2000). The question of how these T cells can be activated in the context of obesity remains to be investigated.

Although expression of activation marker CD25 indicates a recent change in local T cell activation (Poulton et al., 1988), here we have to note that $CD4^+CD25^+$ T cells are a population containing two subpopulations: activated T cells ($FoxP3^-$) and Treg cells ($FoxP3^+$). Human Treg cells comprise 5-10% of peripheral $CD4^+$ T cells (Baecher-Allan et al., 2001). Since intracellular FoxP3 staining was not included in our study, we cannot further differentiate activated T cells and Tregs. Therefore, our data can also partially reflect the recently reported increased number of Treg cell in peripheral blood (van der Weerd et al., 2012). Taken together, T cell activation in the circulation may be another feature of the complex of immunological processes associated with obesity.

Accumulation of adipose tissue macrophages is another hallmark observed in obesity, and diverse lines of evidence show the contribution to the development of insulin resistance and obesity-related disorders (Lee and Lee, 2014). *In vivo*, tissue macrophages are derived from circulating monocytes recruited in the tissues by constitutive or inflammatory signals. The existence of morphologically and functionally distinct monocyte subsets has been defined (Passlick et al., 1989). In principle, monocytes can be distinguished by the expression of the LPS receptor CD14 and the Fcγ receptor CD16. $CD16^+$ monocytes can be further subdivided into $CD14^{++}CD16^+$ and $CD14^+CD16^+$ cells. These later two subsets can be summarized as $CD16^+$ monocytes. $CD16^+$ monocytes were shown to be potent producers of pro-inflammatory cytokines, and an expansion of this subset was noted in multiple inflammatory disorders, e.g. rheumatoid arthritis, cardiovascular disease and atherosclerosis. $CD14^+CD16^-$ monocytes constitute approximately 80% of the circulating population (Ziegler-Heitbrock, 2006). In addition, they differ in the pattern of antigen presentation and migration behavior. Moreover, transcriptomic approaches revealed major differences among diverse monocyte subsets. CD16+ monocyte subsets were linked to Antigen processing, presentation and inflammation (Wong et al., 2011, Zawada et al., 2011). Previous studies indicated that the maturation of monocytes is reflected in increased surface expression of CD16 and diminished expression of CD14, suggesting that $CD14^+CD16^-$ monocytes may mature from $CD14^+CD16^+$ monocytes (Ziegler-Heitbrock, 2006). In 2003, it was appreciated to further subdivide $CD16^+$ monocytes into $CD14^{++}CD16^+$ and $CD14^+CD16^+$ cells (Ancuta et al., 2003), which was not analyzed in this study, as the amount of cells measured in experiments was not sufficient to properly define this subpopulation.

In the present study, potential associations between monocyte subsets and obesity were determined. Staining with anti-CD14 and anti-CD16 monoclonal antibodies distinguished two monocyte subsets: $CD14^+CD16^-$ and $CD14^+CD16^+$, representing 87.30 ± 4.56 and 12.70 ± 4.56 of total monocytes from lean subjects, respectively, consistent with previous reports (Rogacev et al., 2010). Moreover, a significant increase in the percentage of circulating $CD14^+CD16^+$ monocytes in obese women compared to lean women could be demonstrated. There were no differences in total monocyte percentages, indicating a subset-specific effect.

These results are in line with studies published during progression of this thesis. Rogacev et al. could also show a significant association between counts of $CD14^+CD16^+$ monocytes - but not of total monocytes - and obesity as well as subclinical atherosclerosis in a cohort of healthy individuals (Rogacev et al., 2010). A significant increase of $CD14^+CD16^+$ monocytes has also been demonstrated in obese and obese diabetic subjects compared to normal-weight subjects (Poitou et al., 2011). Furthermore, bariatric surgery with subsequent weight loss led to the lowering of pre-interventional elevated $CD14^+CD16^+$ monocyte counts (Cottam et al., 2003). Together, these data support the interpretation that elevated circulating levels of $CD14^+CD16^+$ monocytes are associated with obesity. The high frequency of $CD14^+CD16^+$ cells might be a relevant, valuable predictor for obesity-associated inflammation. However, further investigations should study the mechanisms by which adipose tissue affects monocyte heterogeneity and how monocyte heterogeneity could have an effect on macrophage polarization within adipose tissue.

