Immunometabolism in osteoarthritis

Jong, A.J. de

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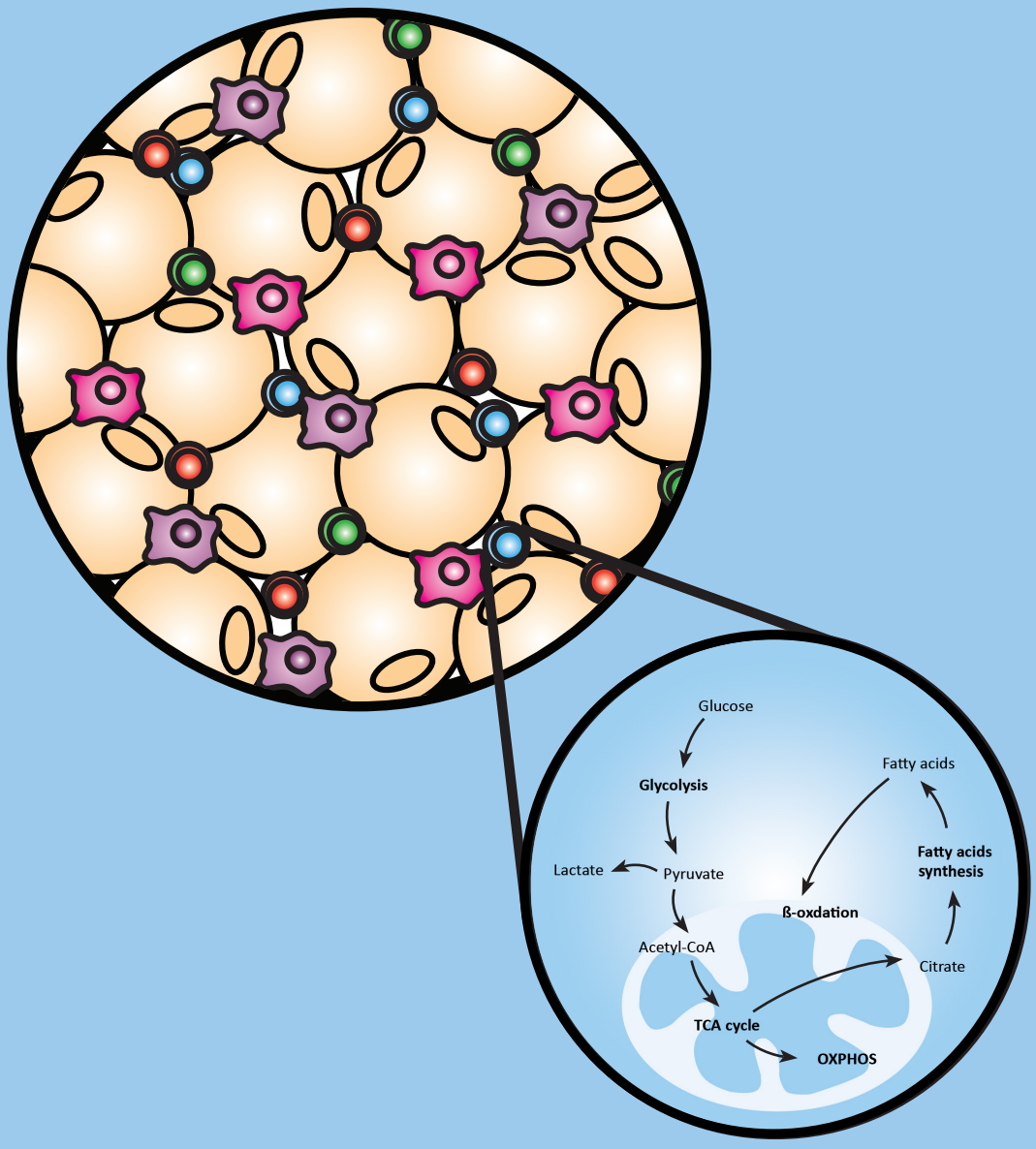

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Cover design: Anja de Jong & Loes Kema
Layout: Ferdinand van Nispen, *my*-thesis.nl
Printing: GVO drukkers & vormgevers B.V.

ISBN: 978-94-6332-304-8

The research described in this thesis was performed at the department of rheumatology of the Leiden University Medical Center.

Printing of this thesis was financially supported by Leiden University.

Immunometabolism in Osteoarthritis

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op dinsdag 20 februari 2018
klokke 15.00 uur

door

Anja de Jong
geboren te Stede Broec
in 1989

Promotores:	Prof. dr. R.E.M. Toes
	Prof. dr. G. Kloppenburg
Co-promotor:	Dr. A. Ioan-Facsinay
Leden promotiecommissie:	Prof. dr. T.W.J. Huizinga
	Prof. dr. ir. J.A.P. Willems van Dijk
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Voor mijn ouders

Table of contents

Chapter 1

Introduction immunometabolism in osteoarthritis

Osteoarthritis

Osteoarthritis (OA) is a heterogeneous joint disorder affecting mostly the hip, knee and hand joints. Clinical characteristics are pain, stiffness and disability, accompanied by cartilage loss and structural abnormalities of the joint such as osteophytes, joint space narrowing and bone sclerosis. Furthermore, soft tissue abnormalities, such as synovitis, and subchondral bone lesions can be present [1]. According to different sets of criteria, developed by the American College of Rheumatology (ACR), OA can be classified based on clinical and laboratory, clinical and radiographic or clinical criteria alone [2]. OA was long thought to be solely a chronic degenerative disease driven by cartilage loss, however, OA is a much more complex disease whereby inflammatory processes and all joint compartments, cartilage, bone and synovium, are involved [3, 4].

