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Obesity weight reduction and inflammatory leukocyte receptor expression

Expression of toll-like receptors and inflammation-linked receptors

Master's thesis in Molecular Medicine

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Abstract

Obesity is defined as accumulation and storage of excessive body fat, and is increasing worldwide. Obesity is associated with diseases as diabetes mellitus type 2 and cardiovascular diseases. A hallmark of obesity is chronic inflammation, with elevated levels of inflammatory markers. Blood leukocytes express inflammatory cell-surface receptors that plays a role in the chronic inflammation associated with obesity. In the present study the focus was on toll-like receptors TLR2 and TLR4, CD14, CD16, CD36, monocyte CD14/CD16, as well as the cell-adhesion receptors CD11b and CD66b.

The aim of this study was to determine if the obese condition following a diet would affect the expression of the inflammation-linked leukocyte receptors in blood. Cell-surface receptor expression was measured by flow cytometry before and after obese weight loss using a very low calorie diet (VLCD), following a stabilization period. In addition, gene expression levels of the receptors (TLR2, TLR4, CD14, CD36) were determined by RT-qPCR, as well as the soluble CD14.

The main findings were an increase in cell-surface receptor expression on leukocytes after weight loss (W8), with maintenance in expression level following the stabilization period (W12). The expression levels were elevated after weight loss; For TLR2 and TLR4, an increase in receptor expression was observed after weight loss, and further on an increased proportion of leukocytes expressing the receptors, which might be of importance in defense. The scavenger receptor for oxLDL, CD36, increased after weight loss, as well as the proportion of monocytes expressing high amounts of CD36. The percentage distribution of CD14/CD16 monocyte subpopulations showed small changes in the inflammatory and intermediate monocytes after weight loss, while the main classical monocyte population increased. The cell-adhesion receptors CD11b and CD66b increased after weight loss. CD11b also contributes in phagocytosis of invading targets, thus an increased expression might have consequences for this function.

In conclusion, there was found an effect of weight loss and diet intervention on the expression patterns of the selected receptors (TLR2, TLR4, CD14, CD36, CD16, CD11b, CD66b and monocytes CD14/CD16) on the monocytes, granulocytes and eosinophils. These changes might have consequences for the defense properties and/or in relation to the inflammatory behaviors.

Table of contents

1 Introduction

1.1 Obesity

Obesity is defined as accumulation and storage of excessive body fat. Body mass index (BMI) over 30 kg/m^2 is characterized as obese, while BMI from 25-30 is defined as overweight[1]. Obesity is increasing worldwide and is associated with the development of diseases as diabetes mellitus type 2 and cardiovascular diseases as hypertension and atherosclerosis[2]. Fat cells, also called adipocytes, consist of a large lipid droplet and regulates energy storage and release. Adipocytes produce pro-inflammatory cytokines as interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) alongside with adipokines, such as the appetite hormone leptin[3]. The release of these factors can be due to intracellular stress from excessive lipid buildup[4]. Macrophages, or monocyte precursors, migrate to adipose tissue and is called adipose tissue macrophages (ATM). Lumen *et al.* has shown that in obese patients, the ATM switch from the anti-inflammatory M2 macrophages to pro-inflammatory M1 macrophages. The M1 macrophages secrete pro-inflammatory cytokines as IL-6 and TNF-α. In this way, the M1 macrophages contribute to the chronic inflammation in obesity[5]. The amount of monocytes is associated with the increase of BMI and the development of atherosclerosis. Monocytes plays a role in innate immunity, including phagocytosis and secretion of cytokines[2].

Obesity is associated with inflammation, and obese subjects have increased levels of inflammatory markers as C-reactive protein (CRP), cytokines and interleukins[2]. A hallmark of inflammation in obesity is chronicity. It appears that the expression of inflammatory cytokines happens gradually and remains unsolved. Compared to lean individuals where the inflammatory response is acute at the site of injury[6].

Obesity is often treated with a very low calorie diet (VLCD) to obtain rapid weight loss. As obesity is associated with the metabolic syndrome, the VLCD may have metabolic consequences. Studies have shown that both long-term and short-term effects of these diets leads to reduction in triglyceride levels, normalization of blood glucose levels and high-density lipoprotein (HDL) cholesterol levels, as well as reduction in systolic blood pressure[7-9]. Diets can also affect the leukocyte levels. A study done on healthy men who were fed on a high fat or high glucose diet for 6 hrs, showed that both diets led to an increase in granulocytes and lymphocytes counts[10]. On the contrary, a study done on anorexic patients compared to their

healthy controls, showed lower levels of leukocytes, suggesting that the calorie restriction in anorexia is very extreme, leading to reduction in immune-cell populations[11].

1.1.2 Blood leukocytes

Leukocytes, also called white blood cells, are circulating cells helping to fight infections in the immune system. The leukocyte population includes lymphocytes, granulocytes, monocytes, macrophages and platelets. Among the leukocytes, the granulocytes are the first responders to infections in the body. They can be divided into neutrophils, eosinophils and basophils. The neutrophilic granulocytes (39-73%) and eosinophilic granulocytes (0-8%) are phagocytic granulocytes, while the basophilic granulocytes (0.2-1.3%) are non-phagocytic. The neutrophilic granulocytes circulate in the blood before they are recruited to the site of infection as a response to inflammatory molecules, such as chemokines. An indication of infection is an increased number in circulating neutrophils. The eosinophilic granulocytes can also migrate from blood to the tissue, but plays the most important role in their defense against parasitic organisms. The basophilic granulocytes are rare in the circulation, but in response to antibody binding, they release for instance histamine which increase blood vessel permeability[4, 12]. The monocytes (5-13%) are a group of cells involved in phagocytosis, cytokine production and antigen presentation. They are found circulating in the blood, or migrated to the tissue in response to infection[12, 13]. Here they differentiate into macrophages and participate in repair or in the innate immune response[4]. Monocytes have also recently been divided into subpopulations based on surface expression of monocyte markers[13]. The lymphocytes (18-48%) are divided into B-cells, T-cells and natural killer cells (NK-cells), and are an important population in the adaptive immune response, compared to the other leukocytes that are important in first line defenses in response to an infection[4, 12].

In response to infection, the leukocytes communicate via signaling pathways. Foreign pathogen molecules are recognized by extracellular or intracellular receptors which initiate a signaling cascade that releases cytokines or other signaling molecules, recruiting leukocytes to the site of infection[14].

1.1.3 FcγRIII (CD16)

CD16 (also called FcγRIII) is a Fcγ receptor involved in binding of antibodies in the IgG class. The immunoglobulin superfamily consists of FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16)

and FcγRIV(CD16-2). CD16 is a low affinity receptor, and bind with low affinity to IgG isotypes[15]. CD16 are present in two isotypes, type A and B. Type A is a transmembrane molecule found on macrophages, activated monocytes and natural killer (NK) cells, among others. Type B is a GPI anchored receptor specific to neutrophils[16].

CD16 links the cellular and humoral immune system. CD16 present on NK-cells binds to IgG antibodies and then activates antibody-dependent cell-mediated cytotoxicity (ADCC). This triggers target cell lysis and cytokine production, which destroys the target[17].

It has recently been found that monocytes have multiple subpopulations based on the expression of CD16 on the surface. The classical monocytes ($CD14^{++}CD16^{-}$) are the largest monocyte population. The non-classical monocytes ($CD14^{+}CD16^{++}$) are smaller in size and are referred to as pro-inflammatory due to secretion of inflammatory cytokines as TNF-α. Last, the intermediate monocytes ($CD14^{++}CD16^{+}$) also produce inflammatory cytokines, such as TNF-α and interleukin-1β (IL-1β), in a larger degree[18].

$CD16^{+}$ monocyte subpopulation is shown to increase due to inflammation. For example, patients with infections, atherosclerosis and rheumatoid arthritis have an increased frequency of $CD16^{+}$ monocytes. It is also shown that obesity increase the $CD16^{+}$ monocyte subpopulations[2]. A large cohort study of healthy volunteers has shown a relation between the $CD16^{+}$ monocyte level and subclinical atherosclerosis and obesity[19]. Research done by Poitou *et al.*, showed that the $CD14^{+}CD16^{++}$ and $CD14^{++}CD16^{+}$ monocytes decreased by drastic fat loss. A fat loss of less than 5% did not reduce the $CD16^{+}$ monocyte population. This was also shown in patients reducing fat mass by gastric surgery[2]. Another study were patients reduced fat mass by gastric surgery showed that the $CD14^{+}CD16^{+}$ monocyte subpopulation level was higher before surgery compared to normal controls. After surgery, the percentage of $CD14^{+}CD16^{+}$ monocytes decreased throughout the study period of 12 months[20]. Exercise has also shown to reduce the percentage of $CD14^{+}CD16^{+}$ monocytes. Both lean controls, and obese insulin-sensitive and insulin-resistant volunteers, had a reduction in the percentage of $CD16^{+}$ monocytes after an exercise session. In this study, only the obese insulin-resistant individuals had a higher level of $CD14^{+}CD16^{+}$ monocytes[21]. A study with less obesity relevance by Timmerman *et al.*, shows that subjects in the age of 65-80 who is physical active had a lower percentage of $CD14^{+}CD16^{+}$ monocytes, compared to the inactive group, in conjunction with aging being characterized with increased systemic inflammation. After the inactive group had been 12 weeks on a training program, the percentage of $CD14^{+}CD16^{+}$ monocytes were reduced[22].

1.2 Toll-like receptors

Toll-like receptors (TLR) are pattern recognition receptors (PRR) that plays a major role in the innate immune system. They recognize pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharide (LPS), or danger-associated molecular patterns (DAMPs), such as proteins from damaged tissue[23].

When TLR binds PAMPs or DAMPs the receptors dimerize. In most cases with each other, while TLR2 forms heterodimers with TLR1 or TLR6. TLRs are present on the plasma membrane if they recognize PAMPs from surface of the extracellular microbe. If the TLRs recognize microbial components such as nucleic acid, they are localized in lysosomes/endosomes. TLR4 is present on both the plasma membrane and the lysosomes/endosomes[24].

1.2.1 TLR4

TLR4, also called CD284, is a member of the transmembrane-1 protein family. This receptor is expressed on monocytes, granulocytes, dendritic cells (DC) and adipose tissue, among others, in humans[25]. Recent discoveries shows that is has also been found to be expressed on eosinophils[26]. TLR4/4 recognizes and binds LPS, which is a part of the structural membrane of Gram-negative bacteria, such as *Escherichia coli (E.coli)*. For this ligand binding to happen TLR4 must make a complex with CD14 and MD-2. The TLR4/CD14/MD-2 complex binds LPS and activate MyD88-dependent or independent signaling pathway. In the MyD88-dependent pathway, the TIR-domain-containing adapter protein (TIRAP) helps to recruit another adaptor protein called myeloid differentiation factor 88 (MyD88). This signaling pathway leads to activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and several cytokines[25]. In the MyD88-independent signaling pathway, the TIR-domain-containing adapter inducing interferon-β (TRIF) helps to recruit TRIF-related adapter molecule (TRAM)**.** This leads to activation of interferon regulatory factor-3 (IRF3) which activates genes encoding type I interferons (IFNs), such as INF-β and INF-α[25]. Several studies have shown that fatty acids, which is in a higher circulating level in obese, binds to and activates TLR4[27-30]. Research done by Strohacker *et al.* showed that diet-induced obese mice had a lower TLR4 receptor expression when fed on high-fat diet compared to low-fat diet[31]. Additionally, mouse studies have shown that mice fed on a high-fat diet with TLR4 deficiency in hepatocytes and adipose tissue reduce the inflammation in the adipose tissue and

enhance insulin sensitivity, suggesting that TLR4 contributes to obesity-associated inflammation and insulin-resistance[29, 32, 33]. Obese and non-obese subjects with established atherosclerosis showed no difference between the two groups when analyzing the expression of TLR4 on circulating monocytes[34].

1.2.2 TLR2

TLR2, also called CD282, is expressed on the plasma membrane of monocytes and granulocytes in humans[35], adipose tissue[36], and it has also recently been found to be expressed on eosinophils[37]. This receptor dimerizes with TLR1 or TLR6 and recognizes Gram-positive bacteria such as *staphylococci*. When TLR2 is in complex with TLR1 or TLR6 it is partly dependent of CD14 as an associated protein, while the TLR2/TLR6 complex are dependent of CD36 as an associated protein. Both the TLR2/TLR1 and TLR2/TLR6 complexes signal through the MyD88-dependent pathway where adaptor proteins TIRAP and MyD88 are involved. This signaling pathway leads to activation of NF-κB and several cytokines[24]. Research has shown that TLR2 can be activated by fatty acids[27, 38]. A study done by Wan *et al.* were young and healthy men were on a high-fat diet for one week, showed that the receptor expression of TLR2 was reduced, and the function impaired[39].It is also shown that subjects with diabetes type 2 have a higher TLR2 expression and higher levels of TLR2 ligands, compared to healthy controls[40]. Obese and non-obese subjects with developed atherosclerosis showed no difference in TLR2 expression on circulating monocytes between the two groups[34]. In addition, the appetite hormone leptin is found to increase the TLR2 expression in human monocytes[41]. TLR2 deleted mice who were fed on an obesity-induced diet showed that loss of TLR2 could lead to prevention from diet-induced obesity and insulin resistance, compared to wild type mice[42].

1.2.3 CD14

CD14 is a GPI-bound membrane protein that works as a co-receptor for TLR4. It is expressed on monocytes[43], and it works as an useful monocyte marker[44]. This receptor binds directly to TLR ligands, as LPS, and chaperones LPS to the TLR4/MD-2 complex[43]. The receptor complex is then internalized and directed to the endosomal compartment, where the MyD88-independent pathway is activated[45]. Mice studies have shown that a high-fat diet leads to increased plasma LPS levels, and that CD14 deficient mice fed on a high-fat diet were more

protected from obesity and metabolic syndromes[46]. Another study done in diet-induced obese mice lacking CD14 showed that CD14 deficiency lead to decreased adiposity, glucose homeostasis and reduced blood pressure, suggesting that CD14 deficiency protects from metabolic syndromes accompanied by obesity[47]. Patients reducing fat mass by gastric by-pass operation had a higher CD14 level before surgery, than normal controls. The CD14 levels were reduced after surgery, reaching the same level as normal controls three months after surgery[20].

CD14 is also found in a soluble form (sCD14) in the circulation. It is secreted from the liver or cleaved from the membrane protein[48]. sCD14 reaches the concentration of 2-6 µg/ml in plasma[49]. The circulating sCD14 is regarded as an acute phase protein and increases due to chronic infections or inflammatory diseases, especially HIV[48]. Research on obese women undergoing surgical-induced weight loss showed that the sCD14 level decreased after the women's weight loss[50]. A similar study showed an increase in sCD14 levels after surgical-induced weight loss, suggesting that obese subjects have reduced sCD14 levels[51].

1.2.4 CD36

CD36 is an integral membrane glycoprotein expressed on monocytes, macrophages, DC, adipocytes, and other cell types. It functions as a scavenger receptor, a receptor recognizing oxidized low-density lipoprotein (oxLDL), and other ligands. This leads to formation of "foam cells" and atherosclerotic plaque, which is the critical step in formation of atherosclerosis[52]. OxLDL is internalized and the expression of CD36 is upregulated, leading to more uptake of oxLDL. The interaction between CD36 and oxLDL leads to secretion of cytokines and recruitment of immune cells. This develops to arterial inflammation, narrowing of the artery and atherosclerosis[53]. Based on this, several studies have shown that knock-out of CD36 in mice leads to less formation of plaque[52]. A study done on obese women compared to lean controls showed that CD36 receptor expression was lower in obese compared to controls[54]. Research done on adipose tissue from CD36 knock-out mice on a high-fat diet showed that the tissue was more insulin sensitive and had lower inflammatory markers compared to the wild type mouse[55]. Recently is has been shown that fatty acids, which is increased in obese, binds to CD36. Binding of fatty acids leads to enhanced uptake of oxLDL by CD36, dependent on the fatty acid dose[56]. Research has showed that subcutaneous adipose tissue had a higher CD36 expression in obese and diabetes type 2 subjects, compared to lean controls[57].

In addition, CD36 works as an accessory protein for toll-like receptors. Especially TLR2 or TLR6, in response to recognition of bacteria[52]. It is also suggested that the TLR4/TLR6 heterodimer works together with CD36 to mediate responses to oxLDL and amyloid-β[58]. It has recently been discovered that CD36 is present in a soluble form in plasma. Both sCD36 and oxLDL is found to be in higher levels in the plasma of obese subjects compared to normal controls[59].

1.3 Adhesion receptors

Integrins are cell-adhesion receptors involved in leukocyte migration and activation in response to an infection. The receptors participate in for instance transendothelial migration, adhesion strengthening, cytokine production and complement-mediated phagocytosis[60]. Integrins can also function as adaptors in activation of GPI-anchored proteins[61]. For neutrophils to reach the site of infection, they have to pass endothelial layers, a process characterized by an increase in CD11b and CD66b expression[62].

1.3.1 CD11b

CD11b is an integrin type alphaM (α_M), that together with CD18 (integrin β_2) forms the CD11b/CD18 heterodimer, also known as the Mac-1, or complement receptor 3 (CR3). This receptor is expressed on monocytes, macrophages, granulocytes and NK-cells, and is known to be involved in adhesion, migration, phagocytosis and chemotaxis[63]. The Mac-1 receptor binds to C3b and iC3b, which are the activated C3 fragment and the inactivated products respectively. The binding leads to phagocytosis, and it also regulates cytokine responses[64]. For the receptors role in adhesion, the most important molecule to bind to is the cell-surface molecule intracellular adhesion molecule 1 (ICAM-1). Other adhesive ligands to Mac-1 is the glycosaminoglycan heparin and human leukocyte elastase (HLE)[65, 66]. Mac-1 is also involved in binding of CRP, which is reported to lead to chemokine secretion and monocyte migration via CD11b[67]. Since CD11b is expressed on monocytes and macrophages, it is an important receptor in the migration of macrophages into the adipose tissue. CD11b deficient mice fed on a high-fat diet showed a reduced migration of blood monocytes into adipose tissue, compared to wild type mice, while an increase in ATM accumulation was observed. It is suggested that proliferation and activation of ATM normally is regulated by IL-4/STAT6 which is inhibited by CD11b through the activity of phosphate SHP-1, meaning that CD11b deficiency

will lead to unregulated ATM proliferation[68]. Another study done on CD11b deficient mice fed on a high-fat diet showed that the numbers of leukocytes were the same in CD11b deficient mice as in the wild type, suggesting that CD11b is not required for leukocyte migration into adipose tissue[69]. It is shown that obese subjects undergoing weight loss by gastric surgery have the same levels of CD11b expression as lean controls, and the levels did not change after surgical-induced weight loss[15, 20]. However, a study done on healthy men being fed on fresh cream for 6 hrs showed that granulocytes and lymphocytes levels increased, as well as CD11b receptor expression on monocytes and granulocytes[70]. Additionally, research done on obese subjects with established atherosclerosis, showed that the CD11b receptor expression on monocytes were higher in the obese subjects, compared to the non-obese subjects with atherosclerosis[34].

1.3.2 CD66b

CD66b, which is also called CEACAM8, CGM6 or NCA-95, is a single chain, GPI-anchored glycoprotein that is a member of the antigen (CEA) family. It is expressed on human granulocytes and functions as a granulocyte marker. It is also expressed on eosinophils, were its function is more unknown. CD66b is located in lipid rafts, were it is involved in interactions and signal transductions with help from Src family kinase, Hck, or the adhesion molecule integrin, such as CD11b. In this way CD66b is involved in cell-adhesion[61]. Since CD66b is clustering with CD11b, it is thought that CD66b does not initiate signal transduction alone, but is dependent on an interaction in the lipid raft, due to the lack of cytoplasmic domains[62]. Research has shown that the expression of CD66b is elevated in obese subjects compared to lean controls[71, 72]. It is also shown that expression is higher in subjects with diabetes type 2 compared to lean controls[73, 74]. Additionally, van Oostrom *et al.* showed that the expression of CD66b increased after healthy men only ate fresh cream for 6 hrs[70]. A less obesity relevant study by Takahashi *et al.*, showed that CD66b receptor expression decreased when subjects in the age of 65-79 years exercised twice a week for 12 weeks[75].

2 Aim

The aim of the current study was to determine if the obese conditions following a diet would affect the expression of inflammation-linked leukocyte receptors in blood. Cell-surface receptor expression was measured by flow cytometry before and after obese weight loss using a very low calorie diet (VLCD), following a stabilization period. More specifically, the leukocyte receptors included were toll-like receptors TLR2 and TLR4, CD14, CD16, CD36, monocyte CD14/CD16, as well as the cell-adhesion receptors CD11b and CD66b. In addition, gene expression levels of the receptors (TLR2, TLR4, CD14, CD36) were determined by RT-qPCR, as well as the soluble CD14.

3 Materials and methods

3.1 Weight loss study

The patients enrolled in the weight reduction study followed a very low calorie diet (VLCD) for 8 weeks to induce rapid weight loss followed by 4 weeks of weight stabilization. The patients were asked to not change their physical activity level during the study. Both women and men were on a commercial very low calorie diet (Allévo, Cederrot Sweden) with 550 kcal/day and 660 kcal/day, respectively, including no-energy fluids and low starch vegetables (max 100g/day). After 8 weeks on VLCD the patients were gradually introduced to normal food, while gradually withdrawing from the VLCD products with 2-3 products/day on week 9 and 1 product/day on week 10. On week 11 the patients were prescribed an individual diet plan by a trained dietician to keep a stable weight. This diet included 15-20% protein, 20-30% fat and 50-60% carbohydrates. Blood was collected at three time points:

W0: Week 0, start of the study

W8: Week 8, after 8 weeks of VLCD

W12: Week 12, after 4 weeks on a restricted healthy diet

The expression of inflammatory receptors at the leukocyte surface was studied at the three different time points. The following receptors were included; TLR2 and TLR4, the co-receptor CD14, the oxLDL-receptor CD36, as well as inflammatory receptors CD16, CD11b and CD66b.

The data analyzed from this study only shows results from patients were blood was collected at all three time points, N=17. For receptor CD36, CD11b, CD16 and CD66b, N=15.

3.1.1 Control group

A control group of five volunteers (age 23-29, both sexes, normal weights) were included to get an impression of receptor expression at different time points under normal feeding conditions, and also experimental variations. Blood was collected at two time points (T1, T2), with 3-6 weeks between.