Similar to the distinction of different monocyte subpopulations, recent studies have also pointed to the heterogeneity of macrophages infiltrated into obese adipose tissue. Macrophages can be divided into at least two subgroups: “classical activate” M1 macrophages and “alternatively activated” M2 macrophages (Mosser, 2003). The phenotypic characterization of monocytes, the circulating precursors of macrophages, might influence the heterogeneity of adipose tissue macrophages. In mice, circulating monocytes can also be distinguished into distinct subpopulations based on specific surface markers. Inflammatory monocytes, $CCR2^{high}$ $Ly6C^+$ monocytes and resident, $CCR2^{low}$ $Ly6C^+$ monocytes are generally thought to preferentially differentiate into M1 inflammatory and M2 anti-inflammatory macrophages, respectively (Ingersoll et al., 2011). Thus, the increased frequency of $CD14^+CD16^+$ circulating monocytes might influence M1 polarization in human adipose tissue in the obese state. This effect remains to be elucidated in more detail, whether and how monocyte phenotype can alter adipose tissue macrophage heterogeneity in obesity.

Chemokines mediate innate and adaptive immune responses by their ability to recruit, activate and costimulate T cells and monocytes. In various mouse models of inflammation, chemokine receptor deficiency could show a lower disease activity (Charo and Ransohoff, 2006). Therefore, investigations of chemokine receptor expression patterns may help to extend our knowledge and understanding of the complex and cell-type-specific functions of immune cells involved in obesity-associated disorders. Regarding adipose tissue infiltration, to date, most studies were dealing with chemokine and chemokine receptor expression in tissue resident cells. For monocyte migration into adipose tissue, it has been supposed to be induced by numerous chemokines, which are upregulated in obese adipose tissue (Ito et al., 2008, Kamei et al., 2006, Kanda et al., 2006, Keophiphath et al., 2009, Lumeng et al., 2007, Weisberg et al., 2006, Wu et al., 2007), and an upregulation of the chemokine receptors CCR1, CCR2, CCR3, and CCR5 expression was described in adipose tissue of obese individuals (Huber et al., 2008). However, it remains unclear whether this upregulation is restricted to adipose tissue or can also be found in peripheral blood. Therefore, in this study the expression of chemokine receptors on various T cell and monocyte subsets was examined by FACS. The analysis included the chemokine receptors CCR2, CCR5, CXCR3 and CCR3.

Comparison of chemokine receptor expression between various cell types reflects the known variability of expression patterns on different leukocyte subsets (Charo and Ransohoff, 2006). Only a minority of $CD4^+$ T cells and $CD4^+CD25^+$ T cells stained positive for CCR5, CCR2 and CCR3, whereas monocytes express higher levels of CCR5 and CCR2. In contrast, a high proportion of T cells in the peripheral blood expressed the chemokine receptor CXCR3 and only a marginal expression was observed on monocytes. Comparing normal-weight and obese subjects, no obvious differences in chemokine receptor expression on T cells could be detected. Moreover, the repertoire of chemokine receptors are differentially expressed on Th1 and Th2 effector cells. Th1 cells predominantly express CXCR3 or CCR5*,* whereas Th2 cells favor CCR3 and CCR4 chemokine receptor expression (Sallusto et al., 1998). A role of imbalance and dynamic switching of Th1/Th2 has been proposed in the obese adipose tissue, with a shift towards inflammatory Th1-polarized cells (Feuerer et al., 2009a, Winer et al., 2009). Furthermore, an association of Th1 to Th2 ratio with weight and weight loss in morbidly obese male subjects with type 2 diabetes or impaired glucose tolerance was demonstrated (Viardot et al., 2010). Also, a predominance of Th1 cells in peripheral blood of obese is shown to be associated with insulin resistance (Viardot et al., 2012). However, in this study no changes in Th1-associated chemokine receptor expression could be observed. Therefore, it is likely that a shift towards Th1-polarized circulating T cells is rather associated with insulin resistance than BMI.