Pathophysiology of osteoarthritis

Articular cartilage consists of chondrocytes producing the extracellular matrix (ECM), containing collagen and proteoglycans. Normally chondrocytes maintain cartilage through normal anabolic (matrix-producing) and catabolic (matrix-degrading) activities, however, in OA this balance is disturbed. Chondrocytes proliferate and form clusters, leading to extensive matrix degradation and loss due to the production of proteases, such as matrix metalloproteinases and members of a disintegrin and metalloproteinase (ADAM) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) families [5, 6]. Lying immediately beneath the cartilage is the subchondral bone, which provides mechanical support for the articular cartilage. However, during the course of OA, the subchondral bone undergoes changes, such as bone remodelling, tissue sclerosis, and the formation of osteophytes at the joint margins [3] and is a source of inflammatory mediators which can affect the cartilage and synovium [7].

In addition, the synovial tissue consists of a small layer of synoviocytes producing components of synovial fluid, such as hyaluronic acid and lubricin, essential for frictionless movement. In the OA joint, the production of these components are altered, which leads to adverse effects on the cartilage integrity [8]. Furthermore, synovial tissue inflammation is present in OA patients [9], characterized by synovial hyperplasia and influx of inflammatory cells. Although it is not completely known,

it is likely that cartilage degradation products induces synovial inflammation in OA, activating synoviocytes to produce pro-inflammatory mediators, such as TNFα and IL-1β, leading to immune cell attraction, such as macrophages and T cells. This will, conceivably, lead to a further phenotype switch of chondrocytes, and eventually a vicious circle of continued joint destruction [10]. As synovitis is associated with symptom severity and cartilage degradation [10], it is suggested that synovitis plays an important role in the pathophysiology of OA.

Obesity and osteoarthritis

Major risk factors for OA are gender and age [1, 11], however, the development and progression of OA are also associated with obesity [1, 11, 12]. The association of OA with obesity was thought to be solely due to mechanical stress caused by the increased or altered mechanical load on the joint. However, obesity is also a risk factor for non-weight-bearing joints such as the hand [12-14], indicating that mechanical factors alone cannot fully explain the association between obesity and OA. It is suggested that in addition to mechanical factors, systemic factors, such as low-grade inflammation, disturbed lipid metabolism and adipokines, contribute to the association between obesity and OA [11]. Both mechanical and systemic factors play a role in the association between OA and obesity, however, the relative contribution differs between weight-bearing and non-weight-bearing joints. In knee OA, mechanical stress is the most important underlying mechanism, whereas in hand OA systemic processes contribute the most [14].

Immunometabolism

Immunometabolism is an emerging field, focussing on the interplay between immunological and metabolic processes, both at a systemic and a cellular level. Immunometabolism on systemic level explores the link between immune cells and their effect on metabolic tissues, such as adipose tissue, which can affect whole-body metabolism. Immunometabolism on cellular level explores the intracellular metabolic pathways in immune cells that alter their function.

Systemic immunometabolism

Adipose tissue

Adipose tissue, or fat, is long thought to solely serve as energy depot, releasing fatty acids in times of energy demand and storing triglycerides (TGs) in periods of energy excess. However, it is also a highly active metabolic and endocrine organ as it can secrete various adipokines and cytokines, together called adipocytokines, which can affect whole-body metabolism [15-17]. Leptin, which was the first adipokine to be discovered is important as metabolic signal for the energy balance by inhibiting hunger [18]. Other adipokines such as adiponectin, resistin, and visfatin are involved in lipid and glucose homeostasis [18]. Cytokines secreted by the adipose tissue are mainly IL-6 and TNFα [18]. Adipokines mediate the crosstalk between adipose tissue and other metabolic organs and thereby affecting whole-body metabolism, however, they can also have an effect on the immune system. Adiponectin, depending on its molecular form can have a pro- or anti-inflammatory effect, while leptin and resistin are both thought to be pro-inflammatory [18-21].

Besides adipocytes, the adipose tissue contains a stromal vascular fraction, which consists of fibroblasts, progenitor cells, nerve cells, endothelial cells and immune cells. Among the immune cells present in adipose tissue, macrophages and T cells are most abundant, however, mast cells, natural killer (NK) cells and B cells can be found as well [22, 23].

Adipose tissue as inflammatory site

Obesity is associated with changes in the adipose tissue, which not only affects the adipocytes, but, also the immune cells present and their secretion profile (see figure 1). Adipocytes enlarge, which results in expansion of the adipose tissue, and results in hypoxia which causes adipocyte cell death [24-26]. This is accompanied by macrophage infiltration and formation of crown-like structures (CLS) by macrophages around dead or necrotic adipocytes [22, 27, 28]. This changes the polarization stage of the macrophages from an anti-inflammatory M1 macrophage to a pro-inflammatory M2 macrophage [29-31], contributing to the pro-inflammatory state of the adipose tissue. Together with macrophages, T cells are among the first cells infiltrating the adipose tissue [32-35]. T cells infiltrating the adipose tissue are mainly pro-inflammatory T helper 1 (Th1) cells and $CD8^+$ T cells, where they contribute to the pro-inflammatory state of the adipose tissue and overcome the anti-inflammatory effects of Th2 and T regulatory (Treg) cells [33, 36-38].

As macrophages and T cells are the most abundant cell types present in the adipose tissue most studies have focussed on these cells, however, other immune cells have also been implicated to be involved in adipose tissue inflammation. It has been shown that obesity leads to accumulation of pro-inflammatory cells such as neutrophils, mast cells and B cells, especially IgG-producing mature B cells, while a decrease in anti-inflammatory cells, such as eosinophils and innate lymphoid cells (ILCs) have been found [39], contributing to the pro-inflammatory state of the adipose tissue.