3.1.2 Receptor staining

Table 3.1 Reagents used in receptor staining of EDTA blood

	PE Anti Human CD282 (TLR2)	PE Anti Human CD284 (TLR4)	CD36 PE	CD14 PE	CD11b PE	PE Mouse Anti Human CD16
Clone	TL2.1	HTA 125	185-1G2	MΦP9	D12	3G8
Cat. No.	309708	312806	Sc-21772	345785	333142	555407
Lot. No.	B195156	B176128	D0805	4106528	4153793	3113810
Conc.	200 µg/ml	400 µg/ml	200 µg/ml	50 µg/ml	50 µg/ml	-
Producer	BioLegend, San Diego, USA	BioLegend, San Diego, USA	Santa Cruz Biotechnology, Dallas, USA	BD Biosciences, New Jersey, USA	BD Biosciences, New Jersey, USA	BD Biosciences, New Jersey, USA

Table 3.2 Reagents used in receptor staining of EDTA blood

	PE Mouse IgG2b, κ Isotype Ctr	PE Mouse IgG2a, κ Isotype Ctr	PE Mouse IgG1, κ Isotype Ctr	APC Mouse IgM, κ Isotype Ctr	Anti-Human CD 66b APC	CD14 (FITC)
Clone	MPC-11	MOPC-173	MOPC-21	MM-30	G10F5	MΦP9
Cat. No.	400312	400212	555749	401616	17-0666	345734
Lot. No.	B174618	B180239 B19537	3046675	B184794	E16847-106	4281575 5132856 5023986
Conc.	0.2 mg/ml	0.2 mg/ml	-	200 µg/ml	5 µl (0.125 µg)/test	25 µg/ml
Producer	BioLegend, San Diego, USA	BioLegend, San Diego, USA	BD Biosciences, New Jersey, USA	BioLegend, San Diego, USA	eBioscience, San Diego, USA	BD Biosciences, New Jersey, USA

EDTA blood was collected in 6 ml vacuette tubes (Greiner Bio-One, Austria), and put on ice for 1 hr +/- 10 min before staining. EDTA blood (25 µl) was stained with the mixtures showed in the table below (Table 3.3) in a 96-well nonpyrogenic plate (Corning incorporated, NY, USA).

Table 3.3 The amounts of reagents added to the EDTA blood

	Antibody (PE)	CD14 FITC	PBS	CD66b APC	Isotype ctr IgM APC
TLR2 (PE)	1.25 µl	2.5 µl	1.25 µl	-	-
TLR4 (PE)	1.25 µl	2.5 µl	1.25 µl	-	-
CD36 (PE)	1.25 µl	2.5 µl	1.25 µl	-	-
CD14 (PE)	2.5 µl	-	2.5 µl	-	-
CD11b (PE)	2.5 µl	2.5 µl	-	1.25 µl	-
CD16 (PE)	2.5 µl	2.5 µl	-	1.25 µl	-
IgG2a (PE) (Ctr TLR2 and CD36)	1.25 µl	2.5 µl	1.25 µl	-	-
IgG2a (PE) (Ctr TLR4)	2.5 µl	2.5 µl	-	-	-
IgG2b (PE) (Ctr CD14 (PE))	0.63 µl	2.5 µl	1.875 µl	-	-
IgG2a (PE) (Ctr CD11b)	0.63 µl	2.5 µl	1.875 µl	1.25 µl	-
IgG1 (PE) (Ctr CD16 and CD66b)	2.5 µl	2.5 µl	-	-	1.25 µl
FMO control 1	-	2.5 µl	2.5 µl	-	-
FMO control 2	-	2.5 µl	2.5 µl	1.25 µl	-

Blood (25 µl) was stained with the antibodies (the same lot. no. throughout the study period) and incubated on ice protected from light for 15 min. BD FACS™ Lysing Buffer (100 µl) (BD Biosciences, New Jersey, USA, Cat. No.: 349202) was added to each well before the samples were transferred to flow vials containing BD FACS™ Lysing Buffer (900 µl). The samples were incubated for another 15 min in room temperature (RT), protected from light. Subsequently, the samples were centrifuged at 1000 rpm and 4°C for 5 min, and re-suspended in PBS without Mg^{2+}/Ca^{2+} (500 µl) (Oxoid Limited, Hampshire, England). The stained samples were run through a flow cytometer (BD FACS Canto II) with 1000 evt., using BD FACSDiva™ software version 6.1.3 (BD Biosciences, San Jose, USA). Raw data was analyzed using FlowJo software version 7.6.5 (TreeStar, Ashland, USA).

3.1.3 Flow cytometer

Flow cytometry is a method that can measure various cellular properties by cells, such as size, granularity, receptor expression and viability with help from light, and/or in combination with fluorescence antibodies or other dye markers. Light that scatters in forward directions is called forward scatter (FSC) and gives information about the size of the cell. The more forward light scatter, the larger is the cell. While light that scatters in a 90-degree angle is called side scatter (SSC) and gives information about the cell granularity[76]. It gives an indication of the cellular complexity and membranous structures as endoplasmic reticulum and mitochondria[4]. The sample tube is placed in the flow cytometer and cells travel up the flow cell in a pressurized steam with rapidly moving sheath fluid, passing one by one through the laser beam. When the cells travel through the laser beam, they scatter laser light and fluorescence from the monoclonal antibodies they are stained with. These antibodies are conjugated to fluorescence dye that labels the cell-surface proteins. The light is sent to the Photo Multiplier Tube (PMT) via longpass mirrors and bandpass filters who only pass certain wavelengths. The fluorescence dye absorbs energy and emits light at different wavelengths, which gives information about the expression of the cell-surface proteins. For instance, the FITC dye only passes through the LP mirror and BP filter that admits light from 515-545 nm, and the PMT will emit green light from FITC dyes. It is also possible to detect multiple fluorochromes at once on a cell[76]. Flow cytometers can also be used to detect intracellular components. Cells permeabilized with detergent to allow antibodies to stain the cell intracellularly emit fluorescence detected by the flow cytometer[4]. The fluorochromes emit light over a range of wavelengths, so a signal from one fluorochrome, for instance FITC, can also appear in a detector for another fluorochrome, as PE. This spillover must be corrected by compensation, so that the FITC population is not present in the PE channel[76].

3.1.4 Gating strategy

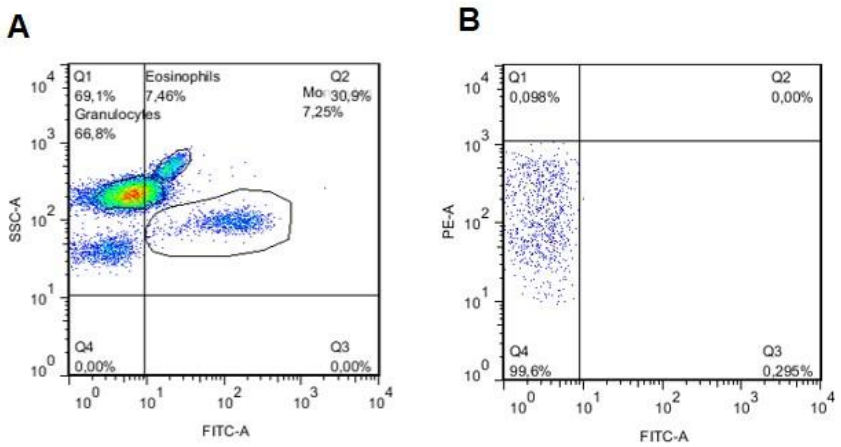

Figure 3.1 Dot plot of gating strategy done in FlowJo version 7.6.5. Gating of monocytes, granulocytes and eosinophils in a CD14/SSC dot plot (A). Threshold of monocyte population in a PE/FITC dot plot (B).

Table 3.4 Receptor expression in eosinophils (W0) measured in median fluorescence intensity (MFI) on the flow cytometer.

	W0					
Eosinophils	Receptor	Isotypectr	Receptor-Isotypectr	FMO control	Receptor-FMO	PE-A+
TLR2	23,7	22,9	0,8	14,1	9,6	32,9
TLR4	30,1	28,8	1,3	14,1	16,0	35,6
CD36	13,5	22,9	-9,4	14,1	-0,6	69,2
CD14(PE)	18,9	19,9	-1,0	X	X	X
CD11b	80,6	20,5	60,1	16,2	64,4	81,3
CD16	17,0	17,3	-0,3	16,2	0,8	33,4
CD66b	102,0	10,9	91,1	X	X	X

Gating was done in FlowJo software version 7.6.5 (version 10.1 was used for the control group) (TreeStar, Ashland, USA). Monocytes were gated based on the expression of CD14 in a SSC/CD14 (FITC) dot plot. The granulocytes were gated based on their high granularity and low expression of CD14, while eosinophils were gated based on their higher autofluorescence/granularity, which in most cases was giving a separate population from the main granulocyte population (Figure 3.1 A). The monocytes were gated based on a threshold on the FITC scale that was set to 10^1, which also included the smaller fraction of monocytes expressing a low amount of CD14, but still more than the other leukocyte populations (Figure

3.1 A,B). The granulocytes and eosinophils was gated with narrow gates to best differ between the granulocytes and eosinophil population. Expression of receptors was measured as median fluorescence intensity (MFI) in each cell population, either by using fluorescence minus one (FMO), or by subtracting an isotype control value. FMO is a control were cells are stained with the same fluorochromes as in the panel, except for that one that is being measured, hence fluorescence minus one. They are used to identify and gate cells, to best differ between positive and negative cells, and to identify any spread of fluorochromes into the channel of interest[77]. The MFI value of CD14 (PE) and CD66b (APC) were obtained by subtracting the MFI value of the isotype controls. Different strategies were initially tested;

1) Subtract isotype control from receptor expression

2) Set a threshold for PE positive cells

3) Subtract FMO control from receptor expression

It was first tried to subtract the MFI value of the isotype controls from the same company as the specific antibody with corresponding concentrations, but this gave negative numbers, especially for the TLRs (Figure A.1, Appendix). Next, it was tried to set a threshold at the end of the FMO control histogram and assume that everything over this threshold was PE positive (Figure A.2, Appendix). This method gave uncertain results, it showed for instance that receptor CD36 had MFI expression on eosinophils even though this receptor is known to only be expressed on monocytes (Table 3.4). Based on this, the best strategy was to subtract the MFI of the FMO control from the MFI of the receptor, which in the case of CD36, gave a value close to zero on its expression on eosinophils (Figure A.1, Appendix) (Table 3.4).

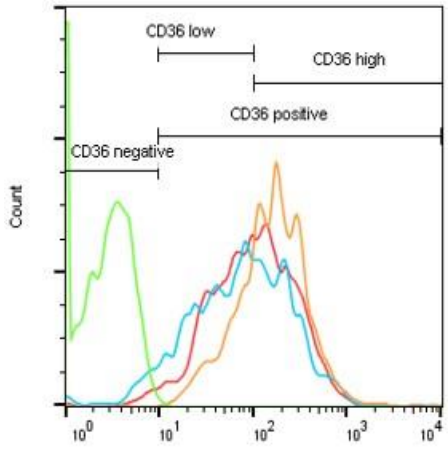

Figure 3.2 An example of a histogram were thresholds was set to analyze the percentage distribution of receptor expressing cells. Green line = FMO control; Red line = expression at time point W0; Blue line = expression at time point W8; Orange line = expression at time point W12.

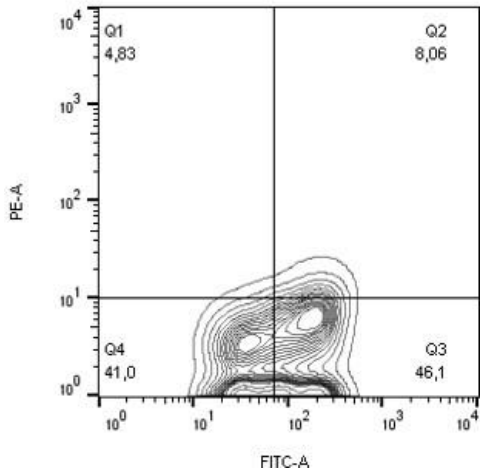

Figure 3.3 An example of a quadrant plot were thresholds was set to analyze the percentage of monocyte subpopulations. Q1= $CD14^{Low}/CD16^{High}$ (Inflammatory); Q2= $CD14^{High}/CD16^{High}$ (Intermediate); Q3= $CD14^{High}/CD16^{Low}$ (Classical); Q4= $CD14^{Low}/CD16^{Low}$.

Next, was the percentage of cells expressing the receptors analyzed, as well as the levels of expression. This strategy was done on the receptors CD36, TLR2 and TLR4. A threshold between positive and negative receptor-expressing cells was set based on the FMO value from W0. This was because the FMO value was stable over time (W0, W8, W12) (Figure A.3 A, Appendix), except for two patients (Figure A.3 B, Appendix). It was tested that even though the FMO value was skewed in expression, it did not affect the percentage distribution. To

distinguish between high and low expression of the receptor, a threshold was set on the histogram based on the best way to present the difference between high and low receptor expression, as shown in figure 3.2. A CD14/CD16 quadrant plot was made to analyze the percentage of inflammatory monocytes associated with obesity. The quadrants showed $CD14^{Low}/CD16^{Low}$, $CD14^{High}/CD16^{Low}$ (classical), $CD14^{High}/CD16^{High}$ (intermediate) and $CD14^{Low}/CD16^{High}$ (inflammatory) monocytes. A threshold was set on the y-axis based on the FMO value, and on the x-axis between two monocyte populations (Figure 3.3).

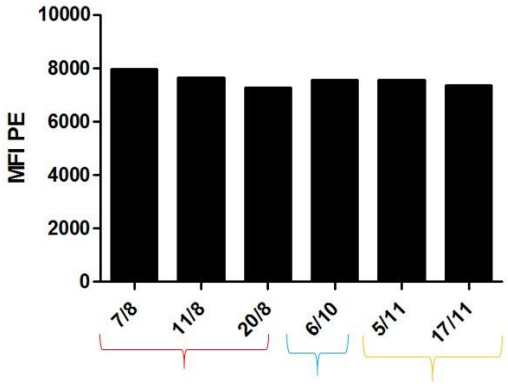

Figure 3.4 A bar chart of the median fluorescence intensity (MFI) of cells in the PE-channel from beads run throughout the study period. Red brace = Beads run at W0; Blue brace; Beads run at W8; Orange brace = Beads run at W12.

Due to some technical issues, samples from the four first patients were run with another compensation in the flow cytometer than the rest, throughout the whole study period (W0, W8, W12) (Figure A.4, Appendix). They were run with the compensation PE – %FITC 16.10 and FITC – %PE 1.03. The compensation was changed so that the rest of the patient samples had the compensation PE - %FITC 16.17 and FITC – %PE 1.16 throughout the study. Before the samples were analyzed in FlowJo software version 7.6.5 (TreeStar, Ashland, USA), the compensation was adjusted, so that all patients had the new compensation. The effect of different compensations on the MFI values was evaluated, showing minimal changes in receptor expression. BD Calibrate ^{TM} Beads (BD Bioscience, CA, USA, Cat. No.: 349502) was also run on the flow cytometer throughout the obesity study as a control that the flow cytometer was stable over time and that the FITC fluorochrome would not leak into the PE fluorochrome channel and affect the results of the PE-marked receptor antibodies. The beads showed that there were minimal changes over time, and with no direction on the small variability between each time point (Figure 3.4).

3.2 Real-time quantitative polymerase chain reaction (RT-qPCR)

Real-time quantitative polymerase chain reaction (RT-qPCR) is a frequently used method in biology to amplify and detect the expression of targeted nucleic acids (DNA). RNA is transcribed into complementary DNA (cDNA), before the cDNA is used as the template in the RT-qPCR reaction. During a PCR reaction the amount of PCR product doubles for each cycle, and DNA-binding dyes, fluorescent primers or probes release an amount fluorescence proportional to the amount of amplified DNA. Fluorescence is detected at the threshold cycle (C_T) in the PCR reaction and is dependent on the amount of DNA present at the start of the reaction. If more DNA is present, fewer amplification cycles are required before fluorescence is detected, leading to a lower C_T value. The measured C_T values are used to determine the expression level of the target gene. C_T values within the range 17-32 are considered as positive gene expression[78].

3.2.1 RNA isolation of whole blood

RNA from 17 of the patients who enrolled in the weight reduction study was isolated using PAX gene® Blood RNA kit 50. V2 (PreAnalytiX, Hombrechtikon, Switzerland, Lot: 151031470).

The PAXgene Blood RNA Tubes containing blood from 17 of the patients were collected from the -80°C freezer and stored at RT the day before RNA isolation. The tubes were centrifuged for 10 min at 4000 x g in a swing-out rotor at RT. The supernatant was removed by decanting. RNase-free water (3 ml) was added and vortexed until the pellet was dissolved. The tubes were centrifuged for 10 min at 4000 x g in the same centrifuge at RT. The supernatant was removed by decanting. Resuspension buffer (300 µl) was added, and the tubes were vortexed until the pellet was dissolved. The sample was pipetted into a 1.5 ml microcentrifuge tube and binding buffer (300 µl) and proteinase K (40 µl) was added. The samples were vortexed for 5 sec and incubated for 10 min at 55°C in a shaker-incubator. The lysate was pipetted into a spin column placed in a 2 ml processing tube, and centrifuged for 3 min at 16,100 x g in RT. The supernatant of the flow-through fractions was carefully transferred to a 1.5 ml microcentrifuge tube without disturbing the pellet. 96-100% ethanol (350 µl) was added to the supernatant, before it was vortexed and centrifuged briefly for 1-2 sec at 600 x g in RT to remove drops from the inside of the tube lid. Sample (700 µl) was pipetted into a spin column placed in a 2 ml processing

tube and centrifuged for 1 min at 14,000 x g at RT. The spin column was placed in a new 2 ml processing tube, and the old processing tube containing flow-through was discarded. This was done after every centrifugation later on. The remaining sample from the 1.5 ml microcentrifuge tube was pipetted into the spin column and centrifuged for 1 min at 14,000 x g at RT. Wash buffer 1 (350 µl) was added to the spin column and centrifuged for 1 min at 14,000 x g at RT. DNase I (10 µl) stock solution was added to DNA digestion buffer (70 µl) in a 1.5 microcentrifuge tube. The sample was gently mixed and DNase I incubation mix (80 µl) was added directly on the spin column membrane and placed on the benchtop in RT for 15 min. Wash buffer 1 (350 µl) was added into the spin column and centrifuged for 1 min at 14,000 x g at RT. Wash buffer 2 (500 µl) was added into the spin column and centrifuged for 1 min at14,000 x g at RT. Wash buffer 2 (500 µl) was again added into the spin column and centrifuged for 3 min at 14,000 x g at RT. The tubes were again centrifuged for 1 min at 14,000 x g. The old processing tube was discarded, and the spin column was placed in a 1.5 ml microcentrifuge tube. Elution buffer (30 µl) was added directly into the spin column membrane and centrifuged for 1 min at 14,000 x g at RT to eluate the RNA. The elution step was repeated. The eluate was incubated for 5 min at 65°C in a heat block before it was chilled on ice. Each sample had 5 µl taken out in a new Eppendorf tube and the RNA concentration was measured in NanoDrop Spectophotometer ND-1000. The RNA samples were stored at -80°C.

3.2.2 cDNA synthesis

The RNA isolated from 17 of the patients who enrolled in the weight loss study was synthesized to cDNA to use as template for RT-qPCR with the High Capasity RNA-to-cDNA kit (Applied Biosystems, CA, USA, Part.No. 4387406).

The kit components 2XRT Buffer and RT Enzyme Mix was thawed on ice. A mix of 2XRT Buffer (10 µl) and 20XRT Enzyme Mix (1 µl) was made in the wells, before RNA sample (9 µl) was added. In two wells 2XRT Buffer (10 µl) and Nuclease-free H_2O (1 µl) was added, before RNA sample (9 µl) was added. This was the no enzyme control. The plates were sealed and briefly centrifuged. The cDNA synthesis reaction was run in an Applied Biosystems 2720 thermocycler (Applied Biosystems, CA, USA) at 37 °C for 60 min, 95 °C for 5 min and 4 °C ∞. Samples were stored at -20 °C.

3.2.3 RT-qPCR

Table 3.5 Reagents used in RT-qPCR

	PerfeCta® qPCR Fast Mix®, UNG, ROX™	TaqMan® Gene Expression Assay Hs01872448_s1 TLR2	TaqMan® Gene Expression Assay Hs00152939_m1 TLR4
Lot. No.	22901	1423400	1423225
Producer	Quanta Biosciences Inc, MD, USA	Applied Biosystems, CA, USA	Applied Biosystems, CA, USA

Table 3.6 Reagents used in RT-qPCR

	TaqMan® Gene Expression Assay Hs02621496_s1 CD14	TaqMan® Gene Expression Assay Hs0016927_m1 CD36	TaqMan® Gene Expression Assay Hs00187842_m1 B2M
Lot. No.	1411811	1446341	1403717 1470490
Producer	Applied Biosystems, CA, USA	Applied Biosystems, CA, USA	Applied Biosystems, CA, USA

To determine gene expression of TLR2, TLR4, CD14 and CD36, RT-qPCR was performed on cDNA samples of 10 patients with a 260/280 purity over 1.80. cDNA samples were thawed and diluted with nuclease-free water to reach the planned cDNA concentration of 0.5 ng/µl. In the DNA-free cabinet a reaction cocktail containing qPCR Fast Mix (10µl), containing all the essential components for PCR, TaqMan® primer/probe (1 µl) and nuclease-free water (4 µl) per well was made. The reaction cocktail (15 µl) was added to the correct wells in the 96-well PCR plate (Applied Biosystems). The plate was moved to the DNA bench and cDNA (5µl) was added to the correct wells. Gene expression of the housekeeping gene β-2-microglobuling was used as a reference. The 96-wells PCR plate was covered with plastic film and centrifuged at 1500 rpm for 30 sec. The samples were analyzed in triplicates on an Applied Biosystems StepOne plus Real-Time PCR System (Applied Biosystems, CA, USA) PCR machine using the StepOne software version 2.3 at; 95 °C for 20 sec, followed by 40 cycles of 95 °C for 1 sec and 60 °C for 20 sec.

The mean cycle threshold (C_T) values from the RT-qPCR run were analyzed using the comparative C_T-method for relative quantification (RQ). The C_T values was normalized to the reference gene β-2- macroglobulin and then compared. Since gene expression was analyzed in whole blood, cell specific gene expression was not possible.

3.3 Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) is a method that is used to measure concentrations of an antibody or antigen in a solution. A capture antibody specific for the antigen is added to the plate. The plate is blocked with a blocking buffer to avoid unspecific binding. The sample is added and the antigens is able to react with the capture antibody. The plate is washed to remove unbound antigen before a detection antibody is added. The detection antibody is an enzyme-linked antibody specific for a different epitope on the antigen than the capture antibody. A substrate for the enzyme is added, which results in a color change in the solution. This is measured in a spectrophotometer[4].