Whereas T cells show no obvious differences in chemokine receptor expression, the chemokine receptor profile on monocytes differed significantly in the obese state. Both receptors CCR5 and CCR2 could show a significant increase in obese subjects. However, the differences in CCR3 and CXCR3 expression on monocytes did not reach statistical significance.

Numerous lines of evidence indicate an essential role for CCR2 in the recruitment of adipose tissue macrophages, which have been shown to be derived from bone marrow precursors (Weisberg et al., 2003). However, it is still unknown on which cell type CCR2 expression exerts its effect. A recently published study demonstrated that CCR2 in bone marrow cells plays an important role in the recruitment of macrophages into obese adipose tissue (Ito et al., 2008). In addition, adipose tissue macrophages that are newly recruited to the adipose tissue during high-fat feeding exhibit higher expression of CCR2 compared with resident adipose tissue macrophages (Lumeng et al., 2007). Adipose tissue macrophages from obese CCR2-/- mice, which display significantly lower inflammatory gene expression compared with their wild-type counterparts, express M2 markers at levels comparable to those in lean mice, suggesting that the MCP-1/CCR2 pathway contributes to macrophage polarization in the adipose tissue (Lumeng et al., 2007). This observation is consistent with the evidence that resident tissue macrophages in normal conditions are derived from CCR2– monocytes, while macrophages recruited to sites of inflammation are derived from $CCR2^{+}$ monocytes (Geissmann et al., 2003). However, although macrophages infiltrated into obese adipose tissue have been reported to be mostly derived from bone marrow (Weisberg et al., 2003), there is no direct evidence for the contribution of CCR2 to the recruitment of bone marrow-derived macrophages into obese adipose tissue. The here-presented results confirm the importance of CCR2 in obesity and underscore the role of CCR2 on bone-marrow-derived cells. Furthermore, the increase in the percentage of CCR2-positive monocytes is in agreement with a previous report demonstrating increased expression levels of monocyte CCR2 and MCP-1 in patients with type 2 diabetes (Mine et al., 2006). A recently published study by Oh et al. also demonstrated the importance of CCR2 expression on monocyte for the recruitment of these cells into the obese adipose tissue. However, the adoptive transfer experiments of CCR2-deficient monocytes in mice suggested a predominant role of adipose tissue signals versus monocyte preactivation for adipose tissue infiltration (Oh et al., 2011); thus, future experiments are needed to further elucidate the contribution of monocyte preactivation for adipose tissue infiltration in humans. Although CCR2/MCP-1 is important for adipose tissue macrophage accumulation, deletion of these molecules only decreases

macrophage recruitment to adipose tissue by ~40%, indicating that there are one or more additional chemotactic signals that are key to this process.

An important role of CCR5 and its ligand RANTES for leukocyte recruitment into adipose tissue and the association with insulin resistance have been shown in diverse studies (Keophiphath et al., 2009, Wu et al., 2007). Nevertheless, its role in obesity is still not well understood. Here, the expression of CCR5 on freshly isolated mononuclear cells was studied in relation to obesity. Significantly increased CCR5 expression on monocytes was detected in obese women when compared with non-obese. These results are very consistent with the studies mentioned above showing increased expression of CCR5 in obesity. However, the surface expression, analyzed by FACS, appeared to be highly cell-type-specific and with a high inter-individual variability in obese women. This issue will be discussed later in more detail.