As the adipose tissue acquires a pro-inflammatory state, the secretion profile of the adipose tissue changes. While the lean state secretes adiponectin, in the obese state these levels decreases, while the levels of leptin, resistin, TNFα and IL-6 increases [18]. Furthermore, basal lipolysis, the breakdown of triglycerides and the release of lipids, in adipocytes is enhanced in the obese state [40]. All these factors contribute to a more pro-inflammatory status of the adipose tissue, which can affect whole-body metabolism, ultimately leading to insulin resistance.

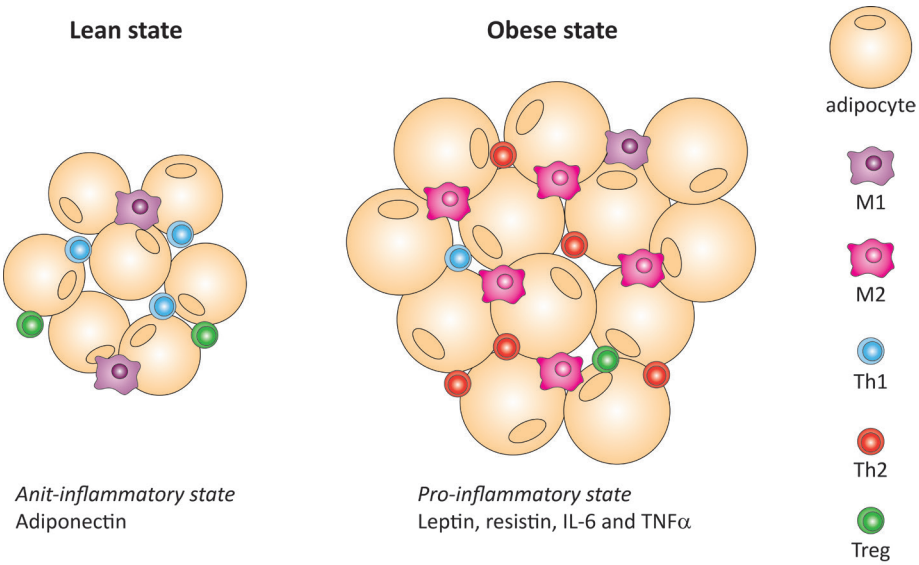

Figure 1. Schematic overview of adipose tissue in the lean and obese state.

Infrapatellar fat pad

The infrapatellar fat pad (IFP), also known as Hoffa's fat pad, is an adipose tissue organ located in the knee. It is intracapsularly and extrasynovially located and in close vicinity

to the synovium, cartilage and bone. Although the exact physiological function is unknown, it is thought that the IFP could involve shock absorption, promoting the free circulation of synovial fluid and protection of adjacent tissues [41, 42]. Furthermore, due to its location, it is considered to play a role in the pathophysiology of OA, through the secretion of soluble factors and the interaction with other joint tissues such as synovium, cartilage and possibly bone [41] (see figure 2).

Several studies have investigated whether the IFP would have a beneficial or detrimental role in knee OA. These studies suggest that the volume or maximal area of the IFP has a beneficial association with pain and structural abnormalities, such as joint space narrowing, osteophytes, cartilage volume and defects and bone marrow lesions [43-45], suggesting a protective role for size of the IFP in osteoarthritis. However, IFP signal intensity alterations on MRI, which may represent pathological changes such as inflammation and oedema in the IFP are associated with the development and progression of OA [46, 47].

Like other adipose tissues, the IFP of OA patients contains both adipocytes and stromal vascular fraction. The immune cells present in the stromal vascular fraction are mainly macrophages and T cells, although mast cells and B cells can be found as well [48]. Furthermore, the IFP is capable of secreting different adipocytokines, among which adiponectin, adipsin, leptin, resistin, visfatin and IL-6 [48-50]. Although the immune cell composition of the IFP is similar to synovium [51], it differs substantially from subcutaneous adipose tissue. The IFP contains more cellular infiltrate, the immune cell composition differs and it secretes higher levels of adipocytokines compared to subcutaneous adipose tissue [48-50, 52], suggesting that together with synovium, the IFP could play a role in the pathophysiological processes in the OA joint.

Whether or not the IFP is affected by obesity like other adipose tissues is largely unknown, although a few studies have investigated possible effects. Two magnetic resonance studies suggest that the size of the IFP is not influenced by obesity [43, 53], however, another study showed that the adipocytes from the IFP were larger in obese persons compared to lean persons and the IFP of obese persons showed more cellular infiltrate [54]. Furthermore, TNFα secretion by the IFP is higher in obese persons compared to lean subjects [48].