3.3.1 sCD14

A human CD14 DuoKit (R&D Systems®, Minneapolis, USA, Cat. No.: DY383) was used to measure the soluble CD14 (sCD14) levels in plasma from 19 of the patients enrolled in the study. Human CD14 Capture Antibody (2.0 µg/ml in PBS, 50 µl/well) was added to a 96-well ELISA plate (Nunc) and incubated over night at RT. The plates were washed using an auto washer were 0.05% Tween® 20 in PBS (300 µl/well) was added in three cycles. This washing step was repeated between every step throughout the procedure. The plates were blocked with Reagent Diluent (RD) (1% BSA in PBS, 100 µl/well) for 1 hr in RT. Samples and standards (50 µl/well) was added. The samples were diluted 1:1500 in RD and added in triplicates. Recombinant human CD14 standard was diluted in RD to 4000 pg/ml as the highest concentration. A seven-point standard curve is made using 2-fold serial dilution and added as replicates. The plates were incubated for 2 hrs at RT. Biotinylated sheep anti-human CD14 Detection Antibody (75 ng/ml in RD, 50 µl/well) was added. The plates were incubated for 2 hrs at RT. Streptavidin-HRP (1:200 in RD, 50 µl/well) was added. The plates were incubated for 20 min in the dark. Equal amounts of TMB substrate A and B (Biolegend, USA, 50 µl/well) was added. The plates were again incubated for 20 min in the dark. Stop Solution (1 M H_2SO_4, 25 µl/well) was added to the plates before the optical density of each well was measured with the microplate reader POLARstar Omega (BMG Labtech, Ortenberg, Germany). The reader was set to 450 nm with wavelength corrections set to 540 nm. There was a high variability in the measured OD values in technical triplicate samples for the patients. Some extreme values were excluded, but the triplicates were kept for most of the patients, due to variability.

3.4 Statistical methods

Statistical analyses were performed by the use of GraphPad Prism software version 7 (California, USA). In the obesity study the MFI expression of the receptors was compared at three different time points, W0, W8 and W12. The statistical analysis was performed using repeated measurements one-way ANOVA, Tukey's multiple comparisons test. The control group was compared at time point T1 and T2, and t-test Wilcoxon matched-pairs signed rank test was performed on the data. For the analyses of percentage expression, the negative, positive, high and low receptor expression was compared (W0, W8, W12) for the overweight patients. A two-way ANOVA, Tukey's multiple comparison test was performed on these data. For the control group, a two-way ANOVA Sidak's multiple comparisons test was performed after the data had been log-transformed, due to low sample size. In the RT-qPCR experiments, a non-parametric ANOVA Friedman test, Dunn's multiple comparisons test was performed on the calculated RQ-values, to compare the fold changes in receptor gene expression (W0, W8, W12). While plasma levels from ELISA was analyzed with one-way ANOVA Tukey's multiple comparison test.

The data was considered significant when $p \leq 0.05$, while when the degree of statistical significance is given in the figures, the stars are given the following values of significance:

Table 3.7 Statistical significant values

ns	$P > 0.05$
*	$P \leq 0.05$
**	$P \leq 0.01$
***	$P \leq 0.001$
****	$P \leq 0.0001$

3.5 Ethical

The project involved working with human whole blood from obese patients, approved by the Regional Ethics Committee (REK) (2012/1901).

4 Results

4.1 Receptor expression on leukocytes in obesity

Flow cytometer was used to analyze the cell surface receptor expression of TLR2 and TLR4, co-receptor CD14, oxLDL-receptor CD36, as well as inflammatory receptors CD16, CD11b and CD66b in blood samples (W0, W8, W12) from obese patients (N=17).

4.1.1 TLR2

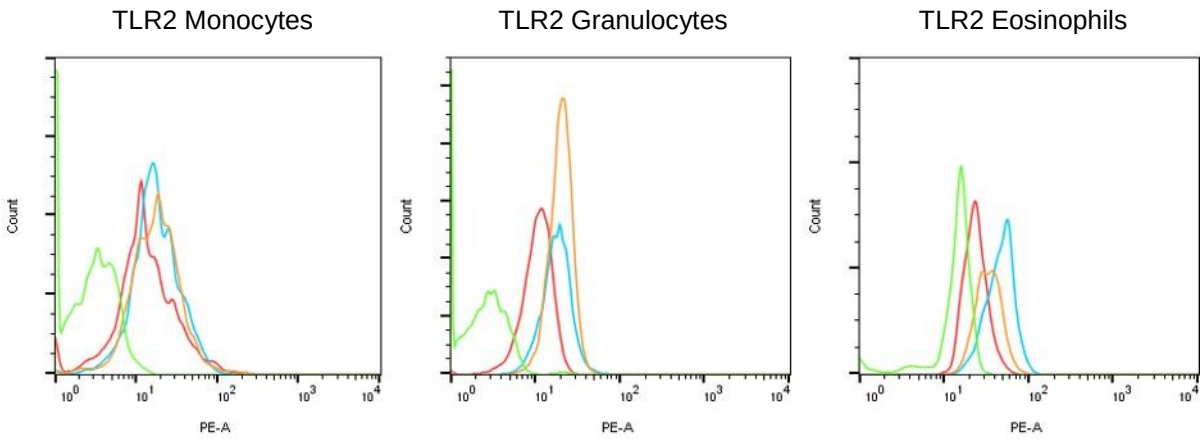

Figure 4.1 A representative histogram of TLR2 surface expression on monocytes, granulocytes and eosinophils in one obese patient. Receptor expression is measured by flow cytometer at time point W0, W8, W12. Green line = FMO control; Red line = expression at time point W0; Blue line = expression at time point W8; Orange line = expression at time point W12.

The histograms showed that TLR2 receptor expression was skewed to the right after W8 and W12, demonstrating an increase in receptor expression after weight reduction (Figure 4.1).

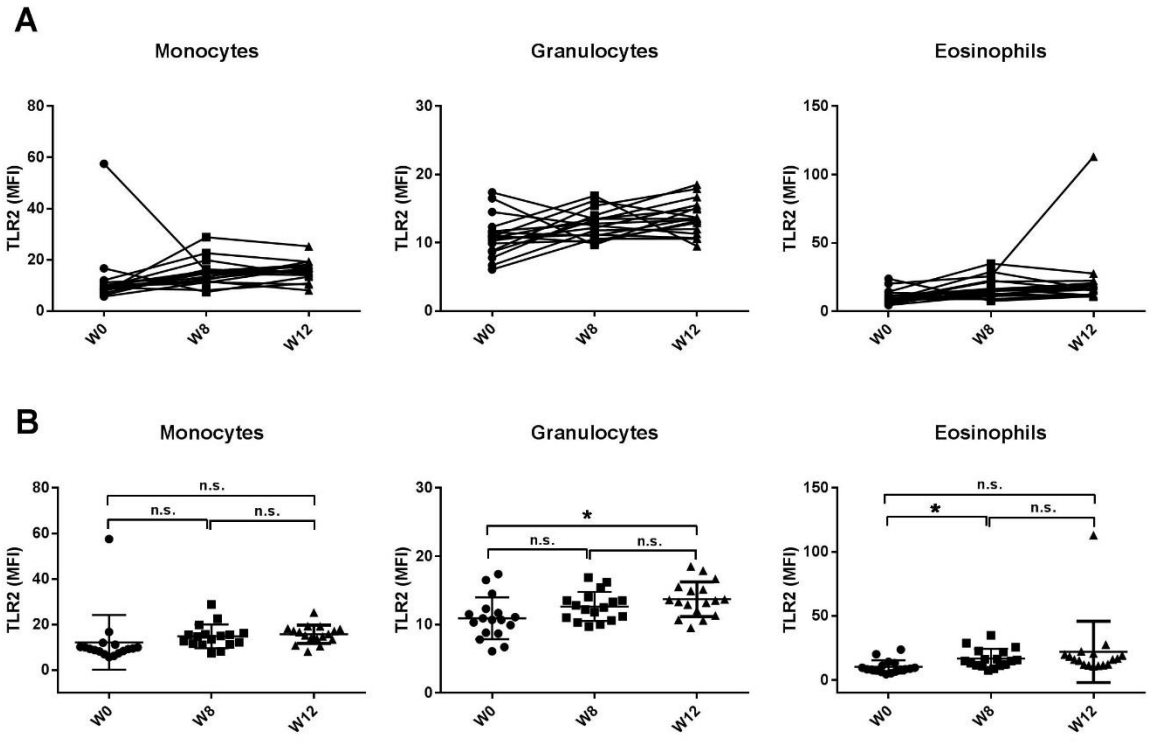

Figure 4.2 Surface expression of TLR2 on monocytes, granulocytes and eosinophils in blood samples (W0, W8, W12) of obese patients (N=17). Expression is measured by flow cytometer as median fluorescence intensity (MFI). Figure A shows the changes in receptor expression for each patient at W0, W8 and W12, while figure B shows individual median data points N=17±SD. N.s. = not significant; * = p≤ 0.05.

TLR2 receptor expression increased between W0 and W8 and then stabilized after W12 in all the cell populations analyzed (Figure 4.2 A,B). Two patients showed a high TLR2 expression on monocytes and eosinophils (Figure 4.2 A,B). A statistical significant (p≤ 0.05) increase was found between W0 and W12 on granulocytes, and between W0 and W8 on eosinophils (Figure 4.2 B).

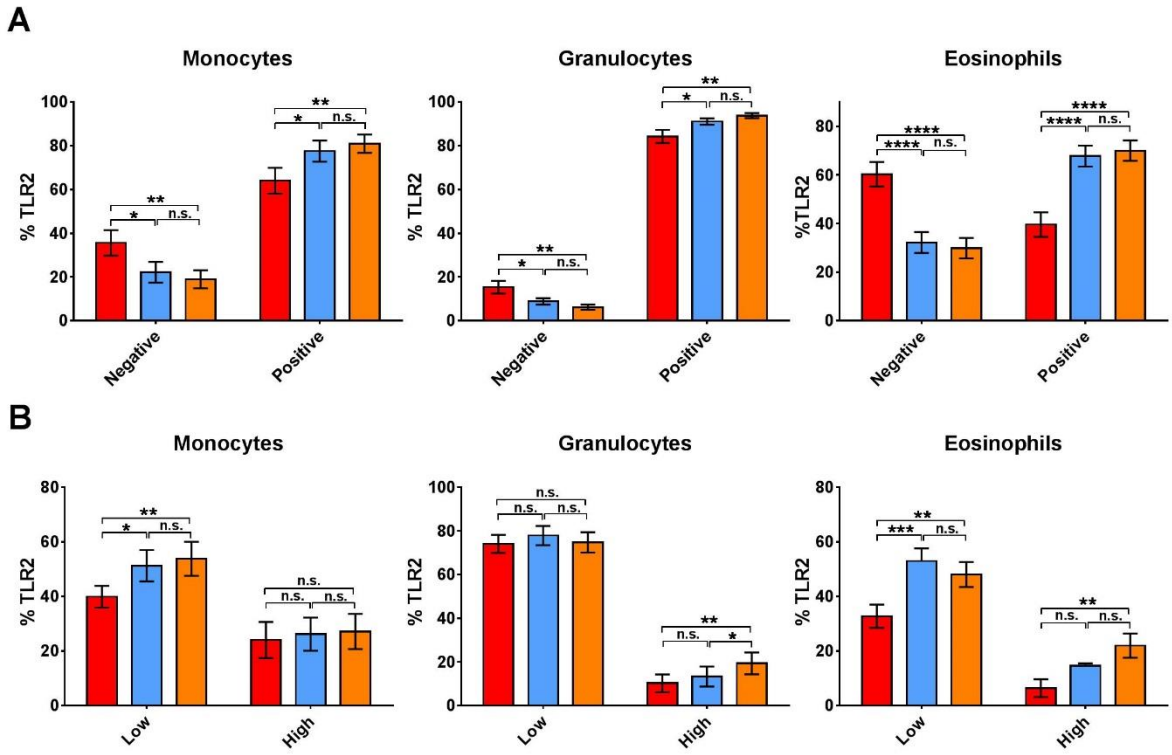

Figure 4.3 Percentage distribution of cells expressing TLR2 on monocytes, granulocytes and eosinophils in blood samples (W0, W8, W12) of obese patients (N=17). Receptor expression is measured by flow cytometer and divided into percentage of negative and positive expressing cells, and percentage of high and low expression of the receptor. Figure A shows the percentage of negative and positive expressing cells at W0, W8 and W12, while figure B shows the low and high expression of the receptor. Red box = W0; Blue box = W8; Orange box = W12. N.s. = not significant; * = p≤ 0.05; ** = p≤ 0.01; *** = p≤ 0.001; **** = p≤ 0.0001.

The percentage of negative TLR2 expressing cells on monocytes and granulocytes statistical significantly decreased between W0 and W8 (p≤ 0.05), and between W0 and W12 (p≤ 0.01) (Figure 4.3 A). A corresponding significant increase was found in the percentage of positive TLR2 expressing cells between W0 and W8 (p≤ 0.05), and between W0 and W12 (p≤ 0.01) (Figure 4.3 A). On eosinophils, there was an excessive decrease in the percentage of negative TLR2 expressing cells between W0 and W8 (p≤ 0.0001), and W0 and W12 (p≤ 0.0001), with a following excessive increase in the percentage of positive TLR2 expressing cells with the same statistical significance (Figure 4.3 A). The percentage of low TLR2 expressing cells increased with a statistical significance between W0 and W8 (p≤ 0.05), and between W0 and W12 (p≤ 0.01) on monocytes (Figure 4.3 B). An increase in the percentage of high TLR2

expressing cells was found on granulocytes between W8 and W12 ($p \leq 0.05$), and between W0 and W12 ($p \leq 0.01$) (Figure 4.3 B). On eosinophils, there was an increase in the percentage of low TLR2 expressing cells between W0 and W8 ($p \leq 0.001$), and W0 and W12 ($p \leq 0.01$) (Figure 4.3 B). An increase was also found in the percentage of high TLR2 expressing cells between W0 and W12 ($p \leq 0.01$) (Figure 4.3 B).

4.1.2 TLR4

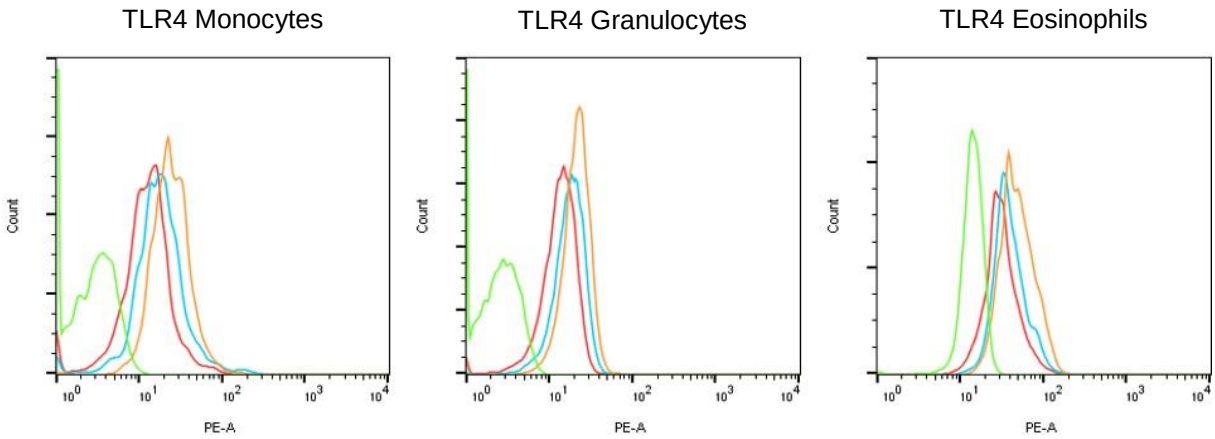

Figure 4.4 A representative histogram of TLR4 surface expression on monocytes, granulocytes and eosinophils in one obese patient. Receptor expression is measured by flow cytometer at time point W0, W8, W12. Green line = FMO control; Red line = expression at time point W0; Blue line = expression at time point W8; Orange line = expression at time point W12.

The histograms showed that TLR4 receptor expression was skewed to the right after W8 and further to the right after W12, demonstrating an increase in receptor expression after weight reduction (Figure 4.4).

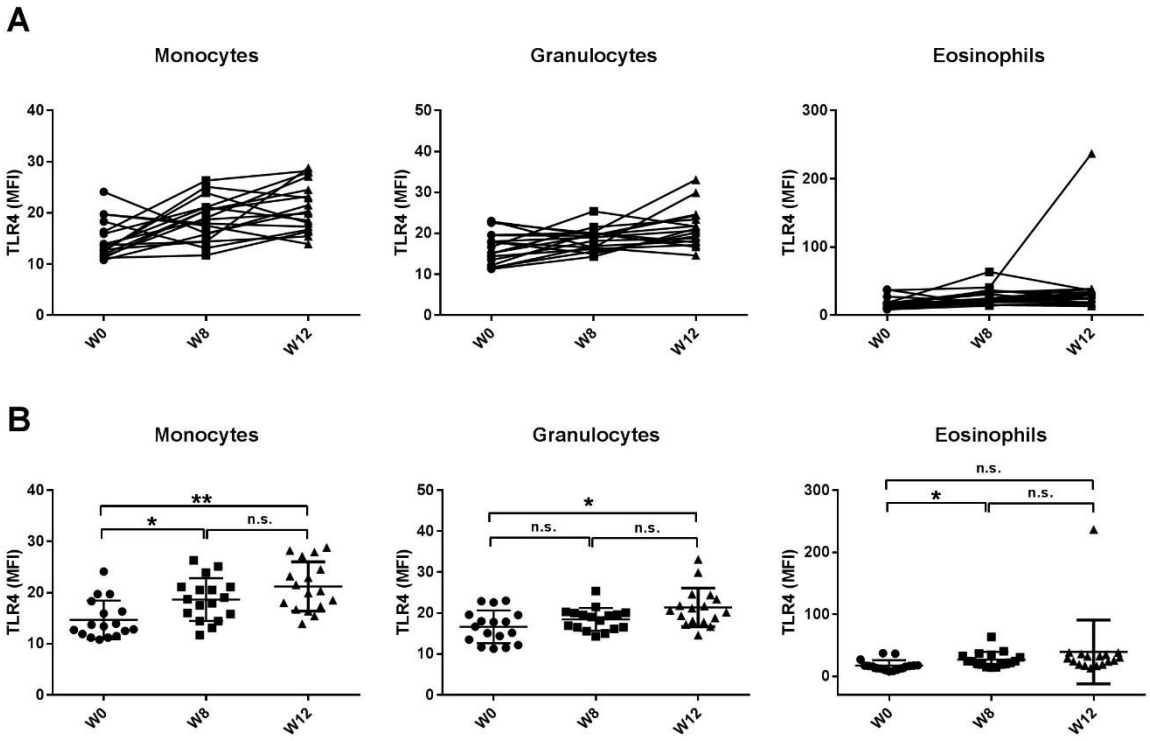

Figure 4.5 Surface expression of TLR4 on monocytes, granulocytes and eosinophils in blood samples (W0, W8, W12) of obese patients (N=17). Expression is measured by flow cytometer as median fluorescence intensity (MFI). Figure A shows the changes in receptor expression for each patient at W0, W8 and W12, while figure B shows individual median data points N=17±SD. N.s. = not significant; * = p≤ 0.05; ** = p≤ 0.01.

TLR4 receptor expression increased between W0 and W12 in all the cell populations analyzed (Figure 4.5 A,B). The expression stabilized after W12 on granulocytes and eosinophils, while on the monocytes, some patients had a further increase in receptor expression (Figure 4.5 A,B). One patient had a high receptor expression at W12 on eosinophils (Figure 4.5 A,B). On monocytes, the increase was statistical significant between W0 and W8 ($p \leq 0.05$), and between W0 and W12 ($p \leq 0.01$) (Figure 4.5 B). A statistical significant increase was also found between W0 and W12 ($p \leq 0.05$) on granulocytes, and between W0 and W8 ($p \leq 0.05$) on eosinophils (Figure 4.5 B).

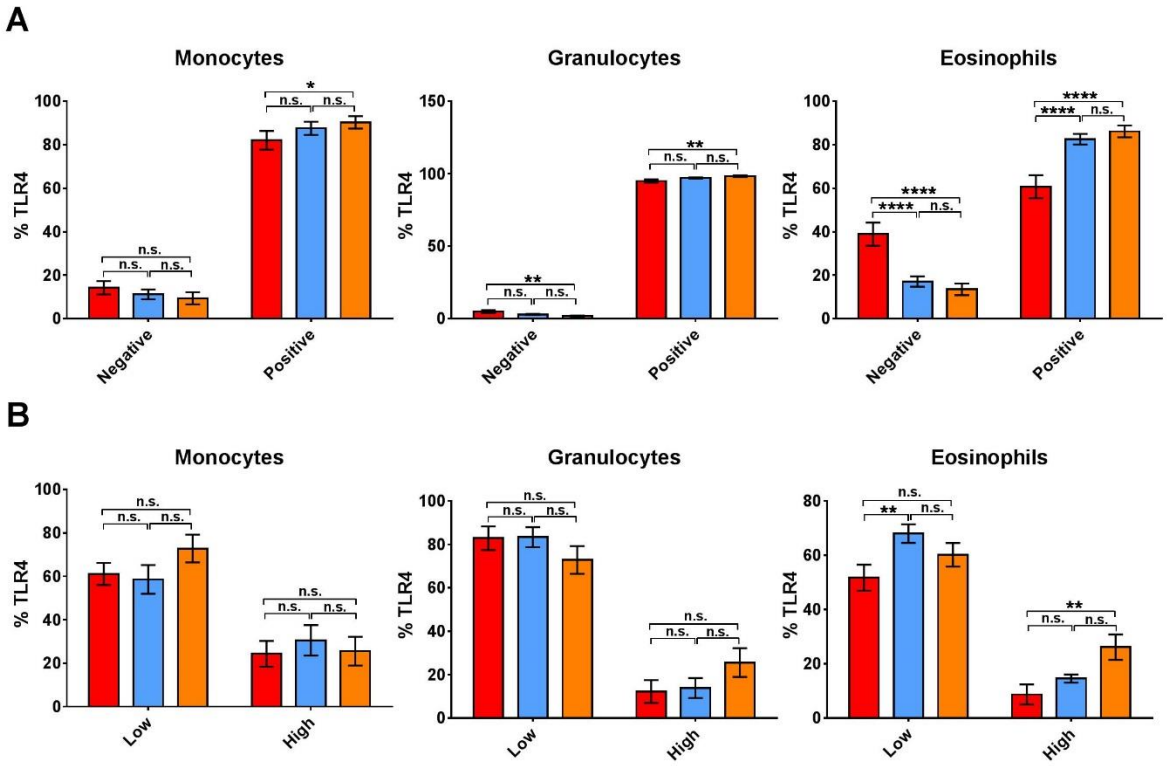

Figure 4.6 Percentage distribution of cells expressing TLR4 on monocytes, granulocytes and eosinophils in blood samples (W0, W8, W12) of obese patients (N=17). Receptor expression is measured by flow cytometer and divided into percentage of negative and positive expressing cells, and percentage of high and low expression of the receptor. Figure A shows the percentage of negative and positive expressing cells at W0, W8 and W12, while figure B shows the low and high expression of the receptor. Red box = W0; Blue box = W8; Orange box = W12. N.s. = not significant; * = p≤ 0.05; ** = p≤ 0.01; *** = p≤ 0.001; **** = p≤ 0.0001.