In this study, only female subjects were included. Since obese female mice exhibited less white adipose tissue inflammation and lower levels of RANTES and CCR5 expression and were also less resistant to insulin than their male counterparts (Wu et al., 2007), it would be interesting to investigate whether such gender-specific effect can also be seen in humans in regard to CCR5 expression on monocytes. Other organs involved in metabolic responses are likely to be affected by similar cellular, molecular or endocrine pathways, as suggested by Lazar (Lazar, 2006). The most striking similarities relate to atherosclerosis. Both are characterized as states of low-grade inflammation with a crucial role of leukocyte infiltration. The data of the here presented study are in accordance with a study by Potteaux showing that CCR5 deficiency in bone-marrow-derived cells reduced macrophage content in atherosclerosis (Potteaux et al., 2006). Moreover, a very recently published study demonstrated that genetic deletion of CCR5 in mice protected against high-fat diet-induced macrophage infiltration, insulin resistance and hepatic steatosis (Kitade et al., 2012).

Both receptors CCR2 and CCR5 modulate functions on monocytes/macrophages and both are involved in the pathogenesis of immunologic disorders, e.g. atherosclerosis, rheumatoid arthritis and Crohn's diseases (Zhao, 2010). However, they are expressed on monocytes/macrophages in a complementary manner. CCR2 is predominantly expressed on blood monocytes (Geissmann et al., 2003), whereas CCR5 is predominantly expressed on tissue macrophages (Wu et al., 1997). Furthermore, recent evidence demonstrated a reciprocal pattern of expression for CCR2 versus CCR5 during differentiation of monocytes. Studies have shown a downregulation of CCR2 expression and an upregulation of CCR5 during monocyte differentiation into macrophages (Fantuzzi et al., 1999, Kaufmann et al., 2001).

This implies that the two receptors exert different effects on monocytes and macrophages. Recent studies have also shown that CCR2 and CCR5 are expressed on separate subsets of blood monocytes. $CD14^+CD16^+$ subset lacks surface expression of CCR2 and shows significantly higher surface expression of CCR5. In comparison to $CD14^+$ monocytes, $CD14^+CD16^+$ cells expressed lower CCR2 but higher CCR5 transcript levels, whereas CCR1 levels were equivalent (Weber et al., 2000). Taking monocyte heterogeneity into account, in this study, $CD14^+$ monocytes were strongly positive for the CCR2, and its expression in $CD14^+CD16^+$ cells was diminished. CCR2 and CCR3/CXCR3 show an inversely distributed surface expression on monocytes. CCR5 expression was slightly decreased in inflammatory monocytes compared to classical monocytes. This aspect is in contrast to other studies where a clear upregulation in inflammatory $CD14^+CD16^+$ monocytes could be observed (Weber et al., 2000). This effect might be due to the, in our study, missing sub-phenotyping of $CD16^+$ monocytes into $CD14^{++}CD16^+$ and $CD14^+CD16^+$ cells.

Comparing chemokine receptor expression of lean versus obese women a significantly increased percentage of $CCR5^+$ cells solely for the $CD14^+CD16^-$ monocyte population was found, whereas the percentage of CCR2 positive cells was specifically increased in the $CD14^+CD16^+$ monocytes. Moreover, a concomitant upregulation of CCR5- and CCR2-receptor surface densities was found on both $CD14^+CD16^-$ and $CD14^+CD16^+$ monocytes. Likewise, the surface density of CCR2 and CCR5 is also significantly increased for both monocyte subsets. In addition to the well-established role of both receptors in adipose tissue from obese subjects, the finding of increased chemokine receptor levels CCR2 and CCR5 on distinct monocyte subpopulations corroborates an obesity-related functional role of these receptors also on peripheral leukocytes. The mechanisms leading to enhanced CCR2 and CCR5 expression in various monocyte subsets remain to be investigated. Leukocyte migration into adipose tissue has been shown to be mediated by chemoattractant substances (Charo and Ransohoff, 2006). Various chemokines including MCP-1 and RANTES, are upregulated in different depots of adipose tissue. Serum levels of these chemokines are dramatically increased in obese versus lean individuals. Expression of chemokine receptors CCR1, CCR2, CCR3 and CCR5 is elevated on inflammatory cells in omental and subcutaneous adipose tissues of obese patients (Yao et al., 2014). Moreover, conditioned medium from visceral adipose tissue of obese subjects or the addition of RANTES induced adhesion and transmigration of human blood monocytes through adipose tissue endothelium (Keophiphath et al., 2009). Therefore, it might be possible that the increased expression of CCR2 and CCR5 on various monocyte subsets is a response to changes in chemokines that are altered during the progression to obesity.