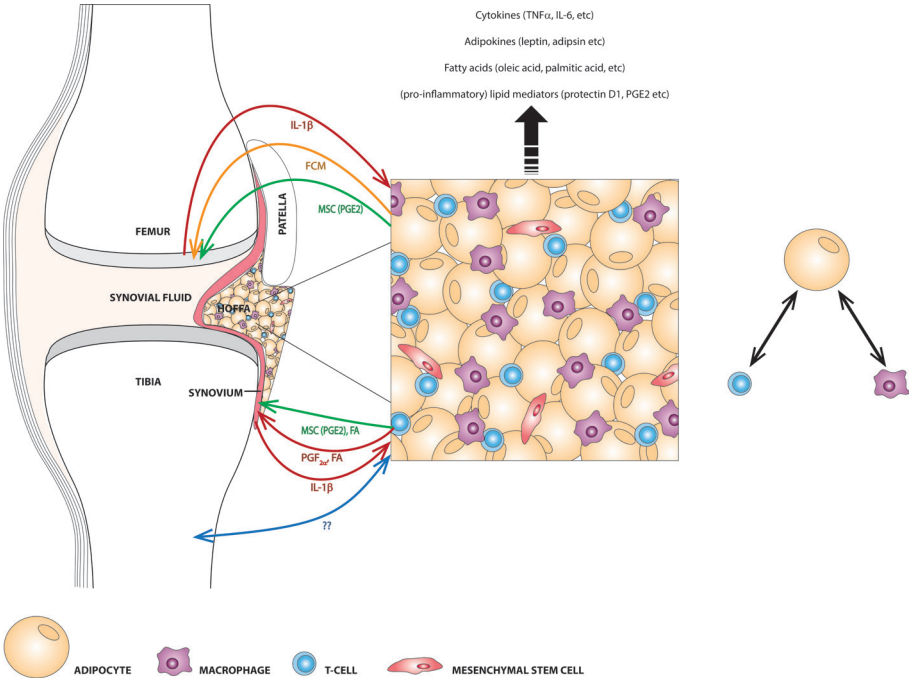

Figure 2. The infrapatellar fat pad and its interactions with other joint tissues.
Adapted from A. Ioan-Facsinay et al. Arthritis Res Ther 2013; 15 (6): 225

Cellular immunometabolism

Cellular metabolic pathways

In general, there are several cellular metabolic pathways active in the cell. These pathways can either be catabolic, obtaining energy and reducing power from nutrients or anabolic, producing new cell components through processes that require energy. Glycolysis converts glucose through several steps into pyruvate, which can then either be converted into lactate (generating 2 ATPs). Pyruvate can also enter the mitochondria where it is converted into acetyl-CoA and can enter the tricarboxylic acid (TCA) cycle (generating 36 ATPs). The TCA cycle generates NADH and FADH2, which will be oxidized by the electron transport chain (ETC) which will lead to ATP production by ATP synthase, also known as oxidative phosphorylation (OXPHOS). The TCA cycle can also be fuelled by glutamine metabolism or by acetyl-CoA derived from β-oxidation, the breakdown of fatty acids in the mitochondria. On the other hand, fatty acid synthesis converts citrate from the TCA cycle, which is transported into the cytosol, to fatty acids. Fatty acids can be converted to lipids such as triglycerides, phospholipids or cholesterol esters [55] (see figure 3). The

metabolic pathways utilized by immune cells and their function is intimately linked. Depending on activation status and function of the immune cell different metabolic configurations are used, however, the metabolic state can also undergo reprogramming and thereby change functional properties of the immune cell.

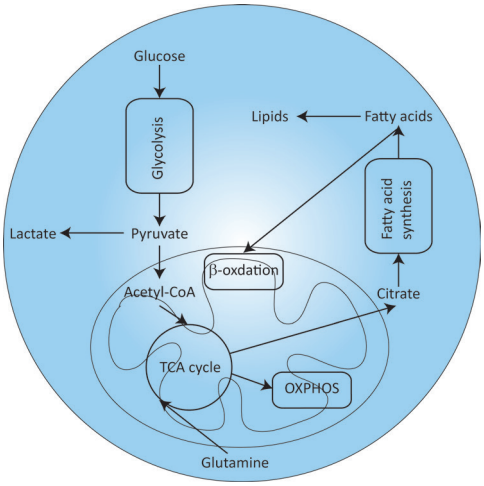

Figure 3. Major cellular metabolic pathways

T cell metabolism and function

T cell function and their metabolism are linked. Naïve or resting T cells have a different metabolic state when compared to activated T cells, and also T effector and Treg cells utilize different metabolic pathways.

Naïve or resting T cells have a relatively low metabolic rate aimed at energy production, rather than biosynthesis. They rely on the TCA cycle, linked to OXPHOS to generate ATP, fuelled by glucose, lipids or amino acids [56]. Activated T cells on the other hand have a high metabolic demand and are aimed at growth-promoting pathways and rely on glycolysis and OXPHOS [57, 58]. They have a high glycolytic rate, mediated by the upregulation of glucose transporter 1 (GLUT1) expression levels [56, 58-61]. Transgenic expression of GLUT1 or failure to elevate the expression of GLUT1 after antigenic stimulation affects the proliferation, survival and cytokine production, indicating that upregulated glucose metabolism is essential for T cell activation [56, 58-61]. Although fatty acids have been shown to influence proliferation of T cells, little is known about the dependence of T cell proliferation on fatty acid uptake. Recently, a study showed that full activation and

proliferation of T cells requires de novo fatty acid synthesis and fatty acid uptake [62]. In line with this, tissue resident memory $CD8^+$ T cells have been shown to take up fatty acids to fuel oxidative metabolism and failure to do so resulted in diminished persistence of these cells [63]. Furthermore, exogenous fatty acids fuelling β-oxidation during T cell activation promotes effector memory $CD4^+$ T cells [64].

Furthermore, T effector and Treg cells also differ in their usage of metabolic pathways. While T effector cells such as Th1, Th2 and Th17 express elevated levels of GLUT1 and rely on glycolysis, Treg cells do not have elevated expression levels of GLUT1 and rely on β-oxidation rather than glycolysis [56, 58, 65, 66]. Manipulation of either pathway has been shown to have an effect on both T effector cells and Tregs. Glycolysis will enhance T effector cells, but will inhibit Tregs, on the other hand lipid oxidation will promote the generation of Tregs, but suppress T effector cell function and survival [58, 65, 67].