The percentage of positive TLR4 expressing cells statistical significantly increase between W0 and W12 (p≤ 0.05) (Figure 4.6 A). There was a slight decrease in the percentage of negative TLR4 expressing cells between W0 and W12 (p≤ 0.01) on granulocytes, with a corresponding increase in the percentage of positive TLR4 expressing cells between W0 and W12 (p≤ 0.01) (Figure 4.6 A). On eosinophils, a decrease between W0 and W8 (p≤ 0.0001), and W0 and W12 (p≤ 0.0001) was found in the percentage of negative TLR4 expressing cells, with a following increase in the percentage of positive TLR4 expressing cells with the same statistical significance (Figure 4.6 A). The percentage of low TLR4 expressing cell on eosinophils increased statistical significantly between W0 and W8 (p≤ 0.01), while the percentage of high TLR4 expressing cells increased between W0 and W12 (p≤ 0.01) (Figure 4.6 B).

4.1.3 CD14

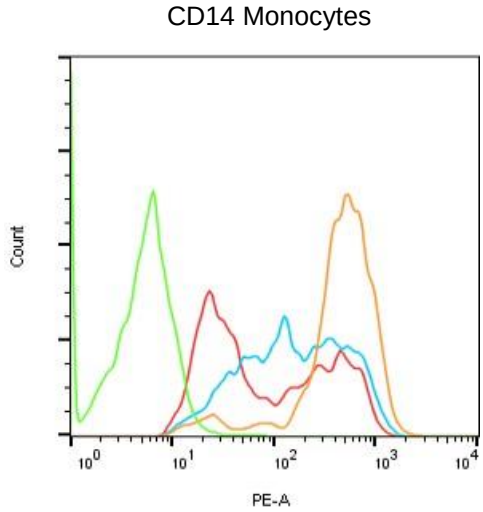

Figure 4.7 A representative histogram of CD14 surface expression on monocytes in one obese patient. Receptor expression is measured by flow cytometer at time point W0, W8, W12. Green line = FMO control; Red line = expression at time point W0; Blue line = expression at time point W8; Orange line = expression at time point W12.

The histogram showed that CD14 receptor expression was skewed lightly to the right after W8, with a following skew after W12, demonstrating an increase in receptor expression after weight reduction, with a further increase after normal diet was introduced (Figure 4.7).

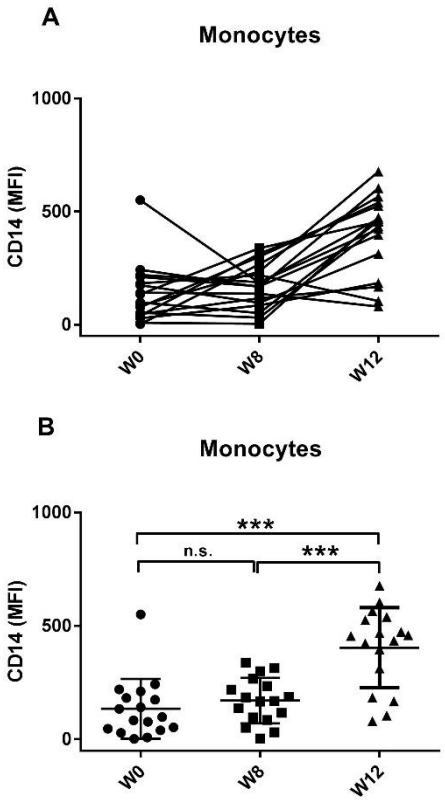

Figure 4.8 Surface expression of CD14 on monocytes in blood samples (W0, W8, W12) of obese patients (N=17). Expression is measured by flow cytometer as median fluorescence intensity (MFI). Figure A shows the changes in receptor expression for each patient at W0, W8 and W12, while figure B shows individual median data points N=17±SD. N.s. = not significant; *** = p≤ 0.001.

CD14 receptor expression did not change or lightly increased between W0 and W8 on monocytes, with a following excessive increase between W8 and W12 (Figure 4.8 A). The increase between W0 and W12, and between W8 and W12 was found to be statistical significant ($p \leq 0.001$) (Figure 4.8 B).

CD14 was also measured in its soluble form in plasma samples (W0, W8, W12) from 19 of the patients enrolled in the study (Figure 4.9).

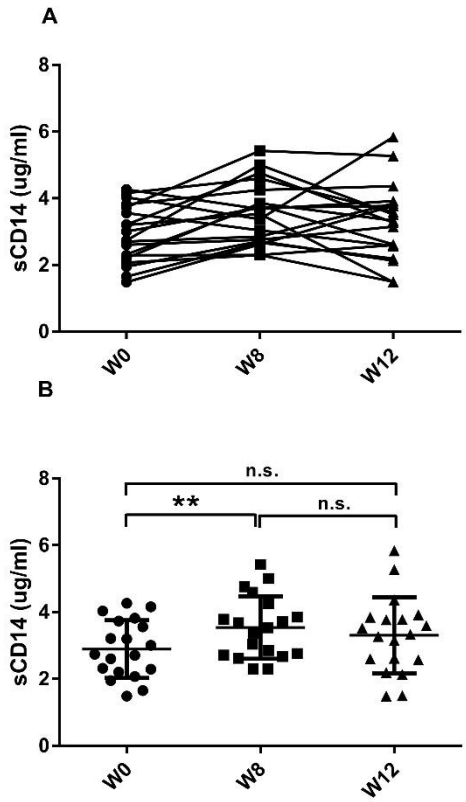

Figure 4.9 Plasma levels of sCD14. Plasma sCD14 concentration (µg/ml) measured by ELISA at time points, W0, W8 and W12 (N=19). Data are presented as average±SD with each line representing an individual patient (A) or as individual data points (B) N.s. = not significant; * = p≤ 0.05; ** = p≤ 0.01; *** = p≤ 0.001; **** = p≤ 0.0001.

sCD14 levels increased significantly (p≤ 0.01) between W0 and W8, and decreased between W8 and W12 in the majority of the patients (n.s) (Figure 4.9 A,B).

4.1.4 CD16

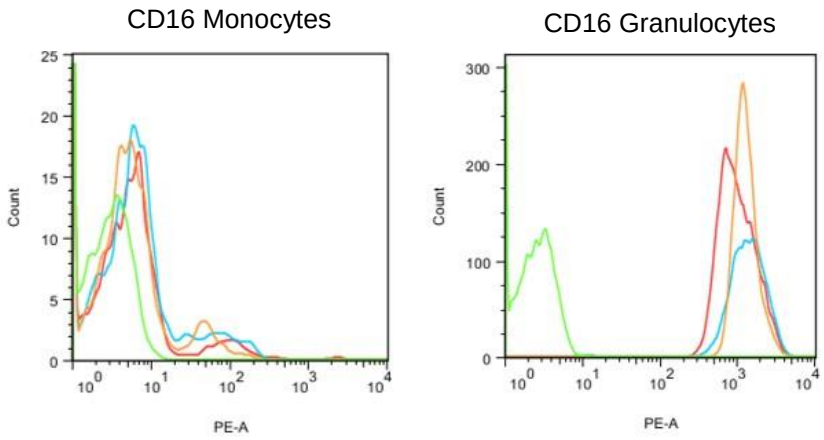

Figure 4.10 A representative histogram of CD16 surface expression on monocytes and granulocytes in one obese patient. Receptor expression is measured by flow cytometer at time point W0, W8, W12. Green line = FMO control; Red line = expression at time point W0; Blue line = expression at time point W8; Orange line = expression at time point W12.

The histograms showed that CD16 receptor expression on monocytes increased from W0 to W8, with a decrease after W12. The green line overlapping with receptor expression indicates that there was a small amount of monocytes expressing CD16. The CD16 receptor expression on granulocytes increased from W0 to W8, with a stabilization in expression after W12. All the granulocytes expressed receptor CD16 (Figure 4.10).

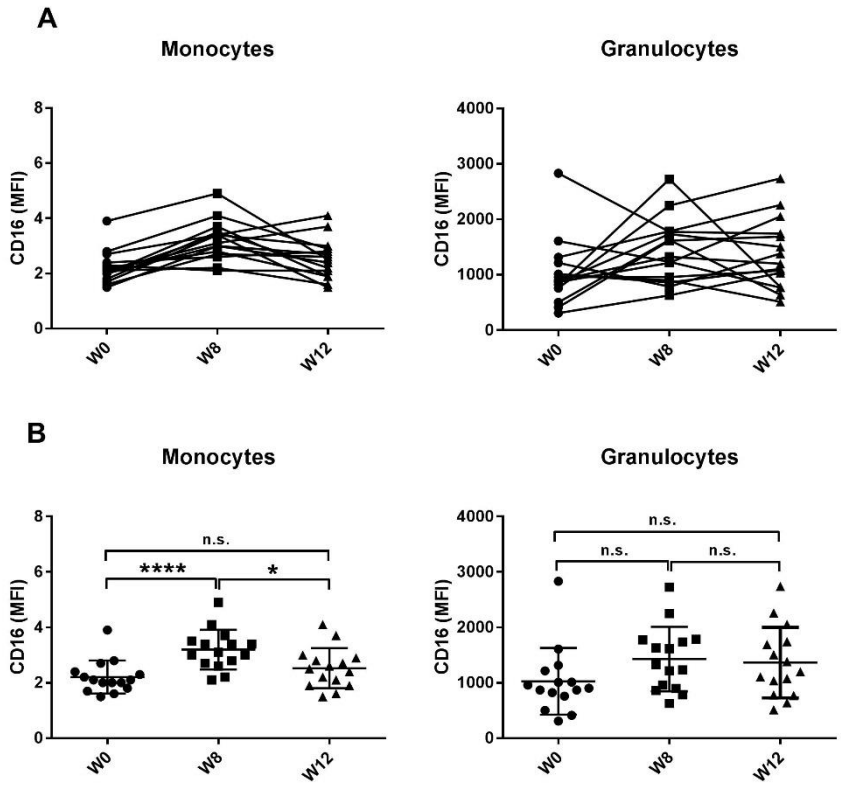

Figure 4.11 Surface expression of CD16 on monocytes and granulocytes in blood samples (W0, W8, W12) of obese patients (N=15). Expression is measured by flow cytometer as median fluorescence intensity (MFI). Figure A shows the changes in receptor expression for each patient at W0, W8 and W12, while figure B shows individual median data points N=15±SD. N.s. = not significant; * = p≤ 0.05; **** = p≤ 0.0001.

CD16 receptor expression on monocytes increased between W0 and W8 (p≤ 0.0001), with a following decrease between W8 and W12 (p≤ 0.05) (Figure 4.11 A,B). The CD16 receptor expression on granulocytes showed some biological variances between the patients (Figure 4.11 A), however, on a general level an increase was found between W0 and W8 with a stabilization in expression after W12 (Figure 4.11 B).

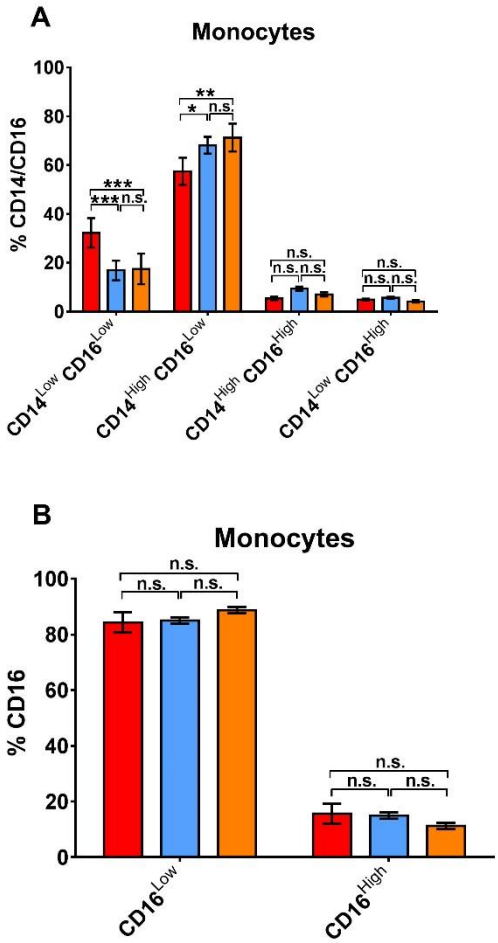

Figure 4.12 Percentage distribution of CD16 monocyte subpopulations in blood samples (W0, W8, W12) of obese patients (N=15). Receptor expression is measured by flow cytometer and divided into high or low CD14/CD16 expression, and high and low CD16 expression. Figure A shows the percentage of CD14/D16 high or low expressing cells at W0, W8 and W12, while figure B shows the percentage of CD16 low or high expressing cells. Red box = W0; Blue box = W8; Orange box = W12. N.s. = not significant; * = p≤ 0.05; ** = p≤ 0.01; *** = p≤ 0.001.

The percentage of $CD14^{Low}CD16^{Low}$ expressing cells statistical significantly decreased between W0 and W8 ($p \leq 0.001$), and between W0 and W12 ($p \leq 0.001$) (Figure 4.12 A). In the percentage of $CD14^{High}CD16^{Low}$ expressing cells (classical monocytes) a statistical significant increase was found between W0 and W8 ($p \leq 0.05$), and between W0 and W12 ($p \leq 0.01$) (Figure 4.12 A). The percentage of $CD14^{High}CD16^{High}$ expressing cells (intermediate monocytes) and $CD14^{Low}CD16^{High}$ expressing cells (inflammatory monocytes) non-significantly increased between W0 and W8, with a following decrease between W8 and W12 (Figure 4.12 A). No

statistical significant changes were found in the percentage of $CD16^{Low}$ and $CD16^{High}$ expressing cells (Figure 4.12 B).

4.1.5 CD36

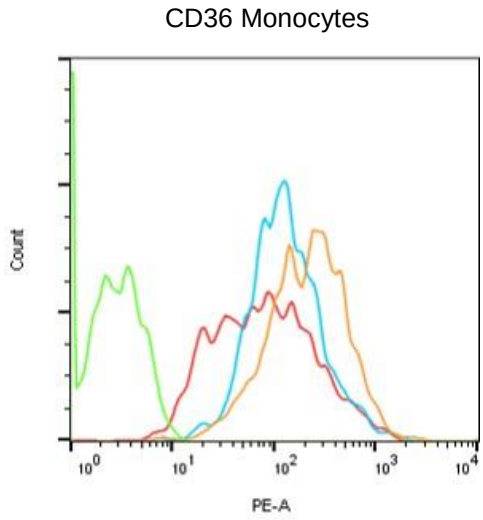

Figure 4.13 A representative histogram of CD36 surface expression on monocytes in one obese patient. Receptor expression is measured by flow cytometer at time point W0, W8, W12. Green line = FMO control; Red line = expression at time point W0; Blue line = expression at time point W8; Orange line = expression at time point W12.

The histogram showed that CD36 receptor expression was skewed to the right after W8 and W12, demonstrating an increase in receptor expression after weight loss (Figure 4.13).

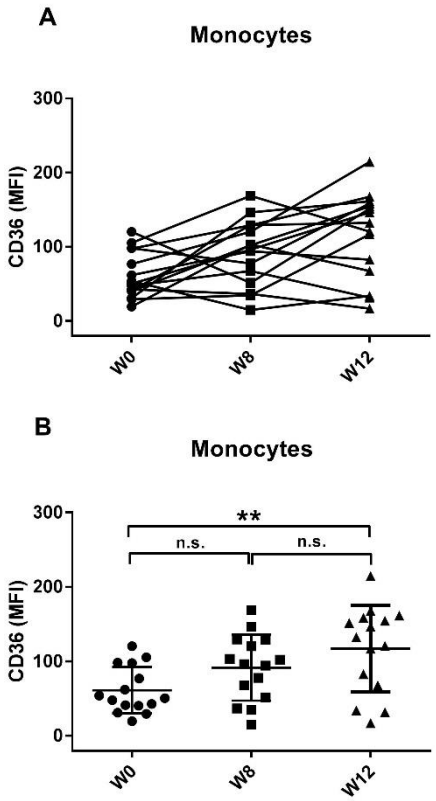

Figure 4.14 Surface expression of CD36 on monocytes in blood samples (W0, W8, W12) of obese patients (N=15). Expression is measured by flow cytometer as median fluorescence intensity (MFI). Figure A shows the changes in receptor expression for each patient at W0, W8 and W12, while figure B shows individual median data points N=15±SD. N.s. = not significant; ** = p≤ 0.01.

CD36 receptor expression showed some biological variance between the patients at time point W0, W8 and W12 (Figure 4.14 A). However, on a general level there was an increase between W0 and W8, with a following increase between W8 and W12 (Figure 4.14 B). The increase between W0 and W12 reached statistical significance (p≤ 0.01) (Figure 4.14 B).

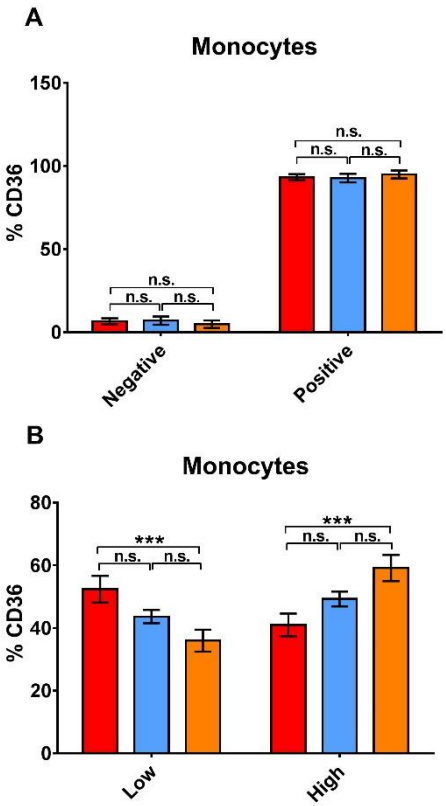

Figure 4.15 Percentage distribution of cells expressing CD36 on monocytes in blood samples (W0, W8, W12) of obese patients (N=15). Receptor expression is measured by flow cytometer and divided into percentage of negative and positive expressing cells, and percentage of high and low expression of the receptor. Figure A shows the percentage of negative and positive expressing cells at W0, W8 and W12, while figure B shows the low and high expression of the receptor. Red box = W0; Blue box = W8; Orange box = W12. N.s. = not significant; *** = p≤ 0.001.

The percentage of low CD36 expressing cells statistical significantly decreased between W0 and W12 (p≤ 0.001) on monocytes, with an equivalent increase in the percentage of high CD36 expressing cells between W0 and W12 (p≤ 0.001) (Figure 4.15 B).

4.1.6 CD11b

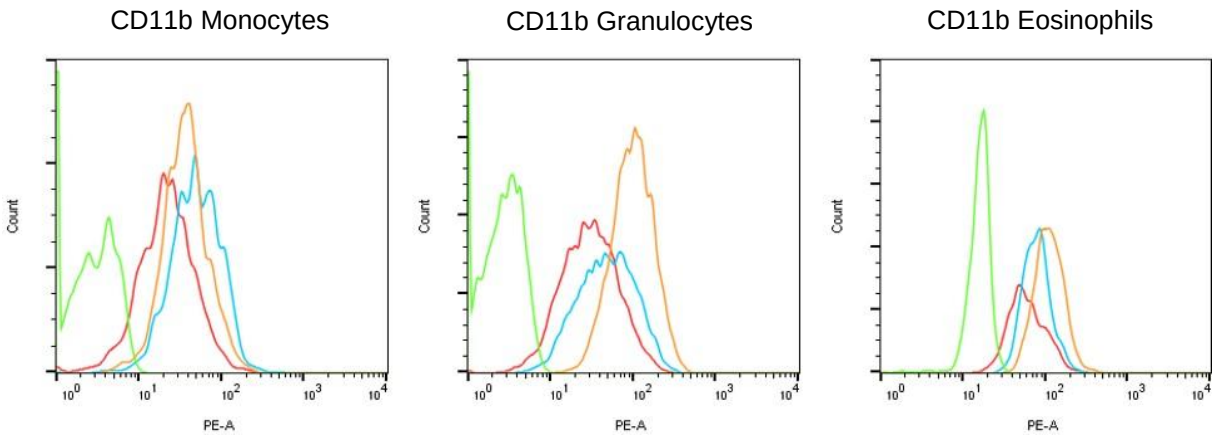

Figure 4.16 A representative histogram of CD11b surface expression on monocytes, granulocytes and eosinophils in one obese patient. Receptor expression is measured by flow cytometer at time point W0, W8, W12. Green line = FMO control; Red line = expression at time point W0; Blue line = expression at time point W8; Orange line = expression at time point W12.

As shown by the representative histograms, the CD11b receptor expression was skewed to the right after W8 and W12, demonstrating an increase in receptor expression after weight reduction (Figure 4.16).

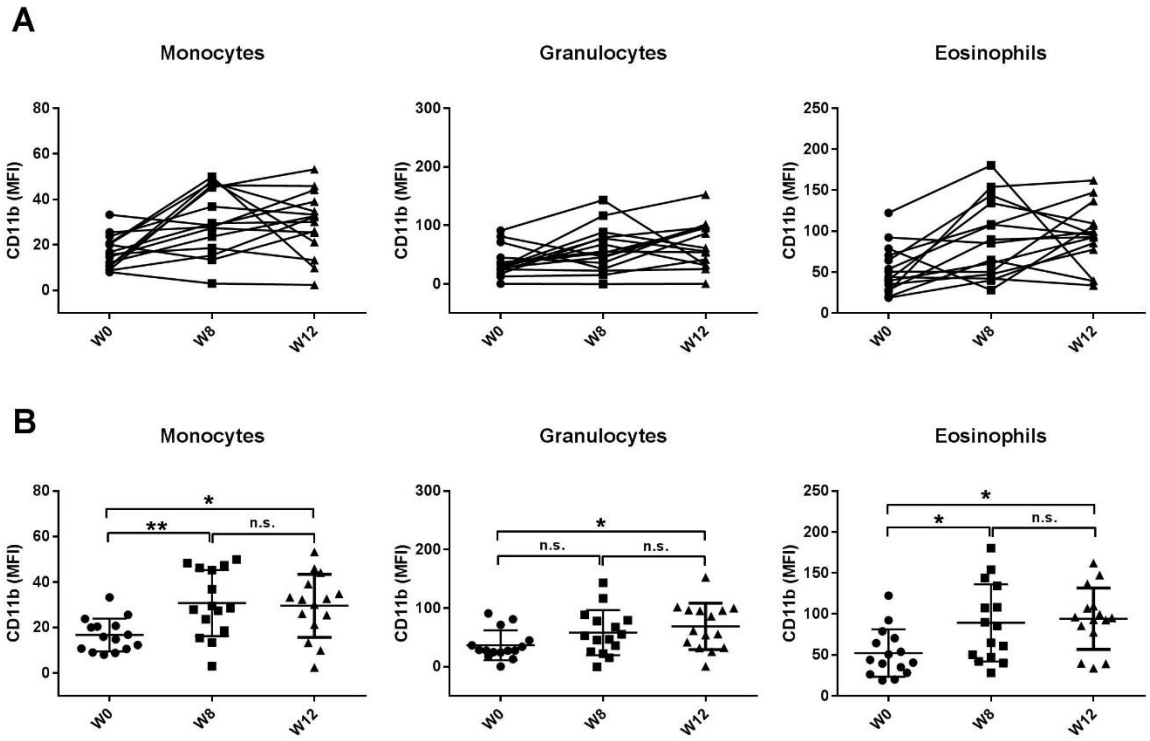

Figure 4.17 Surface expression of CD11b on monocytes, granulocytes and eosinophils in blood samples (W0, W8, W12) of obese patients (N=15). Expression is measured by flow cytometer as median fluorescence intensity (MFI). Figure A shows the changes in receptor expression for each patient at W0, W8 and W12, while figure B shows individual median data points N=15±SD. N.s. = not significant; * = p≤ 0.05; ** = p≤ 0.01.