Due to the increased chemokine receptor expression, it has been further hypothesized that enhanced migration of monocytes into adipose tissue in obesity is not exclusively induced by the elevated production of adipose tissue chemokines. Rather, it was suggested that monocytes from obese subjects also might differ in their ability to migrate compared to monocytes from normal-weight subjects. It has been reported previously that mice harboring a myeloid-specific IKK2 (Arkan et al., 2005) or JNK1 (Solinas et al., 2007) deletion, placed on high-fat diets, become just as obese as wild-type animals but are protected from obesity-induced glucose intolerance and hyperinsulinemia. In addition, hyperinsulinemic/euglycemic clamp studies showed enhanced insulin sensitivity compared with wild-type mice in skeletal muscle, liver and adipose tissue. These knockout animals also display decreased adipose tissue macrophages content and dramatically reduced inflammatory pathway gene expression with decreased tissue cytokine levels in adipose tissue, liver and even muscle tissue. Therefore, these knockout mice are protected from global insulin resistance, probably partly by the reduced macrophage migration into adipose tissue (Arkan et al., 2005, Solinas et al., 2007). Furthermore, Ghanim etl a. could demonstrate that PBMCs from obese humans are in a pro-inflammatory state (Ghanim et al., 2004). These cells express increased amounts of pro-inflammatory cytokines and related factors and show an increased activity of NFκB, which might also affect the migration behavior of lymphocytes. These results together with the increased chemokine receptor surface expression on monocytes of obese women, implicate that monocytes from obese might have increased migratory properties. In order to answer this question, here a chemotaxis assay was performed and the migration behavior of monocytes from lean compared to obese women towards various chemokines was investigated. Furthermore, the association of migration capacity and systemic inflammation and/or insulin resistance was examined.

The different chemokines used in this chemotaxis assay are all known to be secreted by preadipocytes/adipocytes, and their expression and secretion is enhanced in the obese state (Herder et al., 2006b, Kamei et al., 2006, Kanda et al., 2006, Wu et al., 2007). MCP-1 is one of the most reasonable candidate chemokines with regard to its predominant action on monocyte migration and its upregulation in adipose tissue of obese and in atherosclerotic plaques. Additionally, chemokines were selected with different cell-type specificity, i.e. RANTES described to induce both monocyte and T cell migration and IP-10 known to induce predominantly T cell migration (Charo and Ransohoff, 2006). Moreover, a mix of all three chemokines was used, likely to represent more the in vivo situation. The different chemokines varied in their ability to attract monocytes. IP-10 could only show a weak influence on monocyte migration, and there was no significant difference between both groups as