T cells and obesity

In obese persons, the number, subsets and function of T cells seem to be altered although contradictory results are found. Total T cell numbers are elevated [68-70], however, diminished T cells numbers [71] have also been found in obese persons compared to lean persons. Decreased levels of $CD4^+$ T cells [71] and $CD8^+$ T cells [71, 72] have been found, however, also elevated levels of $CD4^+$ T cells [68-70] and normal levels for $CD8^+$ T cells [69, 70]. In addition, T cell function, such as proliferation and cytokine production is also altered in obese persons [68, 70, 73]. The mechanisms underlying these differences between obese and lean persons is unknown, however, free fatty acids levels in plasma are higher in obese persons compared to healthy persons [40, 74, 75], therefore, they could play an important role. There are several indications suggesting that fatty acids can modulate the immune response. Levels of several fatty acids are associated with levels of inflammatory markers in healthy individuals [76]. The type of fatty acids in the diet can influence the risk of development of inflammatory diseases [77-80] and modulate T cell function and phenotype *in vivo* [81-84]. Furthermore, *in vitro* studies suggest that high concentrations of fatty acids are toxic for the cell, while non-toxic concentrations are capable of inducing proliferation and cytokine production [85-90].

Aim of the thesis

Based on the two levels of immunometabolism, this thesis is divided into two parts. The first part focusses on the systemic level of immunometabolism, which explores the link between immune cells and their effect on metabolic tissues, such as adipose tissue. The IFP, an adipose tissue located in the knee, is thought to play a role in the pathophysiology of OA. However, inflammatory processes and obesity related features present in the IFP are unknown. The second part investigates the cellular level of immunometabolism, exploring the link between intracellular metabolic pathways of immune cells and their function. $CD4^+$ T cells and their function are intimately linked, and fatty acids have been described to affect these cells, however, how fatty acids can exert these effects is unknown. Therefore, the aims of the thesis are:

- To characterize the infrapatellar fat pad on the cellular and molecular level and determine its potential role in the pathophysiology of osteoarthritis
- To determine how fatty acids exert their effect on T cells

Outline of the thesis

Part 1 Systemic immunometabolism

OA and rheumatoid arthritis (RA) are both rheumatic diseases in which inflammation can be present, however, RA is in general associated with more inflammation in the synovium [91] and synovial fluid [92-94]. Whether the IFP participates in the inflammatory processes in the joint and thereby contributes to the disease pathogenesis is unknown. Therefore, in **chapter 2** we compared the IFP of OA and RA patients. IFP samples were obtained of OA patients and RA patients with secondary OA undergoing joint replacement surgery. The adipocytokine secretion profile of the adipose tissue (fat-conditioned medium) and adipocytes (adipocyte-conditioned medium) was determined by luminex. Furthermore, the immune cellular infiltrate was counted and the composition was investigated by flow cytometry.

Previously, $CD4^+$ T cells from the IFP of OA patients have been shown to secrete IL-6 directly *ex vivo* [51]. This has led us to the hypothesis that these cells recently have been activated which could suggest that these cells recognize adipose tissue antigens and could play a role in adipose tissue inflammation and thereby contribute to the pathogenesis of OA. Therefore, in **chapter 3,** we extensively characterized the IL-6^+ $CD4^+$ T cell population previously found in the IFP of OA patients. Using flow cytometry, we determined the expression levels of co-stimulatory molecules, activation markers and chemokine receptors. An in-house generated IL-6 capture complex was developed to perform TCRβ gene analysis to determine the clonality of these cells. The localization of the IL-6^+ $CD4^+$ T cells and the effect of adipocytes on $CD4^+$ T cells was determined.

Obesity is usually accompanied by adipose tissue inflammation, characterized by changes in adipocytes and inflammatory cells, however, the effect of obesity on the IFP is unclear. Therefore, in **chapter 4** we extensively investigated the cellular and molecular adipose tissue features typically associated with obesity and determined for all these features whether they associated with BMI, a measurement for obesity. First, the volume of IFP was determined with MRI and linear regression analysis were performed to determine whether the volume associated with BMI or other obesity-related features. Next, the IFP was obtained from OA patients undergoing joint replacement surgery and adipocyte volume and size was determined by light microscopy. The number of adipose tissue immune cells was determined by light microscopy and characterized by flow cytometry and luminex. As macrophages in the IFP expressed anti-inflammatory markers, while producing pro-inflammatory cytokines, we continued by characterizing these macrophages by flow cytometry.

Part 2 Cellular immunometabolism

In part two we focussed on cellular immunometabolism. As first step, we reviewed existing literature regarding the effect of fatty acids and lipid mediators, oxygenized fatty acids, on T cells and their function in **chapter 5**.

As metabolism and function of T cells is linked and fatty acids have been shown to enhance the proliferation of $CD4^+$ T cells, in **chapter 6**, we explored the possible mechanisms underlying this enhanced proliferation. Peripheral $CD4^+$ T cells were incubated with oleic acid for one day and proliferation was assessed after 4 days with incorporation of 3H-thymidine and cell trace violet staining. Functional

metabolic analysis was performed to determine the effect of OA treatment early on and after 24 hrs on the metabolism of $CD4^+$ T cells. Furthermore, by the usage of ^{13}C-OA metabolomics analysis was performed to determine whether oleic acid treatment influences any other metabolic pathway or whether it is used as substrate. In addition, ^{13}C-OA was used to determine the fate of oleic acid in the cell. To determine whether the formation of fatty acids is needed for enhanced proliferation with oleic acid, inhibition assays were performed with C75 and TOFA and proliferation was determined. Furthermore, the influence of oleic acid on signalling was assessed by calcium flux experiments and the degree of phosphorylation of ZAP70 with Western Blot.