CD11b receptor expression showed some biological variance between patients in the cell populations analyzed (Figure 4.17 A). On a general level, there was an increase in receptor expression between W0 and W8, with a following stabilization after W12 in the cell populations analyzed (Figure 4.17 B). On monocytes, granulocytes and eosinophils there was a statistical significant increase between W0 and W12 (p≤ 0.05), and also between W0 and W8 on monocytes (p≤ 0.01) and eosinophils (p≤ 0.05) (Figure 4.17 B).

4.1.7 CD66b

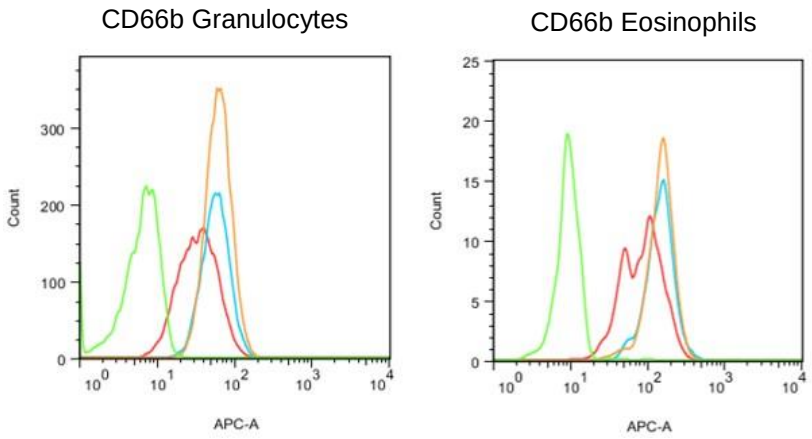

Figure 4.18 A representative histogram of CD66b surface expression on granulocytes and eosinophils in one obese patient. Receptor expression is measured by flow cytometer at time point W0, W8, W12. Green line = FMO control; Red line = expression at time point W0; Blue line = expression at time point W8; Orange line = expression at time point W12.

As shown in the histograms, the CD66b receptor expression was skewed to the right after W8 and W12 on granulocytes, while on eosinophils the receptor expression increased after W8 with a following stabilization in expression after W12 (Figure 4.18).

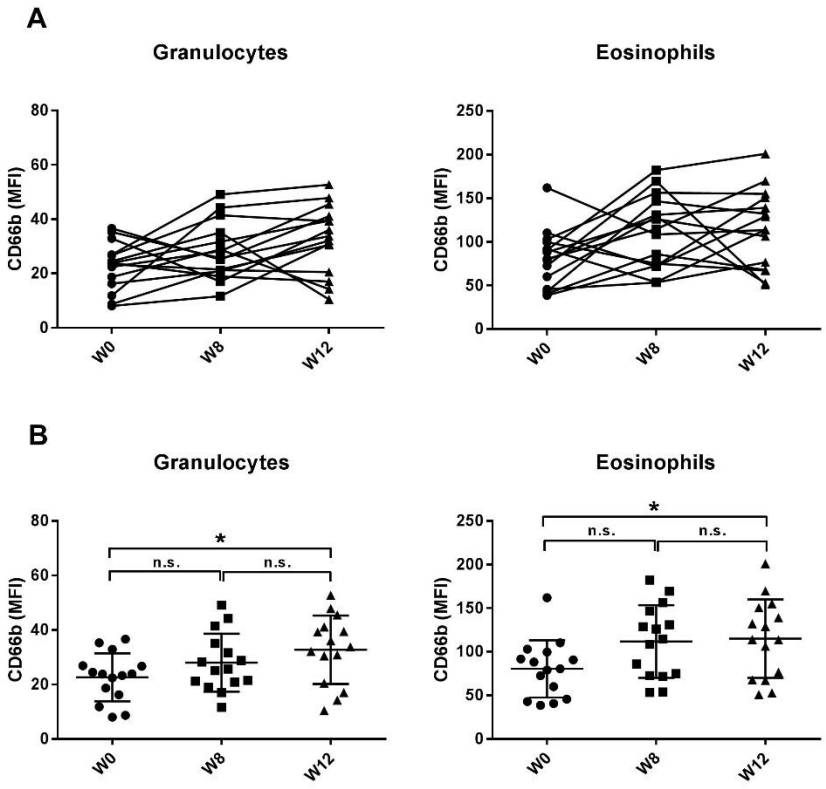

Figure 4.19 Surface expression of CD66b on granulocytes and eosinophils in blood samples (W0, W8, W12) of obese patients (N=15). Expression is measured by flow cytometer as median fluorescence intensity (MFI). Figure A shows the changes in receptor expression for each patient at W0, W8 and W12, while figure B shows individual median data points N=15±SD. N.s. = not significant; * = p≤ 0.05.

CD66b receptor expression showed some biological variance between patients, but an increase in receptor expression between W0 and W8 was observed, with a following increase after W12 on granulocytes and stabilization after W12 on eosinophils (Figure 4.19 A,B). A statistical significant (p≤ 0.05) increase was found between W0 and W12 on both granulocytes and eosinophils (Figure 4.19 B).

4.2 Receptor expression on leukocytes in control group

Flow cytometer was used to analyze the cell surface receptor expression of TLR2 and TLR4, co-receptor CD14, oxLDL receptor CD36, as well as inflammatory receptors CD16, CD11b and CD66b in blood samples (T1, T2) from healthy controls (N=5).

4.2.1 TLR2

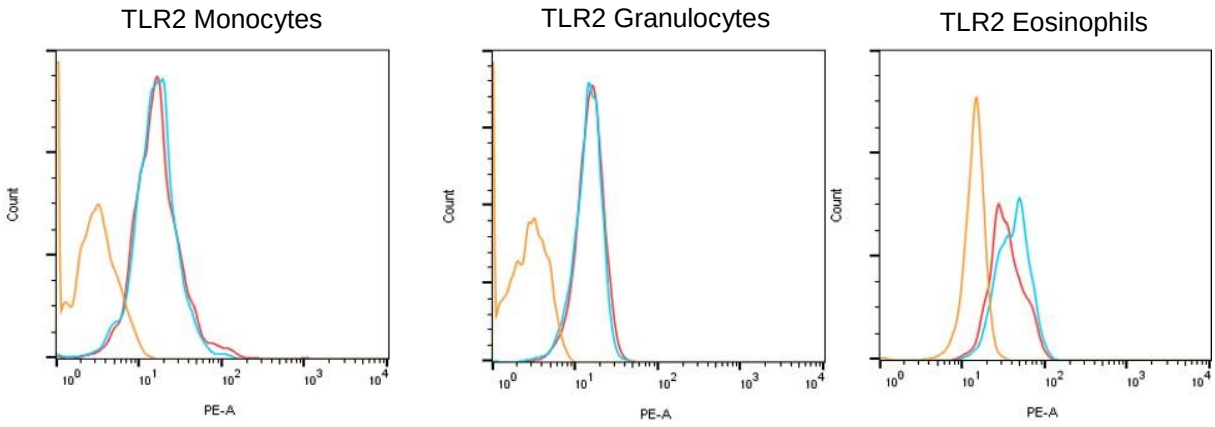

Figure 4.20 A representative histogram of TLR2 surface expression on monocytes, granulocytes and eosinophils in one healthy control. Receptor expression is measured by flow cytometer at time points T1, T2. Orange line = FMO control; Red line = expression at time point T1; Blue line = expression at time point T2.

The histograms showed that TLR2 receptor expression was stable at both time points on monocytes, granulocytes and eosinophils (Figure 4.20).

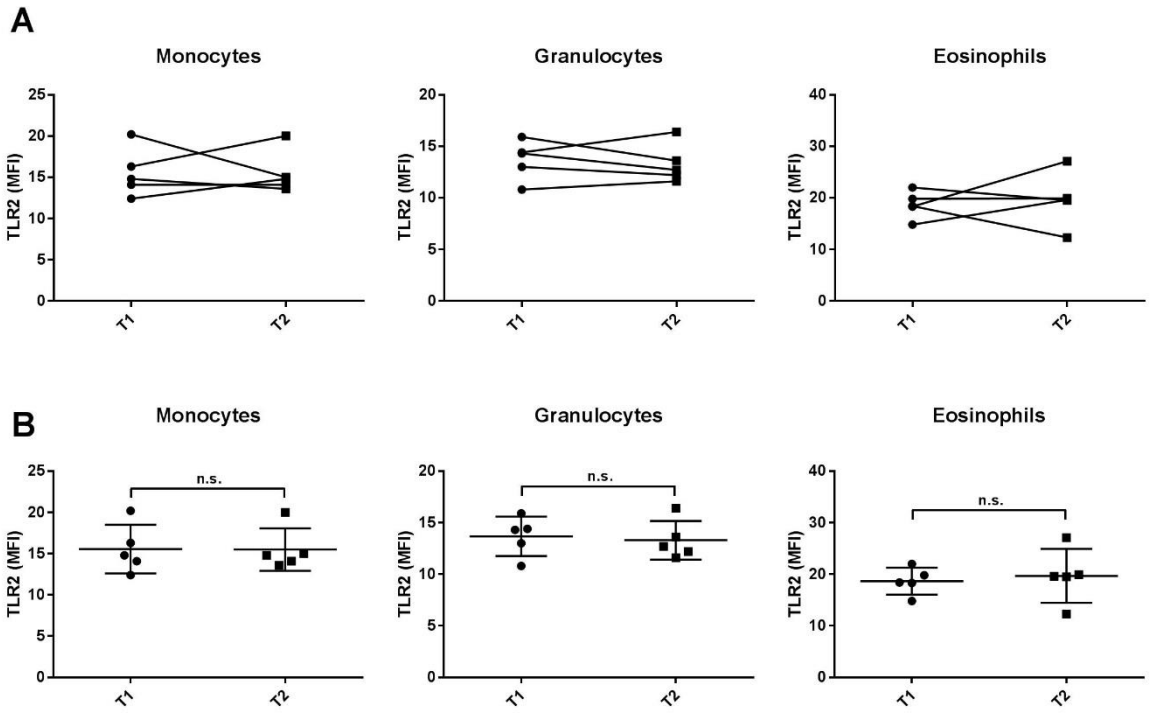

Figure 4.21 Surface expression of TLR2 on monocytes, granulocytes and eosinophils in blood samples (T1, T2) of healthy controls (N=5). Expression is measured by flow cytometer as median fluorescence intensity (MFI). Figure A shows the changes in receptor expression for each patient at T1 and T2, while figure B shows individual median data points N=5±SD. N.s. = not significant.

TLR2 receptor expression varied between T1 and T2 on monocytes, granulocytes and eosinophils, with a decrease in expression on the granulocytes (Figure 4.21 A). There were no statistical significant changes in expression between T1 and T2 in all the analyzed cell populations (Figure 4.21 B).

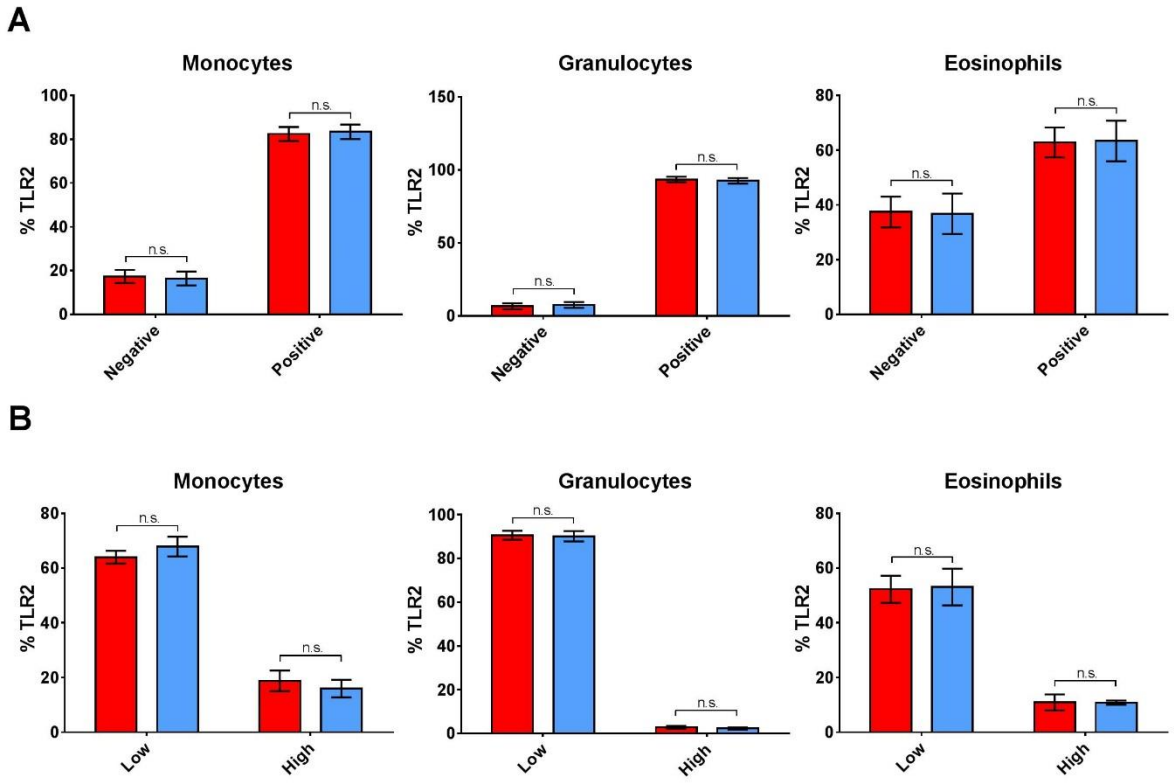

Figure 4.22 Percentage distribution of cells expressing TLR2 on monocytes, granulocytes and eosinophils in blood samples (T1, T2) of healthy controls (N=5). Receptor expression is measured by flow cytometer and divided into percentage of negative and positive expressing cells, and percentage of high and low expression of the receptor. Figure A shows the percentage of negative and positive expressing cells at T1 and T2, while figure B shows the low and high expression of the receptor. Red box = T1; Blue box = T2. N.s. = not significant.

The percentage of negative and positive TLR2 expressing cells, and the percentage of high and low TLR2 expressing cells did not reach any statistical significant changes between T1 and T2 in all the analyzed cell populations (Figure 4.22 A,B).

4.2.2 TLR4

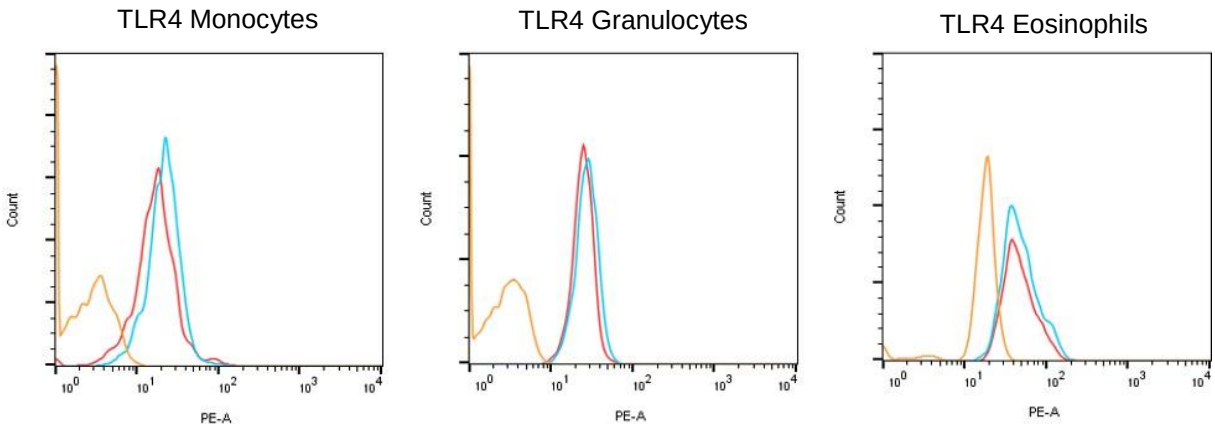

Figure 4.23 A representative histogram of TLR4 surface expression on monocytes, granulocytes and eosinophils in one healthy control. Receptor expression is measured by flow cytometer at time points T1, T2. Orange line = FMO control; Red line = expression at time point T1; Blue line = expression at time point T2.

As shown in the histograms, the TLR4 receptor expression was skewed slightly to the right at T2 compared to T1 in all cell populations analyzed (Figure 4.23).

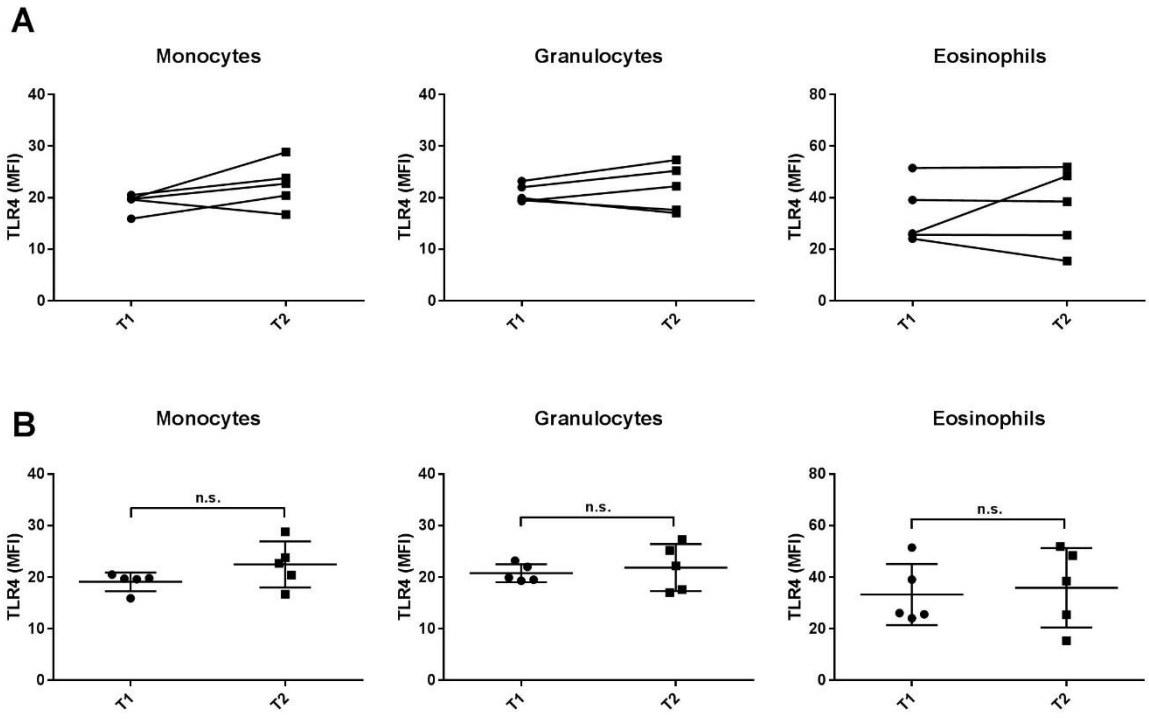

Figure 4.24 Surface expression of TLR4 on monocytes, granulocytes and eosinophils in blood samples (T1, T2) of healthy controls (N=5). Expression is measured by flow cytometer as median fluorescence intensity (MFI). Figure A shows the changes in receptor expression for each patient at T1 and T2, while figure B shows individual median data points N=5±SD. N.s. = not significant.

TLR4 receptor expression slightly increased between T1 and T2 with no statistical significance in all cell populations analyzed (Figure 4.24 A,B). However, on eosinophils there were some biological differences in receptor expression (Figure 4.24 A).

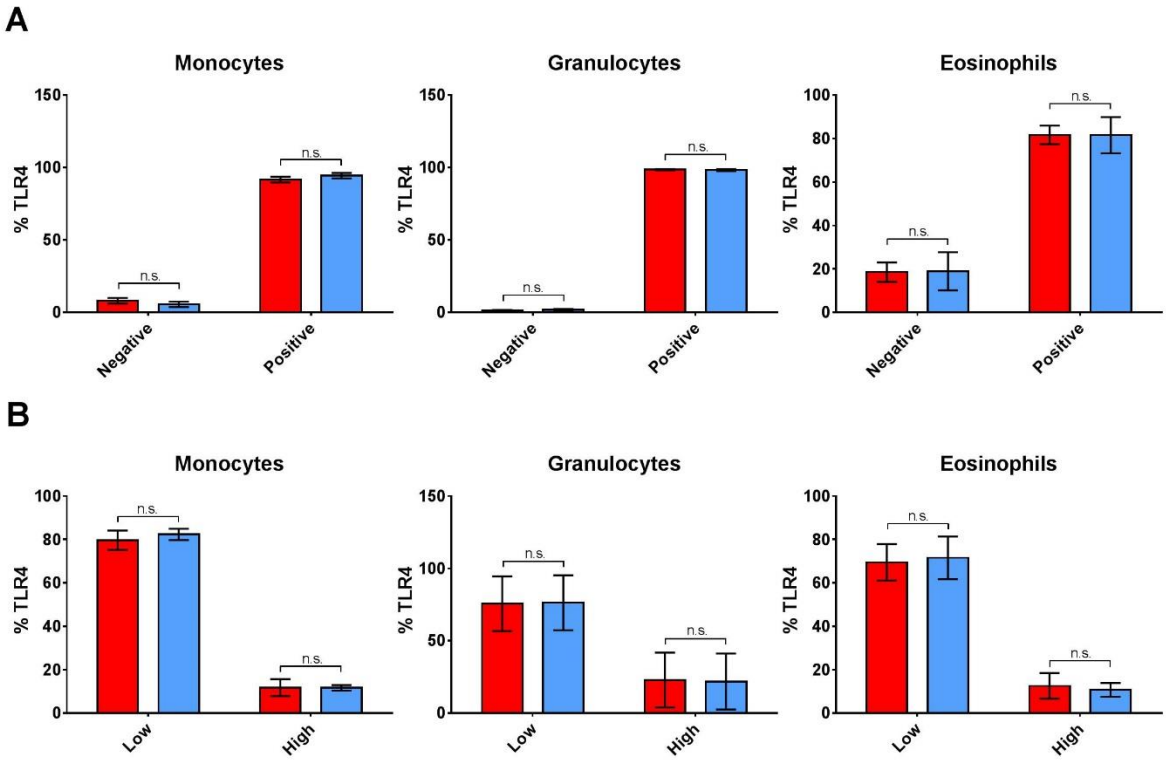

Figure 4.25 Percentage distribution of cells expressing TLR4 on monocytes, granulocytes and eosinophils in blood samples (T1, T2) of healthy controls (N=5). Receptor expression is measured by flow cytometer and divided into percentage of negative and positive expressing cells, and percentage of high and low expression of the receptor. Figure A shows the percentage of negative and positive expressing cells at T1 and T2, while figure B shows the low and high expression of the receptor. Red box = T1; Blue box = T2. N.s. = not significant.