expected, since only a small population of blood monocytes was found to express the IP-10 receptor CXCR3 with no altered expression in obesity. These data confirm the predominant action of IP-10/CXCR10 on T cell migration (Dufour et al., 2002, Janatpour, 2001). The chemokine mix induced the strongest migratory effect, followed by MCP-1 and RANTES. Comparing both groups, a significantly higher chemotactic activity was observed in the migration assay towards the chemokines MCP-1, RANTES and the chemokine mix (including MCP-1, IP-10 and RANTES) in the obese group. For both chemokines, increased expression levels were reported in adipose tissue of obese (Kamei et al., 2006, Kanda et al., 2006, Madani et al., 2009, Skurk et al., 2004). Moreover, these data are in accordance with the enhanced expression of the MCP-1 receptor CCR2 and confirm studies demonstrating that genetic deficiencies of CCR2 and MCP-1 in mouse models reduce macrophage accumulation in adipose tissue in animals fed a high-fat diet (Kamei et al., 2006, Kanda et al., 2006, Weisberg et al., 2003). The increased migratory capacity of monocytes from obese is in line with the enhanced expression of the receptors CCR2 and CCR5. Therefore, the elevated expression of these receptors in obese subjects might cause the increased monocyte migration capacity compared to normal-weight subjects. This activation of peripheral monocytes may contribute to the increased adipose tissue monocytes infiltration in obesity. The relationship of migration capacity and anthropometric and metabolic variables as well as chemokine receptor expression was examined by correlation analyses. Both BMI and fat mass were found to correlate positively with the migratory activities towards MCP-1, RANTES and the chemokine-mix. However, the WHR did not show any association with these parameters, indicating that fat mass might play a more prominent role than body fat distribution in this context. Moreover, it was assumed that the elevated chemokine-induced migration of monocytes into obese adipose tissue might contribute to the elevated circulating inflammation markers (Trayhurn and Wood, 2005), or that the systemic inflammation might enhance the inflammatory state of the PBMCs and thereby intensifies the monocyte migration into adipose tissue. However, no association between monocyte migration and CRP level has been found. The lack of correlation might be due to an inappropriate choice of inflammation marker. CRP is rather a marker of systemic inflammation and is not elevated in cases of localized inflammation (Pfafflin and Schleicher, 2009). Therefore, the inflamed adipose tissue related to monocyte migration might not be displayed by CRP. This is in agreement with the findings of Sourris et al., who did not find an association of CRP and NFκB activity in PBMCs of humans (Sourris et al., 2009). A potential biomarker to examine the inflammatory state could be the NFκB activity in PBMCs measured by EMSA (Ghanim et al., 2004). Furthermore, a relationship between pro-inflammatory PBMCs and insulin resistance has been proposed (Sourris et al., 2009). Insulin resistance in obese mice is associated with the recruitment and

pro-inflammatory activation of adipose tissue macrophages (Cancello et al., 2005, Lumeng et al., 2007, Weisberg et al., 2003). These data are in line with the here-presented results. The migration capacity of monocytes towards the chemoattractants MCP-1, RANTES and the chemokine mix correlated with HOMA-IR, a marker of insulin resistance. In addition, the enhanced migratory ability could be due to an enhanced chemokine receptor expression. Studies demonstrated that a 20% increase of CCR2 on the cell surface almost doubled the chemotactic response to MCP-1 (Han et al., 1998, Tangirala et al., 1997). Furthermore, adipose tissue macrophages that are newly recruited to the adipose tissue during high-fat diet feeding exhibit higher expression of the MCP-1 receptor CCR2 compared with resident adipose tissue macrophages (Geissmann et al., 2003). These findings together with the increased CCR2 expression on monocytes in obesity and its correlation with migratory capacity towards MCP-1, RANTES, IP-10 and the chemokine mix, suggest that the increased macrophage accumulation in obese adipose tissue could be due to, at least in part, an enhanced chemotactic response caused by upregulation of CCR2 expression on monocytes. Since the CCR2 expression on monocytes could show an association with the migratory ability towards all tested chemokines, the chemotactic response seems to be dependent on the presence of CCR2. As mentioned above, there is a significant increase in CCR5 expression on monocytes in obesity, whereas CCR3 could not show any difference. However, there is no correlation between CCR5 expression on monocytes and the migration towards RANTES. Since CCR5 binds further ligands, like MIP-1α, MIP-1β and MCP-2, it would also be interesting to investigate the migratory ability of monocytes towards these chemokines in future. In addition, RANTES is known to act through CCR1, CCR5 and CCR3. Therefore, CCR1 might be the relevant receptor in this context. It is also known to be the most highly expressed one of the three RANTES receptors in human monocytes/macrophages and adipose tissue (Keophiphath et al., 2009). Therefore, it will also be informative to characterize the relationship of this chemokine receptor to the enhanced migration of monocytes from obese towards RANTES. In conclusion, monocytes from obese could also show an enhanced intrinsic migratory capacity probably due to the increased insulin resistance state and enhanced chemokine receptor expression, in particular CCR2.