Finally, in **chapter 7** and **8**, we provide a summary and discussion of our findings.

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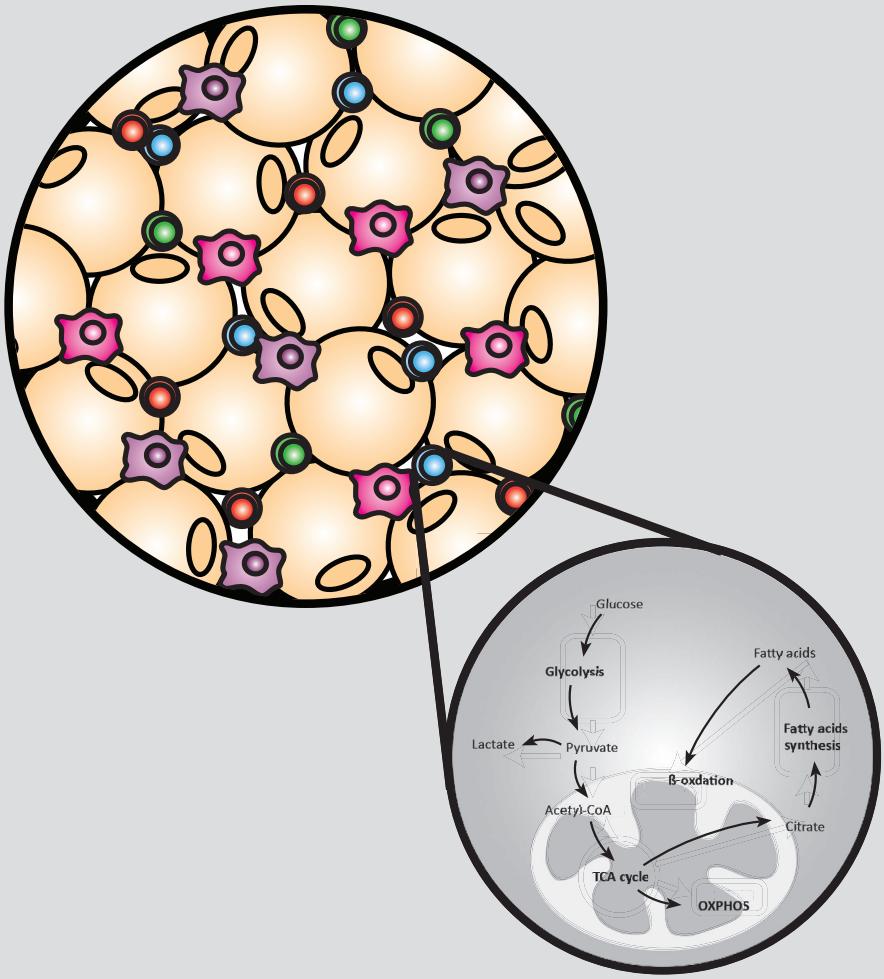
Part 1

Systemic immunometabolism



Chapter 2

Inflammatory features of infrapatellar fat pad in rheumatoid arthritis versus osteoarthritis reveal mostly qualitative differences

Anja J. de Jong, Inge R. Klein-Wieringa, Joanneke C. Kwekkeboom,
René E.M. Toes, Margreet Kloppenburg, Andreea Ioan-Facsinay

Annals of the Rheumatic Diseases. 2017

Rheumatoid arthritis (RA) and osteoarthritis (OA) are characterised by joint destruction. In both diseases, inflammation is implicated; however, RA is generally associated with more inflammation in synovium[1] and synovial fluid (SF)[2-4] compared with OA. The infrapatellar fat pad (IFP), an adipose tissue located in the knee joint, has been proposed to contribute to disease progression in OA. Although IFP of OA has been extensively characterized and identified as a source of inflammation in the joint,[5-7] only scarce information is available on IFP in patients with RA. Therefore, our aim was to get a first insight into the possible contribution of IFP to the inflammatory processes in the RA joint.

To this end, we compared leftover IFP and synovium from patients with RA (N=20: 80% women, mean (SD) age 64 (10.7) years, median (range) body mass index (BMI) 26.9 (18) kg/m^2) and patients with primary knee OA (N=51: 62% women, mean (SD) age 66 (8.9) years, median (range) BMI 28.9 (15) kg/m^2); all were undergoing knee joint replacement surgery. The study was approved by the local medical ethical committee. Fat-conditioned medium (FCM) and adipocyte-conditioned medium (ACM) were generated as previously described.[5] Adipokines and cytokines were measured in FCM and ACM with Milliplex MAP Human Adipocyte kit (Millipore) and Milliplex MAP Human Adipokine kit (Millipore), using the Bio-Plex array reader and Bio-Plex software according to the manufacturer's instructions. Stromal vascular fraction (SVF) was isolated and cells were identified by flow cytometry as previously described.[5] The number of mast cells in IFP was determined based on fluorescence-activated cell sorting (FACS) and the number of SVF cells counted on digestion. Synovitis score and number of synovial CD117$^+$ cells was determined as previously described[1]. OA and RA groups were matched for age, gender and BMI on group level for FCM, ACM, FACS analysis, immunohistochemistry and immunofluorescence analysis.