The percentage of negative and positive TLR4 expressing cells, and the percentage of high and low TLR4 expressing cells did not reach any statistical significant changes between T1 and T2 in all cell populations analyzed (Figure 4.25 A,B).

4.2.3 CD14

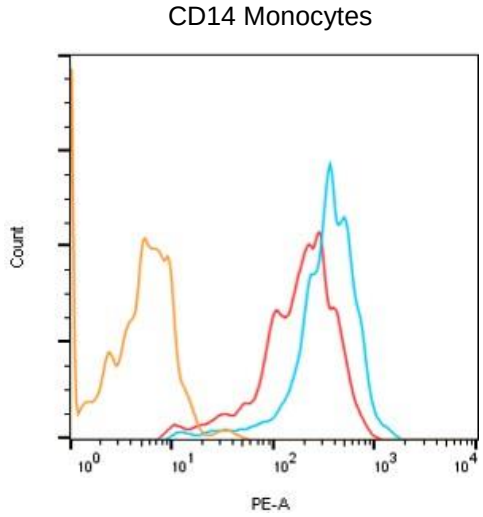

Figure 4.26 A representative histogram of CD14 surface expression on monocytes in one healthy control. Receptor expression is measured by flow cytometer at time points T1, T2. Orange line = FMO control; Red line = expression at time point T1; Blue line = expression at time point T2.

The histogram showed that CD14 receptor expression was skewed to the right at T2 compared to T1 on the monocytes (Figure 4.26).

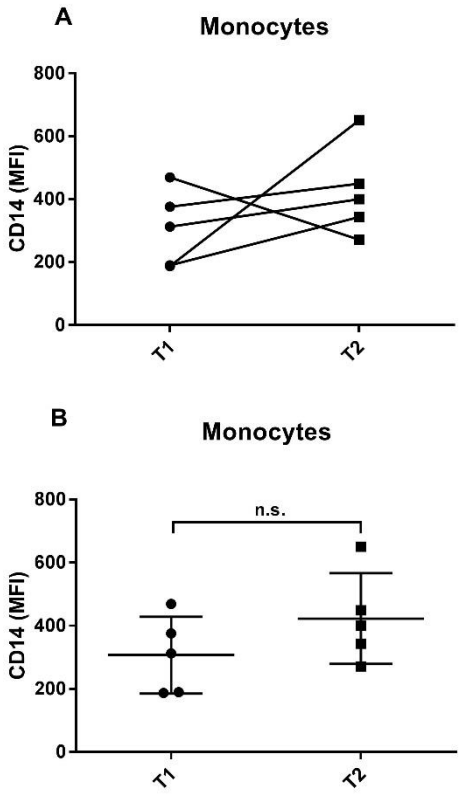

Figure 4.27 Surface expression of CD14 on monocytes in blood samples (T1, T2) of healthy controls (N=5). Expression is measured by flow cytometer as median fluorescence intensity (MFI). Figure A shows the changes in receptor expression for each patient at T1 and T2, while figure B shows individual median data points N=5±SD. N.s. = not significant.

CD14 receptor expression non-significantly increased between T1 and T2 on monocytes (Figure 4.27 A,B).

4.2.4 CD16

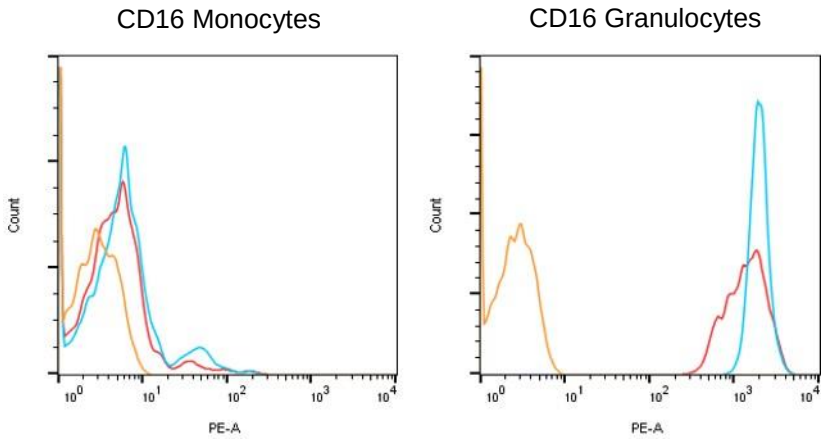

Figure 4.28 A representative histogram of CD16 surface expression on monocytes and granulocytes in one healthy control. Receptor expression is measured by flow cytometer at time points T1, T2. Orange line = FMO control; Red line = expression at time point T1; Blue line = expression at time point T2.

As shown in the histograms, the CD16 receptor expression was higher on monocytes and granulocytes at T1, compared to T2 (Figure 4.28). As indicated by the orange line overlapping with receptor expression, there are a small amount of monocytes expressing CD16, compared to granulocytes (Figure 4.28).

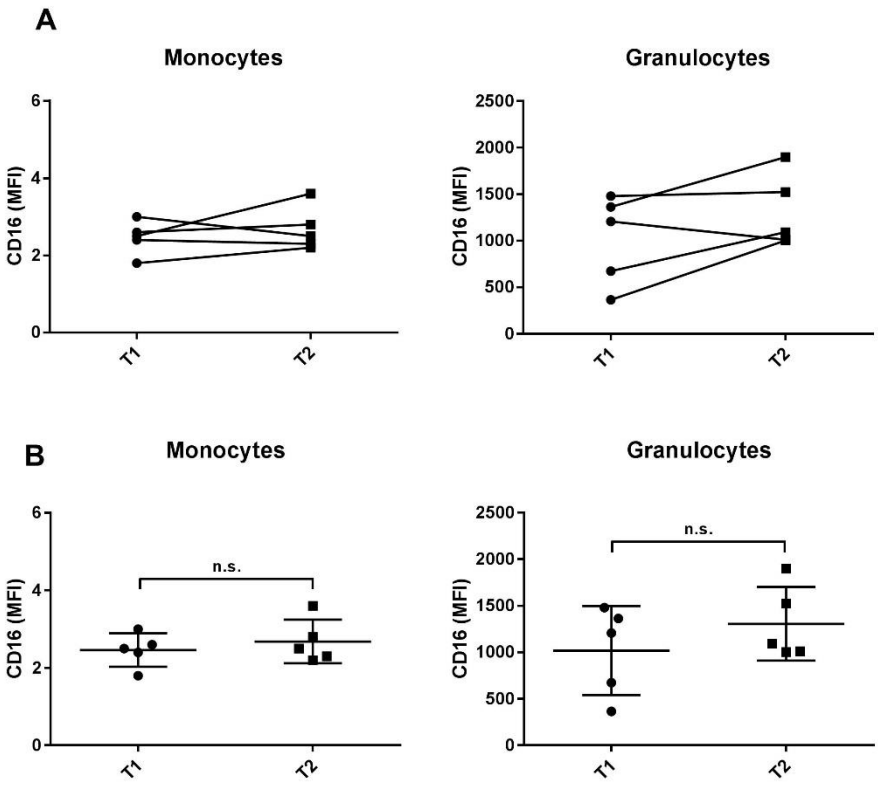

Figure 4.29 Surface expression of CD16 on monocytes and granulocytes in blood samples (T1, T2) of healthy controls (N=5). Expression is measured by flow cytometer as median fluorescence intensity (MFI). Figure A shows the changes in receptor expression for each patient at T1 and T2, while figure B shows individual median data points N=5±SD. N.s. = not significant.

There was some biological variance in CD16 receptor expression between T1 and T2 on monocytes and granulocytes (Figure 4.29 A). However, on a general level there was a lightly non-significant increase between T1 and T2 on monocytes and granulocytes (Figure 4.29 B).

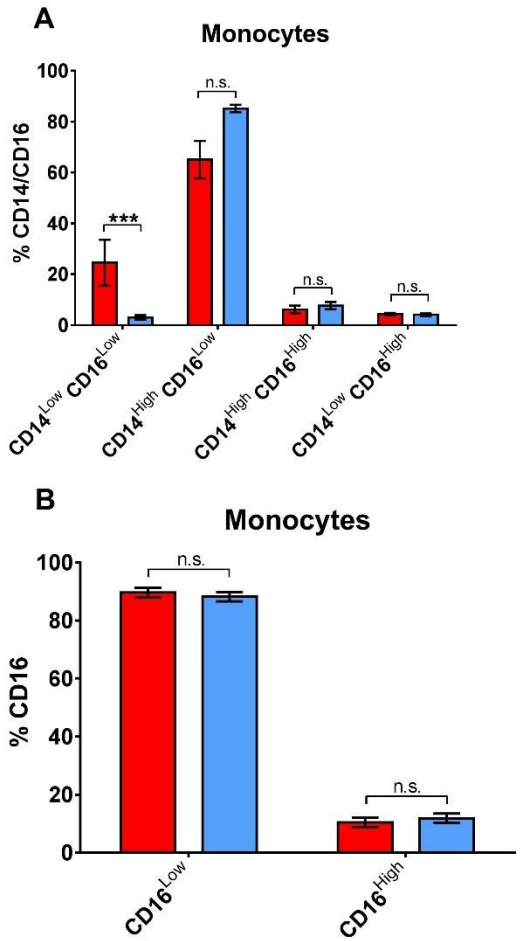

Figure 4.30 Percentage distribution of CD16 monocyte subpopulations in blood samples (T1, T2) of healthy controls (N=5). Receptor expression is measured by flow cytometer and divided into high or low CD14/CD16 expression, and high and low CD16 expression. Figure A shows the percentage of CD14/D16 high or low expressing cells at T1 and T2, while figure B shows the percentage of CD16 low or high expressing cells. Red box = T1; Blue box = T2. N.s. = not significant; *** = p≤ 0.001.

The percentage of $CD14^{Low}CD16^{Low}$ expressing cells statistical significantly (p≤ 0.001) decreased between T1 and T2 (Figure 4.30 A). There was a non-significant increase in the percentage of $CD14^{High}CD16^{Low}$ expressing cells (classical monocytes) and $CD14^{High}CD16^{High}$ expressing cells (intermediate monocytes) (Figure 4.30 A). A non-significant decrease was found in the percentage of $CD14^{Low}CD16^{High}$ (inflammatory monocytes) expressing cells (Figure 4.30 A). There were no statistical significant changes in the percentage of $CD16^{Low}$ and $CD16^{High}$ expressing cells (Figure 4.30 B).

4.2.5 CD36

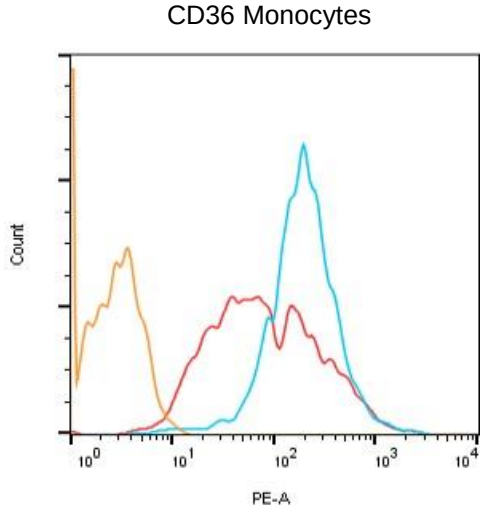

Figure 4.31 A representative histogram of CD36 surface expression on monocytes in one healthy control. Receptor expression is measured by flow cytometer at time points T1, T2. Orange line = FMO control; Red line = expression at time point T1; Blue line = expression at time point T2.

The histogram showed that the CD36 receptor expression was lower at T1 compared to T2 on monocytes (Figure 4.31).

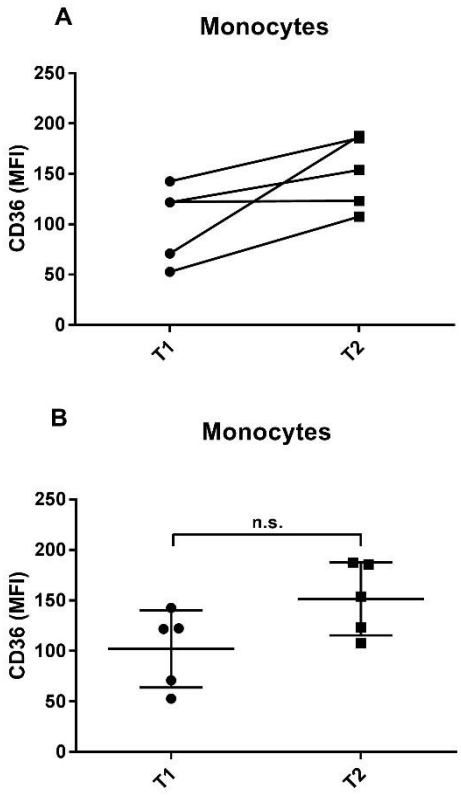

Figure 4.32 Surface expression of CD36 on monocytes in blood samples (T1, T2) of healthy controls (N=5). Expression is measured by flow cytometer as median fluorescence intensity (MFI). Figure A shows the changes in receptor expression for each patient at T1 and T2, while figure B shows individual median data points N=5±SD. N.s. = not significant.

CD36 receptor expression increased non-significantly between T1 and T2 on monocytes (Figure 4.32 A,B).

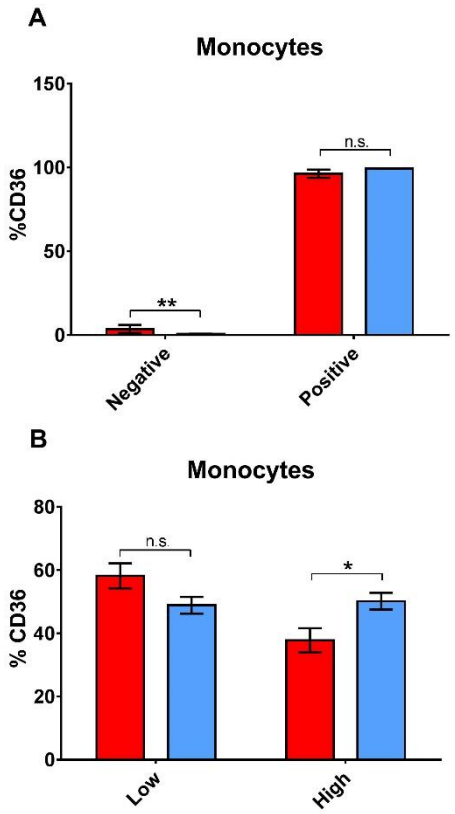

Figure 4.33 Percentage distribution of cells expressing CD36 on monocytes in blood samples (T1, T2) of healthy controls (N=5). Receptor expression is measured by flow cytometer and divided into percentage of negative and positive expressing cells, and percentage of high and low expression of the receptor. Figure A shows the percentage of negative and positive expressing cells at T1 and T2, while figure B shows the low and high expression of the receptor. Red box = T1; Blue box = T2. N.s. = not significant; * = p≤ 0.05; ** = p≤ 0.01.

The percentage of negative CD36 expressing cells statistical significantly (p≤ 0.01) decreased between T1 and T2 (Figure 4.33 A). The percentage of low CD36 expressing cells decreased (n.s) between T1 and T2, with a corresponding statistical significant (p≤ 0.05) increase in the percentage of high CD36 expressing cells (Figure 4.33 B).

4.2.6 CD11b

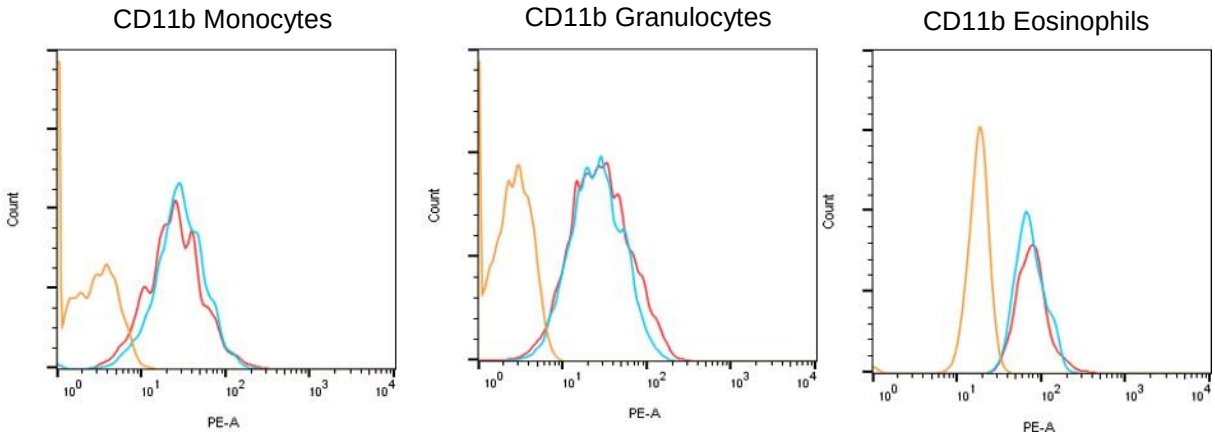

Figure 4.34 A representative histogram of CD11b surface expression on monocytes, granulocytes and eosinophils in one healthy control. Receptor expression is measured by flow cytometer at time points T1, T2. Orange line = FMO control; Red line = expression at time point T1; Blue line = expression at time point T2.

As shown in the histograms, the CD11b receptor expression was stable at both time points on monocytes, granulocytes and eosinophils (Figure 4.34).

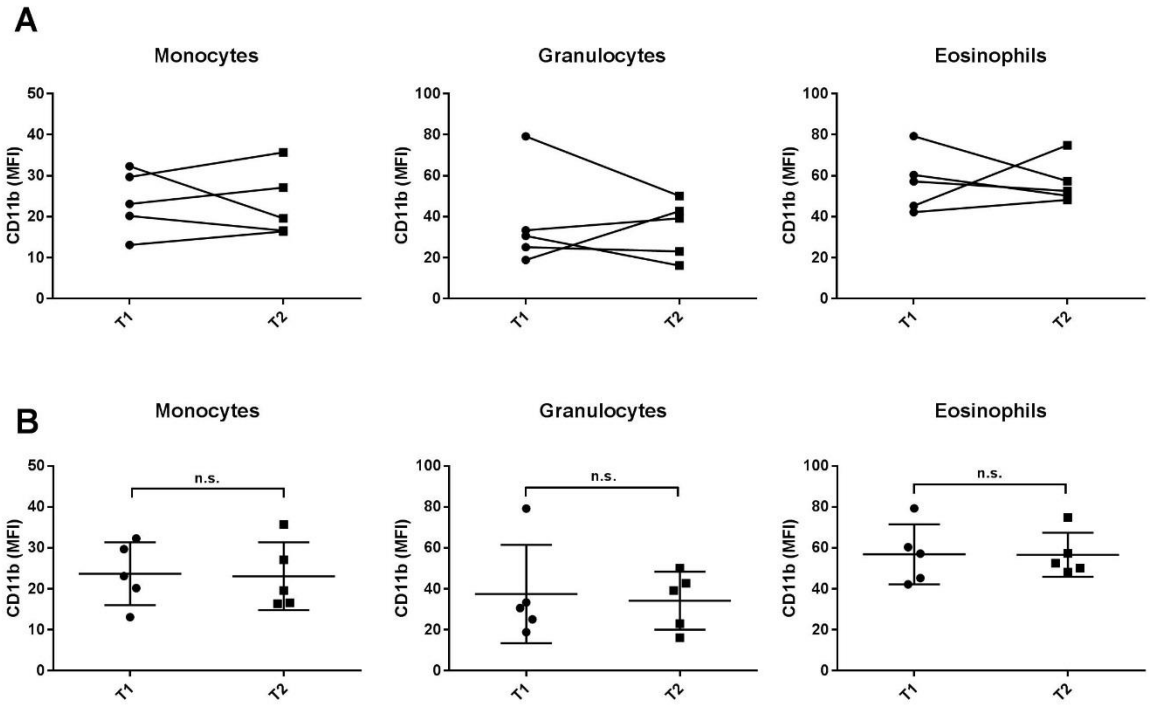

Figure 4.35 Surface expression of CD11b on monocytes, granulocytes and eosinophils in blood samples (T1, T2) of healthy controls (N=5). Expression is measured by flow cytometer as median fluorescence intensity (MFI). Figure A shows the changes in receptor expression for each patient at T1 and T2, while figure B shows individual median data points N=5±SD. N.s. = not significant.

There was some biological variance in CD11b receptor expression in the cell populations analyzed (Figure 4.35 A). However, on a general level, no significant changes were found (Figure 4.35 B).

4.2.7 CD66b

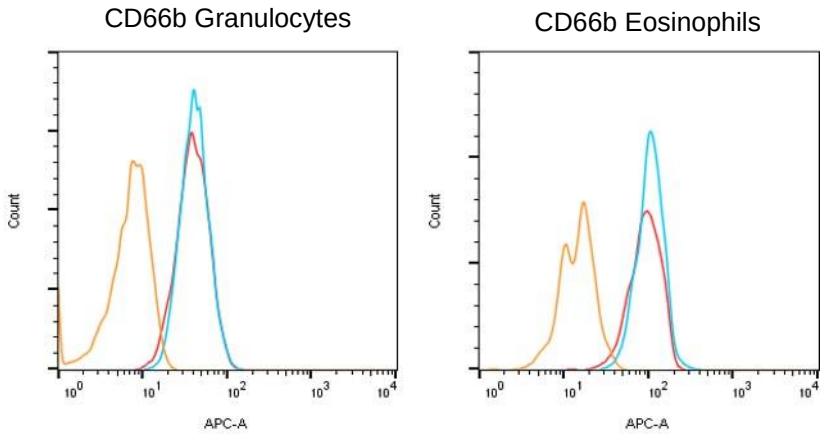

Figure 4.36 A representative histogram of CD66b surface expression on granulocytes and eosinophils in one healthy control. Receptor expression is measured by flow cytometer at time points T1, T2. Orange line = FMO control; Red line = expression at time point T1; Blue line = expression at time point T2.

The histograms showed that the CD66b receptor expression was stable at both time points on granulocytes and eosinophils (Figure 4.36).