The high inter-individual variability seen for most of the results in the obese group might be due to different obesity subgroups. The majority of individuals with obesity develops insulin resistance, type 2 diabetes, dyslipidemia, gout, hypertension and cardiovascular disease. However, approximately 10–25% of obese individuals are metabolically healthy. Despite the well-known associations of obesity with related biomarkers and health complications, it is recognized that not all obese individuals show such laboratory abnormalities or develop

metabolic complications (Stefan et al., 2013). One subset of individuals called " the metabolically healthy but obese" are characterized by high levels of insulin sensitivity, no signs of hypertension, as well as a favorable lipid, inflammation, and immune profile However, the mechanisms that could explain the favorable metabolic profile of metabolically healthy but obese individuals, is still poorly understood (Seo and Rhee, 2014). Recent studies suggest that inflammation of visceral adipose tissue, ectopic fat deposition and adipose tissue dysfunction mediate insulin resistance in human obesity independently of total body fat mass (Bluher, 2010). These research data can explain our high inter-individual variability found in the obese group.

However, due to the limited number of subjects, it has not been possible to allow subgroup analysis. In future studies it would be interesting to define such subgroups and to further investigate the impact of ectopic fat deposition on the here-investigated effects.

7.4 Conclusion and perspectives

In conclusion, this study demonstrates that obesity is associated with activated phenotypes in both adaptive and innate immune cells. These data highlight a significant upregulation of CCR2 and CCR5 expression on peripheral monocytes and monocyte subtypes in obese women, which provides further evidence for an important role of these chemokine receptors in the development of obesity-associated low-grade inflammation. The mechanisms leading to enhanced CCR2 and CCR5 expression in monocyte are still unknown.

Further studies are needed to fully dissect the differential impact of obesity, insulin resistance and hyperglycemia on chemokine receptor expression. Since an essential role of both receptors CCR2 and CCR5 in the tissue recruitment of monocytes/macrophages has been demonstrated, the differential expression profile of chemokine receptors in obesity may contribute to differences in the pathogenesis of type 2 diabetes. Moreover, CCR2 and CCR5 expression on monocytes could serve as early biomarkers to predict obesity-associated complications. In accordance with our data, a very recently published study demonstrated increased CCR2, CCR5 and CX3CR1 mRNA expression in various monocyte subpopulations. Moreover, monocyte subsets from obese patients display a more inflammatory phenotype with a higher response to TLR4 and TLR8 stimulation (Devevre et al., 2015). These findings together with our data further support the hypothesis that the increased infiltration of macrophages into the obese adipose tissue is not only caused by an increased secretion of chemokines. Monocytes from obese could also show an enhanced migratory ability associated with increased insulin resistance and enhanced chemokine

receptor expression. The mechanisms by which obesity modulates the changes in monocytes migration require further clarification.

Given its nature as a case-control study, this study can show associations but not prove causal relations. Moreover, the major limitation of this study was the small sample size. In the future, larger studies with more statistical power are needed. However, even with a small number of participants, it was possible to observe significant changes and associations. Note that the study is restricted to one gender in order to avoid an influence of sex-specific differences in the small cohort. Thus, the findings cannot be generalized for men.

This work provides evidence that in obesity peripheral immune cells response to the altered internal and external and crucially contributes to the inflammatory process. Peripheral immune cells provide an easy and representative view of the obesity-associated changes in terms of activation of pro-inflammatory transcription factors, expression of pro-inflammatory genes, migratory ability and correlation with systemic markers of inflammation and metabolic changes in the circulation. Together, this study supports further interrogation of circulating immune cells in understanding the pathogenesis of inflammation in obesity.