The IFP (FCM) and adipocytes (ACM) of patients with OA and RA secreted various adipocytokines; however, no significant differences were observed (table 1). The IFP of patients with RA contained a higher number of cells than IFP of patients with OA (figure 1A), but the percentages of T cells (CD3$^+$, figure 1B), CD4$^+$ or CD8$^+$ T cells (figure 1C), monocytes (CD14$^+$, figure 1D) and endothelial cells (CD31$^+$, figure 1E) were comparable between patients with RA and OA. The percentage of B cells was below 1% in both populations (data not shown). The only significant difference was observed in the percentage and abundance (CD117$^+$, figure 1F) of mast cells, which were higher in RA IFP. Interestingly, although RA synovium had

a higher synovitis score (figure 1G), lower numbers of mast cells were found in RA synovium compared with OA synovium (figure 1H and de Lange-Brokaar *et al.* [1]), suggesting that mast cell numbers are controlled by different signals in OA and RA.

Table 1. Levels of adipocytokines in FCM and ACM.

Depicted are median (Q1-Q3). Difference between RA and OA group determined with Mann-Whitney are indicated with p value.

			RA		OA		p value
			Median	(Q1-Q3)	Median	(Q1-Q3)	
FCM			N=11		N=22		
	Adipsin	(ng/ml)	382.9	(226.3-457.9)	349.9	(215.2-446.3)	0.667
	Adiponectin	(ng/ml)	103.1	(87.5-124.8)	97.7	(64-193.8)	0.585
	Leptin	(ng/ml)	3.3	(0.7-4.1)	1.4	(0.7-2.4)	0.233
	Resistin	(pg/ml)	66.8	(36.5-248.6)	90.6	(57.4-186.6)	0.618
	IL6	(ng/ml)	29.7	(8.5-63.5)	101.4	(1.9-19.8)	0.076
	IL8	(ng/ml)	18.5	(3.4-74.5)	8.3	(1.6-20.7)	0.133
	MCP-1	(ng/ml)	11.6	(9.9-12.2)	9.2	(5.5-13.1)	0.349
	TNFα	(pg/ml)	2.0	(0.8-12.6)	1.8	(0.9-8.3)	0.873
	IL6R	(pg/ml)	13.6	(5.7-22.3)	20.9	(11.2-31.4)	0.182
ACM			N=13		N=21		
	Adipsin	(ng/ml)	22.6	(10.8-34.1)	30.3	(20.5-44.2)	0.129
	Adiponectin	(ng/ml)	21.8	(11.8-41.0)	34.5	(22.6-51.3)	0.076
	Leptin	(pg/ml)	255.8	(98.9-797.9)	318.5	(149.4-658.5)	0.821
	Resistin	(pg/ml)	2.7	(2.7-7.0)	2.7	(1.2-4.3)	0.255
	IL6	(ng/ml)	0.4	(0.2-1.5)	1.1	(0.3-2.2)	0.344
	IL8	(ng/ml)	0.6	(0.4-2.0)	2.1	(0.6-3.2)	0.193
	MCP-1	(ng/ml)	0.3	(0.1-0.6)	0.7	(0.3-1)	0.089
	TNFα	(pg/ml)	3.4	(1.4-9.8)	8.3	(3.5-13.5)	0.156
	IL6R	(pg/ml)	N.D.		N.D.		

ACM, adipocyte-conditioned medium; FCM, fat-conditioned medium, IL, interleukin; MCP, monocyte chemoattractant protein-1; ND, not detectable; OA, oste

Despite the small sample size, our data indicate that the higher cellular infiltrate in RA compared with OA IFP does not result in clear differences in secretory profile of these tissues, indicating a limited contribution of infiltrating immune cells to secretion of the investigated adipocytokines in these patients. However, our data await further replication and should not be extrapolated to earlier stages of RA as inflammation in all joint tissues could be different at an earlier disease stage. Moreover, as patients with RA usually display a higher SF and synovial inflammatory cytokine load than OA patients,[4, 8] this is not readily apparent for IFP in this study. Although the lack of SF is a limitation of our study, these data suggest little contribution of IFP to SF cytokines in RA. The increased cellular infiltration of RA IFP

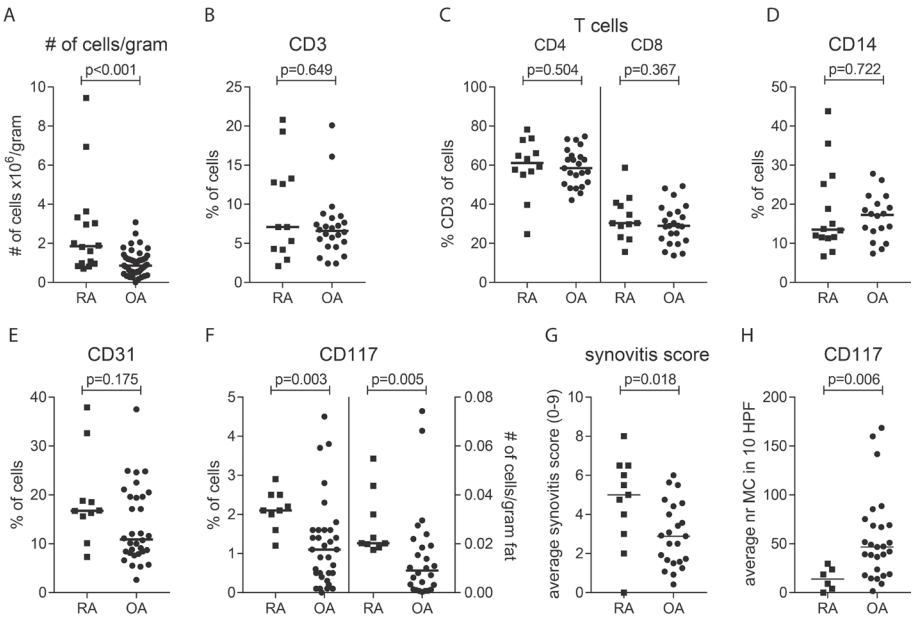


Figure 1. Immune cell quantification and characterization of the stromal vascular fraction (SVF) of the infrapatellar fat pad (IFP) of rheumatoid arthritis (RA) and osteoarthritis (OA) patients.