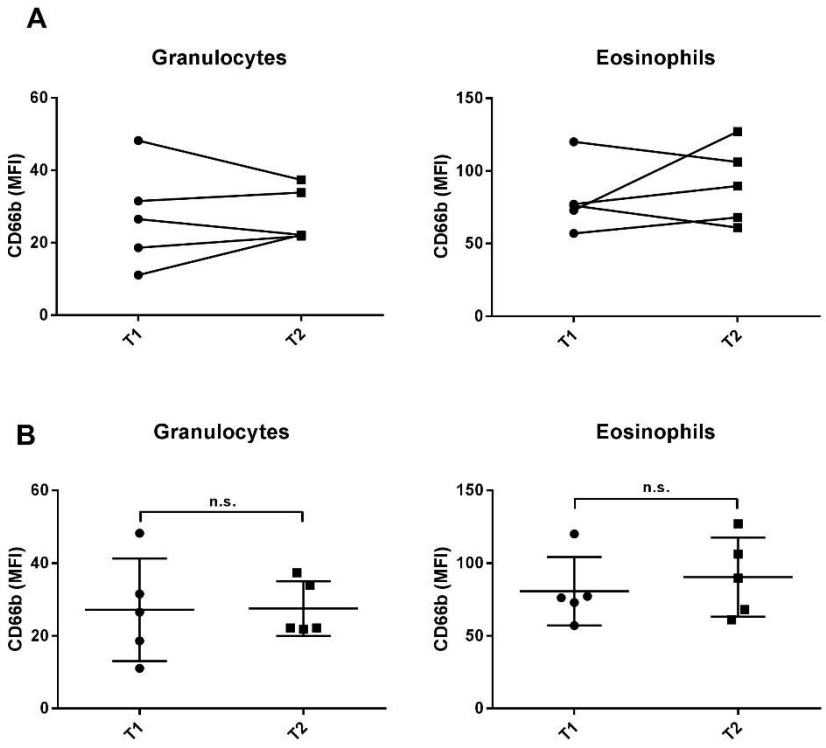

Figure 4.37 Surface expression of CD66b on granulocytes and eosinophils in blood samples (T1, T2) of healthy controls (N=5). Expression is measured by flow cytometer as median fluorescence intensity (MFI). Figure A shows the changes in receptor expression for each patient at T1 and T2, while figure B shows individual median data points N=5±SD. N.s. = not significant.

There was some biological variance in the CD66b receptor expression between the two time points on granulocytes and eosinophils (Figure 4.37 A). On a general level, no statistical significant changes were found (Figure 4.37 B).

4.3 Gene expression of leukocyte receptors in obesity

RT-qPCR was performed to analyze gene expression of TLR2 and TLR4, co-receptor CD14 and the oxLDL-receptor CD36 in cDNA samples (N=10) obtained from whole blood samples (W0, W8, W12).

4.3.1 TLR2

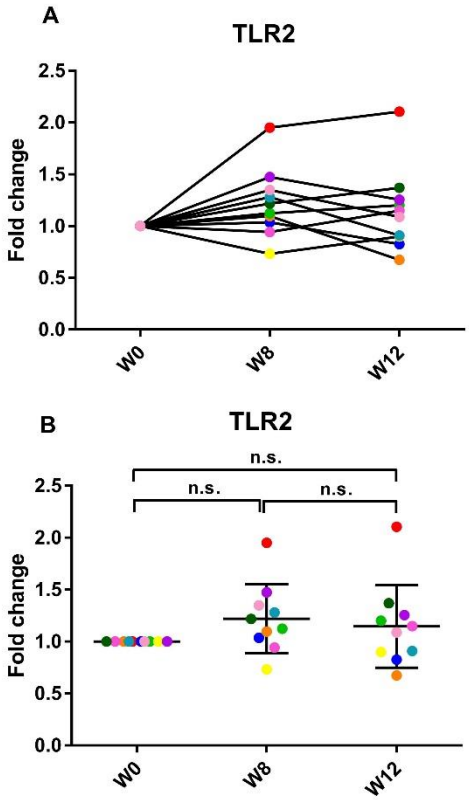

Figure 4.38 TLR2 gene expression levels in whole blood samples (W0, W8, W12) of obese patients (N=10). Gene expression levels are shown as fold changes (RQ values) of positive TLR2 expression relative to the reference gene β-2-microglobulin and W0 samples as estimated by the comparative C_T-method for relative quantification. Figure A shows the changes in receptor expression for each patient at W0, W8 and W12, while figure B shows individual median data points N=10±SD. N.s. = not significant.

TLR2 gene expression increased between W0 and W8, and then decreased between W8 and W12 (Figure 4.38 A,B). Some patients had an increase in gene expression between W8 and

W12, and also a higher level of gene expression was observed in one patient (Figure 4.38 A). No changes reached statistical significance.

4.3.2 TLR4

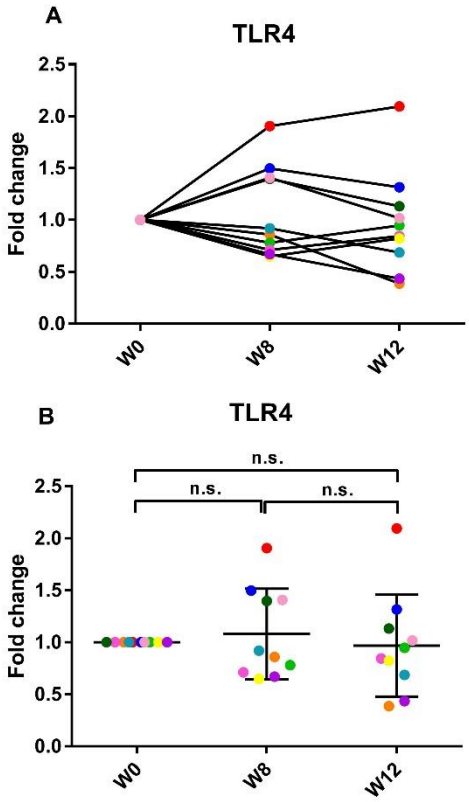

Figure 4.39 TLR4 gene expression levels in whole blood samples (W0, W8, W12) of obese patients (N=10). Gene expression levels are shown as fold changes (RQ values) of positive TLR4 expression relative to the reference gene β-2-microglobulin and W0 samples as estimated by the comparative C_T-method for relative quantification. Figure A shows the changes in receptor expression for each patient at W0, W8 and W12, while figure B shows individual median data points N=10±SD. N.s. = not significant.

TLR4 gene expression increased between W0 and W8, and then decreased between W8 and W12 (Figure 4.39 A,B). A group of patients had a decrease in gene expression between W0 and W8, and then a further decrease or an increase (Figure 4.39 A). One patient showed a higher level of gene expression (Figure 4.39 A). No changes were statistical significant.

4.3.3 CD14

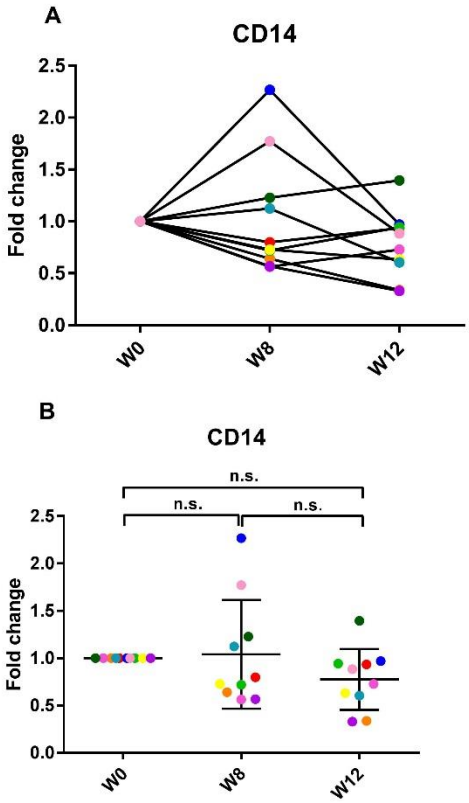

Figure 4.40 CD14 gene expression levels in whole blood samples (W0, W8, W12) of obese patients (N=10). Gene expression levels are shown as fold changes (RQ values) of positive CD14 expression relative to the reference gene β-2-microglobulin and W0 samples as estimated by the comparative C_T-method for relative quantification. Figure A shows the changes in receptor expression for each patient at W0, W8 and W12, while figure B shows individual median data points N=10±SD. N.s. = not significant.

CD14 gene expression decreased between W0 and W8 in one group of patients, and then continued to decrease or increase between W8 and W12 (Figure 4.40 A). Some patients had a higher gene expression level, and increased between W0 and W8, with a following decrease after W12 (Figure 4.40 A). However, the decrease between W0 and W8 was not observed when analyzed on a general level, only a lightly increase with a following decrease between W8 and W12 (Figure 4.40 B). No changes reached statistical significance.

4.3.4 CD36

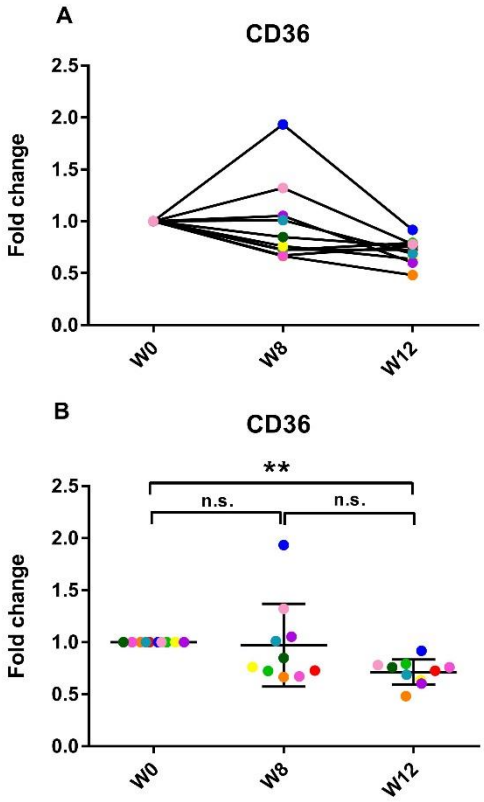

Figure 4.41 CD36 gene expression levels in whole blood samples (W0, W8, W12) of obese patients (N=10). Gene expression levels are shown as fold changes (RQ values) of positive CD36 expression relative to the reference gene β-2-microglobulin and W0 samples as estimated by the comparative C_T-method for relative quantification. Figure A shows the changes in receptor expression for each patient at W0, W8 and W12, while figure B shows individual median data points N=10±SD. N.s. = not significant; * = p≤ 0.05; ** = p≤ 0.01; *** = p≤ 0.001; **** = p≤ 0.0001.

CD36 gene expression decreased between W0 and W8, and continued to further decrease between W8 and W12 (Figure 4.41 A,B). Two patients had a higher gene expression level and an increase in gene expression between W0 and W8 (Figure 4.41 A). A statistical significant (p≤ 0.01) decrease in gene expression was found between W0 and W12 (Figure 4.41 B).

5 Discussion

Obesity is known to be associated with chronic inflammation and an increase in inflammatory markers as CRP, cytokines and interleukins[2]. The present study has focused on the receptor and gene expression of inflammation-linked receptors on human blood leukocytes before and after weight loss by a very low calorie diet (VLCD), followed by a stabilization period.

Studies have shown that diet can affect obesity-associated inflammation. Treatment with VLCD leads to reduction in the signs of metabolic syndrome[7-9], while a high fat or high glucose diet leads to an increase in immune-cell populations as granulocytes and lymphocytes[10]. Studies comparing leukocyte levels of obese subjects and lean controls, shows that obese have higher levels of granulocytes and eosinophils, compared to controls[15, 20], while the level of monocytes have been found to be elevated or unchanged[79]. However, our findings did not see any significant changes in the percentage of eosinophils, monocytes and lymphocytes before and after weight loss, neither after the stabilization period. Still, the granulocytes had a significant increase between W8 and W12 (Figure A.5, Appendix). Additionally, excessive energy intake associated with obesity leads to chronic inflammation[80]. Weight loss by calorie restriction, as in the present study, has been shown to reduce the inflammation in the body by decreasing inflammatory markers produced, as well as gene expression of inflammation-associated genes[80]. In contrast, a study has shown that surgical-induced weight loss does not improve obesity-associated metabolic abnormalities, such as CRP concentration, blood pressure and cytokine concentrations[81].

5.1 Receptor expression on leukocytes and gene expression of leukocyte receptors in obesity

The present study revealed that cell-surface receptor expression of TLR2 and TLR4, co-receptor CD14, oxLDL-receptor CD36, as well as inflammatory receptors CD16, CD11b and CD66b on leukocytes increased after VLCD weight loss, with a maintenance in expression level following the stabilization period.

Further on, the present study did not find any significant changes in gene expression of receptor TLR2, TLR4 and co-receptor CD14 in leukocytes after weight loss followed by a stabilization period. However, oxLDL-receptor CD36 showed a significant decrease in gene expression levels in monocytes. Since gene expression was analyzed in whole blood, cell-specific gene expression was not possible.

5.1.1 TLR2

TLR2 cell-surface receptor expression increased after weight loss, and continued to increase or stabilize following the stabilization period. Additionally, an increased proportion of leukocytes expressing TLR2 was observed. This indicates that more TLR2 expressing cells are present, which leads to a more robust immune system that is ready for an attack by for instance bacteria, than before weight loss. Our findings are supported by a study were men fed on a high-fat diet for one week had a decrease in TLR2 receptor expression on leukocytes[39]. Based on that, TLR2 is found to bind to, and be activated by fatty acids[27, 38], which are elevated in obesity. Hospital analyzes showed that the level of fatty acids significantly decreased after weight loss (Figure A.5 Appendix), suggesting that TLR2 may have a lower surface expression level in obesity or when fed on a high-fat diet for a short time, due to binding of fatty acids and internalization of the receptor into the cell. Contradictory, subjects with diabetes type 2 have a higher TLR2 expression in leukocytes compared to lean controls[40]. Obese subjects with established atherosclerosis also had the same TLR2 expression level as non-obese subjects with atherosclerosis[34]. In addition, mouse studies suggest that deletion of TLR2 in adipose tissue could prevent diet-induced obesity[42].

TLR2 gene expression increased or decreased after weight loss, with a following decrease or increase in gene expression, respectively. Different expression patterns were observed, which indicates a variance in gene expression. These findings contradict with studies suggesting that obese subjects undergoing surgical-induced weight loss have a reduction in TLR2 gene expression in monocytes after weight loss[82]. However, since the present study did not analyze cell specific gene expression, comparison is questionable. Further on, the gene expression of TLR2 in monocytes decreased in obese subjects with diabetes type 2 after two weeks on a very low calorie diet[83], and after surgical-induced weight loss[84]. Additionally, studies have analyzed the TLR2 gene expression in peripheral blood mononuclear cells (PBMC) in obese with diabetes type 2 and metabolic syndrome. They showed that these subjects have higher gene expression than non-diabetic obese and obese without metabolic syndrome[85, 86], suggesting that the inflammation in relation to the diseases affected the gene expression level.

5.1.2 TLR4

TLR4 cell-surface receptor expression increased after weight loss, and continued to increase or stabilize following the stabilization period. Also an increased amount of cell expressing the receptor after weight loss was observed. Our findings indicate that the levels of TLR4 receptor expression elevates after weight loss, meaning that the immune system is better prepared, and the receptor is ready for defense, compared to before weight loss. To our knowledge, there are no studies done on TLR4 receptor expression on leukocytes in human obese subjects. One study showed that obese subjects with atherosclerosis had no change in TLR4 expression level on leukocytes compared to non-obese subjects with atherosclerosis[34]. However, studies done in mice supports our findings by suggesting that diet-induced obese mice have a lower TLR4 receptor expression on leukocytes when fed on a high-fat diet, compared to a low-fat diet[31]. Also TLR4 is found to bind to and be activated by fatty acids[27, 38], which is elevated in obesity. Based on the hospital analyzes of fatty acids (Figure A.5 Appendix), it may also be suggested for TLR4 that the low receptor expression in obesity is due to binding of fatty acids, which activates the receptor and leads to internalization into the cell.

TLR4 showed two different gene expression patterns with an increase or decrease after weight loss, with a following decrease or increase, respectively. Again, this indicates that the cells have a random gene expression pattern. These observations are in contrast to previous studies, were the focus has been on obesity in conjunction with diabetes type 2 and metabolic syndrome. As for TLR2, the gene expression of TLR4 in monocytes decreased in obese subjects with diabetes type 2 or metabolic syndrome after weight loss[83-86]. Additionally, de Mello *et al.* showed that overweight subjects with metabolic syndrome had a reduction in TLR4 gene expression in PBMCs after weight loss by diet[87], which is contradicting with our observations. However, the comparison is as mentioned questionable, since the present study did not analyze cell specific gene expression.

5.1.3 CD14 and CD14/CD16 monocytes

CD14 cell-surface receptor expression on monocytes increased after weight loss, with an excessive continuous increase following the stabilization period. Elevated levels of CD14 makes this receptor more present as a co-receptor for TLRs, strengthening the immune system after weight loss. Our findings is in contrast to a study done by Cottam *et al.*, who discovered

that surgical-induced weight loss lead to decreased levels of CD14, reaching the same levels as normal controls three months after surgery[20]. However, the receptor might respond differently to surgical weight loss, compared to diet restrictions. To our knowledge, the literature has not focused on CD14 receptor expression on blood monocytes, so more research has to be done to see how receptor CD14 respond to weight loss.

CD16 cell-surface receptor expression on monocytes increased after weight loss, with a following decrease. In the percentage distribution of CD14/CD16 monocyte subpopulations small changes was observed in the intermediate and inflammatory monocytes after weight loss, while the main classical monocytes increased. A decrease was observed in the $CD14^{Low}CD16^{Low}$ monocyte subpopulation after weight loss. Research done on the percentage distribution of monocyte subpopulations showed that the intermediate and inflammatory monocytes decreased with diet or surgical-induced weight loss[2, 20]. These studies contradict with our findings, showing that the intermediate and inflammatory monocytes slightly increase after weight loss, with a following small decrease. From literature, the intermediate and inflammatory monocytes are found to be elevated in obesity, secreting cytokines involved in inflammation[2, 18]. Based on that, obesity might not be related to chronic inflammation in the same degree as thought. The same studies also showed that the percentage of intermediate and inflammatory monocytes were lower in healthy controls compared to obese[2, 20], which is not found in the present study were the percentage level is similar between obese and healthy controls. However, Poitou *et al.* showed that the percentage level of intermediate monocytes was lower in obese subjects on diet, compared to controls[2], suggesting that weight loss with a strict diet can affect the percentage distribution of monocytes. This may explain why surgical-induced weight loss showed a decrease in the percentage of monocytes, while the present study showed an increase with diet-induced weight loss. Additionally, studies have also shown that exercise reduce the percentage of intermediate and inflammatory monocytes[21, 22], suggesting that diet-induced weight loss is not the only factor affecting the subpopulations. As mentioned, in the present study there was an increase in the classical monocytes after weight loss, as well as a decrease in the $CD14^{Low}CD16^{Low}$ subpopulations. An increase in the percentage of classical monocytes again indicates that more cells express the CD14 co-receptor after weight loss, making the immune system more ready for attack. To our knowledge, the classical monocytes have not been focused on in relation to obesity and weight loss, as well as

the $CD14^{Low}CD16^{Low}$ subpopulation, which is not identified as a subpopulation in research done.

Plasma levels of sCD14 was measured of the obese patients, which showed an increase in plasma levels after weight loss, with a following decrease. This finding is supported by a study observing that the sCD14 levels increase after surgical-induced weight loss[51] Contradictory, a similar study showed that sCD14 levels decreased in obese women undergoing surgical-induced weight loss[50], which is in line with literature suggesting that sCD14 is an inflammatory-marker increasing in response to chronic inflammation[48]. However, due to high variability in the measured triplicates, more studies need to be done to better measure the sCD14 levels in plasma from obese patients.

CD14 gene expression increased or decreased after weight loss, with a following decrease or increase in gene expression, respectively. These findings are specific for gene expression of CD14 in monocytes, since CD14 is known from literature to only be expressed on monocytes[43]. These observations are supported by Monte *et al.,* who found that obese subjects with diabetes type 2 had a reduction in CD14 gene expression in PBMCs after surgical-induced weight loss[84]. Additionally, studies have showed that obese subjects who lose weight by a very low calorie diet, or a diet and exercise program, has a decrease in CD14 gene expression in macrophages in adipose tissue[88, 89]. Based on this, it is suggested that the gene expression levels are reduced due to a reduction in chronic inflammation, meaning that inflammatory receptors do not need to be expressed in the same degree.

5.1.4 CD16

CD16 cell-surface receptor expression on granulocytes increased after weight loss, with a maintenance in expressing levels following the stabilization period. These findings indicate that after weight loss, elevated levels of CD16 leads to a more robust immune system, ready to destroy the invading targets. To our knowledge, only one study has looked at the CD16 receptor expression on granulocytes in relation to obesity and weight loss. This study is in contrast to our observations, suggesting that the receptor expression did not change with surgical-induced

weight loss[20]. Additionally, the CD16 receptor expression did not differ between obese subjects and normal controls, [15, 20], which is also observed in the present study.

5.1.5 CD36

CD36 cell-surface receptor expression on monocytes increased after weight loss, with a continuous increase in receptor expression. Additionally, the proportion of monocytes expressing high amounts of CD36 increased, indicating that the immune system is more ready for defense after weight loss. A study done by Kuliczkowska-Plaksej *et al.* supports this observation by suggesting that obese women have lower levels of CD36 on monocytes compared to lean controls[54]. Studies done in adipose tissue suggest a higher CD36 expression in obese and diabetes type 2 subjects, compared to lean controls[57]. CD36 is also found to bind fatty acids[56], which is increased in obese subjects, suggesting that the CD36 levels may be lower in obese because the receptor is bound to fatty acids and internalized into the cell. Hospital analyzes shows that the level of fatty acids significantly decreased after weight loss (Figure A.5 Appendix), supporting that obese have higher levels of fatty acids binding to CD36. Additionally, oxLDL plasma levels, the ligand of CD36, was measured by our group, observing a decrease in levels after weight loss, with a following stabilization (Figure A.6 Appendix). These findings may indicate that CD36 receptor levels was lower in obese due to bindings of ligands as oxLDL and fatty acids, and internalization into the cell.

CD36 gene expression decreased or increased after weight loss, with a following increase or decrease, respectively. Since CD36 is known from the literature to only be expressed on monocytes[52], these findings must be regarded as specific for monocytes. To our knowledge, no studies have been done on gene expression of CD36 in blood monocytes to support or contradict our findings. However, a study done in placenta of obese pregnant subjects showed that gene expression levels of CD36 was higher in obese pregnant compared to pregnant controls[90]. Obese mice fed on either a very low calorie diet or ad libitum (the mouse eat as much as it desires), shows that gene expression of CD36 in adipose tissue is higher in obese mice compared to controls, with no difference in expression between the two diet groups[91]. Additionally, obese mice undergoing calorie restriction showed that the CD36 gene expression in adipose tissue increased after three days on the diet, with a following decrease after 42 days, reaching gene expression levels lower than when the mice were obese[92]. The significant

reduction in CD36 gene expression level between W0 and W12 might indicate that after weight loss and the stabilization period, the chronic inflammation in the body is reduced, and expression of inflammatory receptors are not necessary in the same degree.