8. Abbrevations

Ad	Adipocytes
AdipoRiSc	Adiposity Risk Screening
Akt	Protein kinase B
aP2	Adipocyte protein 2
ApoE	Apolipoprotein E
AT	Adipose tissue
Bay117	Bay117082 ((E)3-[(4-methylphenyl)-sulfonil]-2-propenenitrile)
Bcl 3	B cell leukemia 3 protein
BMI	Body-Mass-Index
C57BL/6J	C57 black 6
CC	Chemokine (C-C motif)
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CM	Conditioned medium
ConA	Concanavalin A
CRP	C-reactive Protein
CX3C	Chemokine (C-X3-C motif)
CX3CR	Chemokine (C-X3-C motif) receptor
CXC	Chemokine (C-X-C motif)
CXCL10	Chemokine (C-X-C motif) Ligand 10
CXCR	Chemokine (C-X-C motif) receptor
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
EMSA	Electrophoretic Mobility Shift Assay
Epi	Epididymal
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinases
FACS	Fluorescence-activated cell sorting
Fc	Fragment crystallisable

FFA	Free fatty acid
Foxp3	Forkhead box P3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA-3	GATA Binding Protein 3
GLUT	Glucose transporter
GOT	Glutamic oxaloacetic transaminase
GPT	Glutamic pyruvate transaminase
h	Hour
HDL	High-density lipoprotein
HFD	High-fat diet
HLA	Human leukocyte antigen
HOMA-IR	Homeostatic model assessment of insulin resistance
hsCRP	High sensitive C-reactive protein
I-TAC	Interferon-inducible T-cell alpha chemoattractant
ICAM	Intercellular adhesion molecules
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IKK	Inhibitor of kappa B kinase
IL	Interleukin
Ing	Inguinal
IP-10	Interferon gamma-induced protein 10
IRAK	IL-1R–associated kinase
IRF	Interferon regulatory factor protein
IRS-1	Insulin receptor substrate
IκB	Inhibitor of kappa B kinase
IκBα-mut	IkappaBalpha mutant protein
JNK	c-Jun N-terminal kinases
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LPS	Lipopolysaccharides
MCP	Monocyte chemotactic protein
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MIG	Monokine induced by interferon-gamma

MIP-1α or LD78β	Macrophage inflammatory proteins
Mock	Empty vector as control
mRNA	Messenger ribonucleic acid
mTOR	Mechanistic target of rapamycin,
MyD88	Myeloid differentiation primary response gene 88
NEMO	NF-kappa-B essential modulator
NFκB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NK cells	Natural killer cells
NKT cells	Natural killer T cells
ob/ob mice	Leptin deficient mice
Oct	Octamer
OGTT	Oral glucose tolerance test
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PHA	Polyhydroxyalkanoate
PPAR	Peroxisome proliferator-activated receptor
Pre	Preadipocytes
qRT-PCR	Quantitative real time polymerase chain reaction
RANTES	Regulated on activation normal T cell expressed and secreted
RT-PCR	Real time polymerase chain reaction
S6K	S6 kinase
SCF-bTRCP	Skp1-Cul1-Fbox ligase containing the F-box protein b-transducin repeat-containing protein
SD	Standard deviation
SDF-1α	Stromal cell-derived factor 1
SE	Standard error
SVF	Stromal–vascular fraction
TAB-1	TAK1 Binding Protein
TAK-1	TGF (transforming growth factor)-beta-activating kinase-1
TG	Triglyceride
TIR domain	Toll interleukin 1 receptor domain
TLR	Toll like receptor
TNFα	Tumor necrosis factor alpha
TRAF	Tumor necrosis factor receptor-associated factor

Treg cell	Regulatory T cell
TSH	Thyroid-stimulating hormone
Vs	Versus
w	Week
WAT	White adipose tissue
WC	Waist circumference
WHO	World health organisation
WHR	Waist-to-hip ratio
αIP-10	Anti-IP-10 antibody
β-actin	Beta-actin
β-cell	Beta-cell
γ-GT	Gamma-Glutamyl transpeptidase

9. List of Figures

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11. Literature

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