5.1.6 CD11b

CD11b cell-surface receptor expression increased after weight loss, continuing to slightly increase or decrease following the stabilization period. This increase in receptor expression after weight loss leads to a more robust immune system, which for instance is more ready for phagocytosis of invading targets. However, an increase in cell-adhesion receptors might also have consequences, for instance in relation to the development of atherosclerosis. Contradictory to our findings is studies that have shown that obese patients undergoing surgical-induced weight loss had the same CD11b receptor expression levels on leukocytes as lean controls[15, 20], and the levels did not change after weight loss[20]. A study done by van Oostrom *et al*., showed that healthy men fed with fresh cream for 6 hrs had an increase in CD11b expression levels on leukocytes compared to controls fed with water[70]. The study suggests that high-fat diet leads to an increase in CD11b receptor expression, which is the opposite of the present study, suggesting that the increase in receptor expression is due to weight loss. However, van Oostrom *et al.* also showed that CD11b expression levels were higher in men fed with fresh cream compared to controls[70]. This is not shown in the present study were the expression levels of obese have a similar expression levels as healthy controls, suggesting that the increase in receptor expression after weight loss is due to the strict diet. Additionally, a study done on obese and non-obese subjects with atherosclerosis showed that the CD11b receptor expression on monocytes were higher in obese subject with atherosclerosis, compared to non-obese[34], suggesting that the expression level is affected by obesity.

5.1.7 CD66b

CD66b cell-surface receptor expression increased after weight loss, with a following increase or stabilization in receptor expression, leading to more expression of the cell-adhesion receptor. However, this increase might have consequences, for instance in relation to the cell-adhesion receptors role in development of atherosclerosis. To our knowledge, no studies have looked at CD66b receptor expression before and after weight loss. It is however shown that CD66b receptor expression is higher in obese subjects compared to lean controls[71, 72], and receptor

expression increase in healthy men after being fed with fresh cream for 6 hrs, compared to controls[70]. This is in contrast to the present study were the receptor expression level is similar in obese and healthy controls, again suggesting that the increase in receptor expression after weight loss is due to the strict diet. Additionally, a study showed that aged subject who exercised twice a week for 12 weeks had a decrease in CD66b receptor expression[75]. Although exercise and weight reduction might give different effects on metabolism, these findings contradict to our findings of increased CD66b receptor expression after weight loss.

5.2 Receptor expression on leukocytes in control group compared with obese patients

As mentioned, a control group were included, but it is important to note that these volunteers have unknown BMI, no restriction in diet or exercise, and was included as a control of biological and experimental variations. It was expected that the cell-surface receptor expression would be stable at the two time points, which was the case for all the receptors analyzed, except for CD14, CD16 and CD36. Additionally, significant changes were found in the percentage of CD36 expressing cells, and in the $CD14^{Low}CD16^{Low}$ monocyte subpopulation. It can be speculated in that these receptors may be sensitive to for instance external influences, and change a lot in expression over time. Remarkably, the obese patients have the same increase in receptor expression at the same time period, which might be due to the fact that blood was collected in October and November, when there is an influenza epidemic. Research has shown that the percentage of $CD14^{High}$ and $CD14^{Low}$ monocytes decreased after infection with influenza virus[93], as well as a decrease in CD16 receptor expression on granulocytes[94]. The same study found that expression of the cell-adhesion receptors CD11b and CD66b increased in leukocytes after influenza virus infection[94], while our study showed that CD11b and CD66b receptor expression was stable over time, suggesting that influenza virus may not affect the receptor expression. The difference in receptor expression over time could also be due to different lot numbers in the antibodies used for receptor staining. However, in our study the same lot number was used at all time points except for some exceptions, but this was found to not affect the receptor expression.

When comparing the cell-surface receptor expression in the control group with the obese patients, receptors TLR2, TLR4, CD36 and CD14 showed the same expression level in controls

as the patients after diet stabilization (W12), suggesting that the patients have reach a normal receptor level after weight loss. However, receptor CD11b, CD66b and granulocyte CD16 showed the same expression level in controls as the obese patients (W0). As already mentioned, for receptor CD36 this is supported by the literature, suggesting that CD36 levels are lower in obese compared to controls[54]. Further on, literature on receptor CD14 also suggested that the receptor levels are lower in obese compared to controls[20, 95]. No relevant comparison was done for TLR2, but for TLR4 it was again suggested that the receptor level was lower in obese compared to controls[95]. For receptor CD11b and CD66b, the literature contradicts with our findings, as already mentioned above, suggesting that obese have higher receptor levels than normal controls[70-72]. However, the receptor expression of CD16 on granulocytes were the same in the control group as the obese patients, as implied in the literature[15, 20]. In addition, the percentage of inflammatory and intermediate monocytes had a similar level in the control group compared to obese patients, which contradicts with previous studies[2, 20]. The control group had a higher level of classical monocytes compared to obese patients, while the $CD14^{Low}CD16^{Low}$ subpopulation was lower in healthy controls, compared to obese. However, both of these subpopulation varied between the two time points in the control group. Altogether, this might indicate that the different receptors are regulated differently in response to obesity and the very low calorie diet.

5.3 Study limitations and challenges

In the present study it was decided to use FMO control to measure the cell-surface receptor expression due to issues using isotype control, as previously mentioned. However, isotype control was used to measure the receptor expression for CD14 and CD66b. Ongoing debate about which control is best to use, led to further analyzes of the isotype controls. As already mentioned, the use of isotype control on TLR2 and TLR4 gave negative MFI values. Further on, figure A.7 and A.8 in appendix shows that the isotype control is not stable over time. The isotype control expression increased over time both in obese patients and healthy controls, with the same trend as seen when analyzing TLR2 expression in obese patients (Figure A.7 A,B, Appendix). The fact that the isotype control expression in healthy controls increase more than the TLR2 receptor expression, raises uncertainties in the use of isotype control (Figure A.8 A,B, Appendix). It may be that blood have variations in unspecific binding over time, such as binding to Fc receptors and cell “stickiness”, which could explain the increase in isotype control expression. The Fc-receptor CD16 was measured, and was found to increase in expression after

weight loss. Additionally, there are a lot of unspecific binding in blood compared to for instance a stable cell line, which leads to more conflicts in the use of isotype control. It has also been shown that the same isotype control clone from different manufacturers leads to highly variable levels of unspecific binding[96]. However, the FMO control was found to be stable over time (Figure A.3, Appendix). Based on this, the choice to use FMO control to measure the receptor expression is supported.

The fact that blood was collected in October (W8) and November (W12) could be considered as a reason for the increase in receptor expression. The patients could be affected by external influences, such as bacterial infections or influenza virus. CRP values are mostly used to measure a bacterial infection in the body, and are not a good marker to detect a viral infection[97]. Based on that, a viral infection could affect the receptor expression in the patients, without being detected in the present study. However, bacterial infections could be excluded, since CRP values from hospital analyzes (not shown) showed that even though the patients had elevated CRP values, the measured MFI values were at the same levels as for the other patients.

A larger, age-matched control group with more restrictions should be included to easier compare the cell-surface receptor expression levels of the obese patients with the healthy controls. Further on, a follow-up of the obese patients should be done after a while on a normal diet, to see if the strict diet did affect the receptor expression. However, this would lead to uncertainties in the results, due to the fact that the flow cytometer would not be guaranteed to be stable with the same conditions over a long time period. Several studies have looked at the receptor expression up to 6 months after weight loss, which could lead to uncertainties in their results.

As already mentioned, cell-specific gene expression should be performed to get an impression of the gene expression pattern in the different cell populations after weight loss.

6 Conclusions and future perspectives

Cell-surface receptor expression of the inflammation-linked receptors analyzed in this study increased after weight loss with VLCD (W8), with a maintenance in expression levels following the stabilization period (W12). Additionally, the gene expression of the receptors showed varying gene expression patterns, while on the monocytes, a decrease was observed. Based on this, an effect of the diet-induce weight loss was found on the receptor expression patterns on the monocytes, granulocytes and eosinophils. An increase in receptor expression after weight loss indicates that the immune system is improved, with elevated levels of receptors ready for defense. The low levels of receptor expression in the obese patients suggests that the inflammation-linked receptors were not present to attack the chronic inflammation in the body, associated with obesity. Previous studies observed a decrease in receptor expression after weight loss, which contradicts with our observations. These studies suggest that a decrease in receptor expression is associated with a decrease in inflammation in the body, but our findings suggest the opposite. Based on this, our results indicate that the receptor expression levels are low in obese subjects because the receptors are in use. They bind ligands, for instance fatty acids, leading to internalization of the receptor into the cell. After weight loss, the receptor does not bind ligands to the same degree, leading to more receptors on the cell-surface, ready for immune defense. This is also connected to the decreased gene expression level observed in monocytes, suggesting that less receptors need to be produced, due to a reduction in chronic inflammation. Further on, future studies should include lean controls to easier compare receptor expression in lean and obese subjects, more inflammation-linked receptors should be included to see if the same trend is observed, as well as analyzation of cell-specific gene expression.

7 References

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A Appendix

A.1 Gating strategy

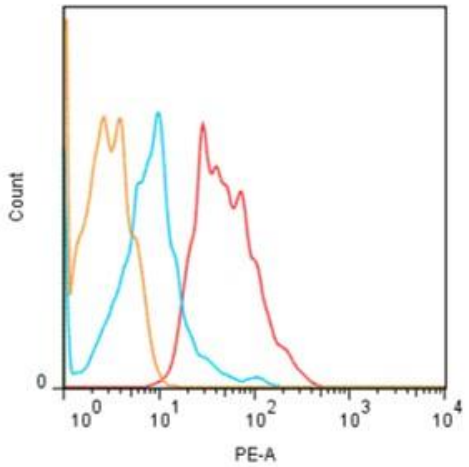

Figure A.1 An example of a histogram of receptor, isotype control and FMO control expression. Expression is measured by flow cytometer. Orange line = FMO control; Blue line = Isotype control; Red line = Receptor expression.

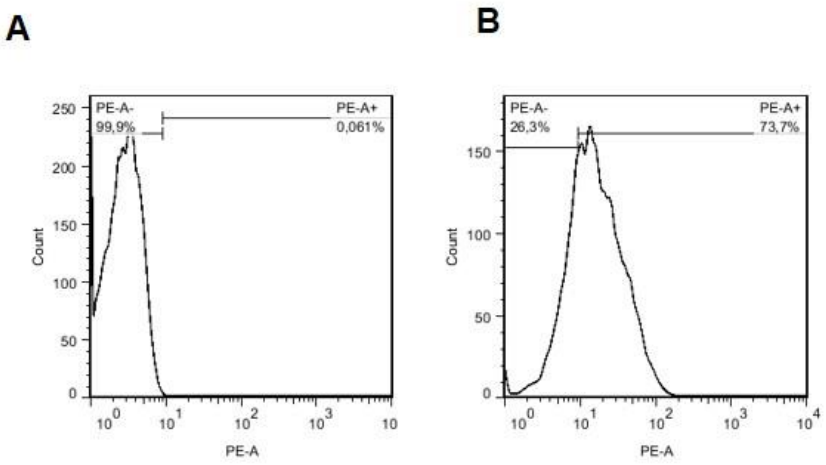

Figure A.2 An examples of a histogram were a threshold was set at the end of the FMO control (A). The threshold was set on the receptor expression, and receptor expression above this threshold was assumed as positive (B).

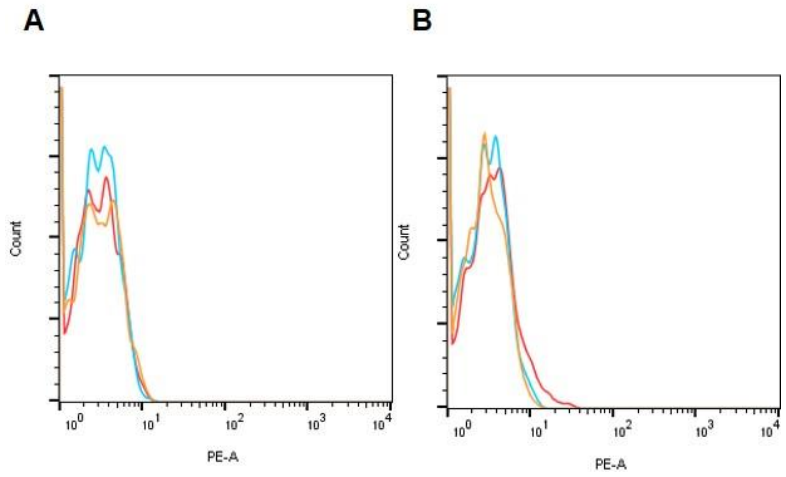

Figure A.3 An example of a histogram were the FMO control was stable over time (W0, W8, W12) (A), and were it changed over time (B). Red line = FMO control at time point W0; Blue line = FMO control at time point W8; Orange line = FMO control at time point W12.

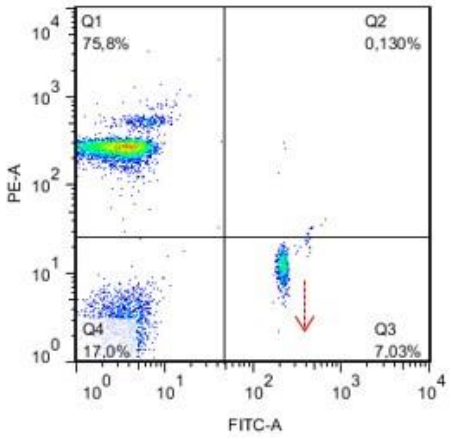

Figure A.4 A FITC/PE dot plot of beads run on the flow cytometer. The arrow indicating that the FITC-channel was under compensated. Q1 = PE-marked cells (PE-channel); Q3 = FITC-marked cells (FITC-channel); Q4 = Unmarked cells.

A.2 Percentage of cells

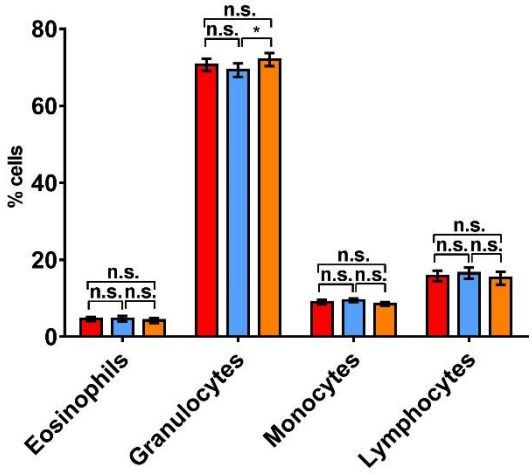

Figure A.4 The amount of cells measured in percent in the different leukocyte cell populations (eosinophils, granulocytes, monocytes and lymphocytes) (W0, W8, W12) (N=17). Red box = W0; Blue box = W8; Orange box = W12. N.s. = not significant; * = p≤ 0.05 (RM two-way ANOVA, Tukey's multiple comparisons test).

A.3 Additional experiments

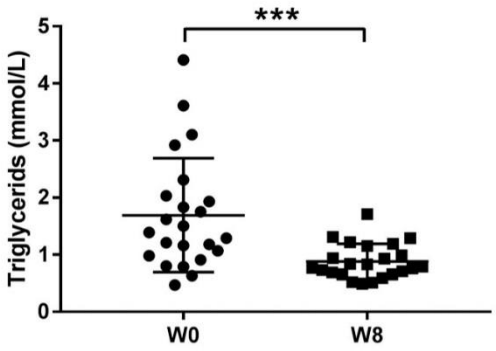

Figure A.5 Hospital analyzes of triglycerides levels in blood measured in mmol/L (W0, W8) (N=23). Data is presented as individual median data points N=23±SD. **** = p≤ 0.0001 (Paired t-test).

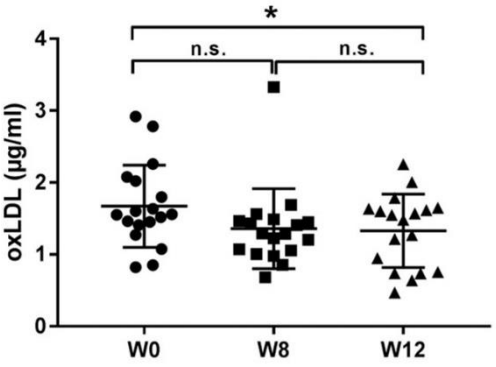

Figure A.6 Plasma levels of oxLDL measured by our group. Plasma oxLDL concentrations (µg/ml) measured by ELISA at time points W0, W8, W12 (N=18). Data is presented as individual median data points N=18±SD. N.s. = not significant; * = p≤ 0.05 (RM One-Way ANOVA, Tukey's multiple comparison test).

A.4 Isotype Control

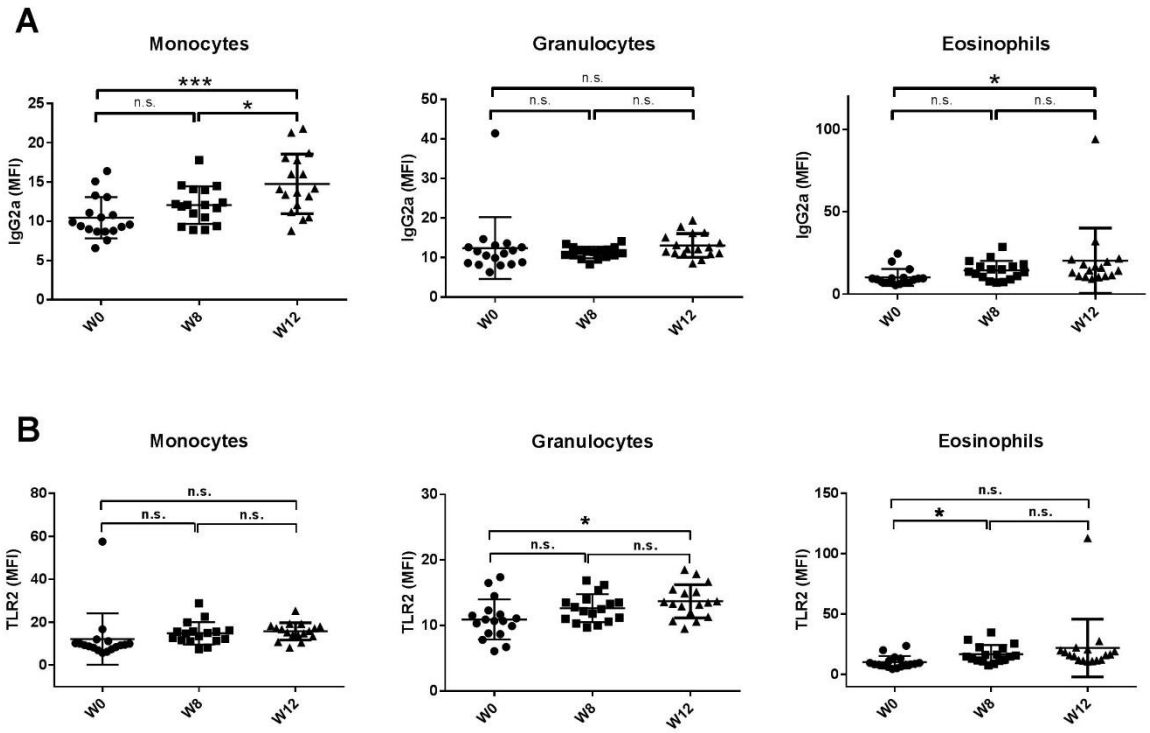

Figure A.7 Surface expression of isotype control IgG2a on monocytes, granulocytes and eosinophils (A), and surface expression of receptor TLR2 (B). Surface expression is measured in blood samples (W0, W8, W12) of obese patients (N=17). Expression is measured by flow cytometer as median fluorescence intensity (MFI) minus FMO control, showing individual median data points N=17±SD. N.s. = not significant; * = p≤ 0.05; *** = p≤ 0.00 (RM One-Way ANOVA, Tukey's multiple comparison test).

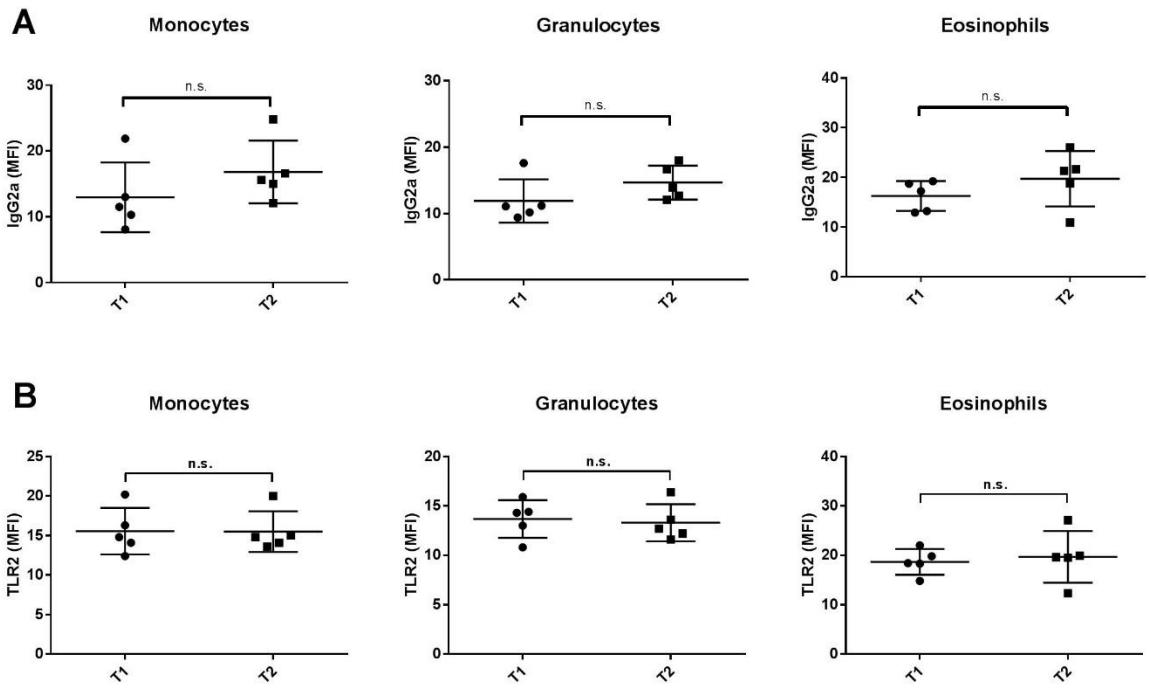

Figure A.8 Surface expression of isotype control IgG2a on monocytes, granulocytes and eosinophils (A), and surface expression of receptor TLR2 (B). Surface expression is measured in blood samples (T1, T2) of healthy controls (N=5). Expression is measured by flow cytometer as median fluorescence intensity (MFI) minus FMO control, showing individual median data points N=5±SD. N.s. = not significant (T-test, Wilcoxon matched pair signed-rank test).