SVF was isolated and number of cells per gram fat tissue was determined (A). Using flow cytometry, the percentage of $CD3^+$ (B), $CD4^+$ and $CD8^+$ (C), $CD14^+$ (D), $CD31^+$ (E) and $CD117^+$ (F) cells were determined. The number of $CD117^+$ was calculated using the percentage of $CD117^+$ cells and the number of cells per gram of tissue (F). Synovitis score (G) and the number of $CD117^+$ cells (H) was determined using immunohistochemistry or immunofluorescence, respectively. Median is represented and each dot represents one patient. According to Bonferroni correction for multiple testing a p value of <0.01, determined by Mann-Whitney was considered statistically significant for (A)-(F), while a p value of 0.025, determined by Mann-Whitney was considered statistically significant for (G)-(H). MC=Mast cell; HPF=high power field.

could be a reflection of the generally higher inflammatory load present in the joint of patients with RA.

Acknowledgements

The authors would like to acknowledge the support of the cooperating hospital LUMC, Leiden, the Netherlands and orthopaedic surgeons and nurse practitioners. Furthermore, we thank Annemarie L. Dorjée for her help with scoring synovitis.

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Chapter 3

IL-6 secreting T cells in adipose tissue: a novel human $CD4^+$ T cell population

Anja J. de Jong, Sabrina Pollastro, Joanneke C. Kwekkeboom, Stefan N. Andersen, Annemarie L. Dorjée, Fawaz Alzaid, Nicolas Venteclef, Niek de Vries, Margreet Kloppenburg, René E.M. Toes, Andreea Ioan-Facsinay

In revision

Abstract

Emerging evidence indicates that a dynamic interplay between the immune system and adipocytes contributes to the disturbed homeostasis in adipose tissue of obese subjects. Recently, we observed IL-6-secretion by CD4$^+$ T cells from the stromal vascular fraction (SVF) of the infrapatellar fat pad (IFP) of knee osteoarthritis patients directly *ex vivo*. We now show that IL-6$^+$ CD4$^+$ T cells from SVF seems to have an activated phenotype as evidenced by the expression of the activation markers, CD69, CD25 and HLA-DR. Co-staining for different cytokines upon polyclonal stimulation revealed that IL-6-secreting CD4$^+$ T cells are distinct from the T cells secreting other cytokines. In addition, analysis of chemokine receptor expression revealed that these IL-6 producing T cells could not be categorized as a conventional T helper subset. TCRβ gene analysis revealed that IL-6$^+$ and IL-6$^-$ CD4$^+$ T cells appear clonally unrelated to each other, suggesting a specific clonal expansion of IL-6$^+$ CD4$^+$ T cells. In line with these observations, adipocytes are capable of enhancing IL-6 production by CD4$^+$ T cells. Thus, IL-6$^+$ CD4$^+$ T cells are TCRαβ T cells expressing an activated phenotype potentially resulting from an interplay with adipocytes that could be involved in the inflammatory processes in adipose tissue.

Introduction

Obesity is an increasing problem in the Western world, and is associated with several diseases. These include the long-known metabolic and cardiovascular disorders [1, 2], but also the more recently described associations with inflammatory diseases, such as rheumatoid arthritis, osteoarthritis and inflammatory bowel diseases [3-5]. Although for most of the associations, the underlying mechanisms are unclear, adipose tissue inflammation is believed to play an important role in obesity-related disorders.

The expansion of adipose tissue during weight gain and development of obesity is accompanied by a switch from an anti-inflammatory state to a pro-inflammatory state of the adipose tissue [1]. The precise sequence of events leading to this switch are incompletely understood, but several studies suggest that changes in both adipocytes and immune cells are involved in this process. Obesity is accompanied by an increase in adipocyte number, size and death [6-8] resulting in hypoxia and infiltration of immune cells, including T cells [9, 10]. Mouse studies revealed an accumulation of Th1 and CD8+ T cells in the obese adipose tissue, at the expense of Treg and Th2 cells [9, 11-13]. Moreover, depletion studies indicated that Th2 and Treg are involved in maintaining insulin sensitivity, while Th1 and CD8 T cells contribute to insulin resistance and adipose tissue inflammation [9, 11, 12].

Although limited knowledge is available about adipose tissue in humans, both $CD4^+$ and $CD8^+$ T cells were shown to be present and more abundant in obesity [13-15]. The observations regarding Th subsets and their association with obesity are, however, less clear in humans. Similar to observations in mice, Th1 cells have been shown to be enhanced in obesity [11, 13] and to outnumber Treg cells [11] in some studies, while in another study Th17 cells were enhanced with no enhancement of Th1 or Th2 in adipose tissue [15]. Moreover, the percentage of Th1 cells correlated positively with insulin resistance [13] and the percentage of Th2 cells correlated inversely with insulin resistance [16] in some studies. Overall, studies in humans support a possible role for pro-inflammatory Th cells, such as Th1 in adipose tissue inflammation and the subsequent insulin resistance. Although the mechanisms underlying the accumulation of certain Th subsets in adipose tissue and their downstream effects are still unclear, some murine studies showed that $CD4^+$ T cells in adipose tissue have a limited TCR-repertoire [9, 11,

12, 14], suggesting that they underwent clonal expansion. Whether these cells have expanded in the adipose tissue and which antigen these cells recognize is unknown. Moreover, it is unknown what the phenotype of these cells is and which cytokines they secrete. Recently, we demonstrated that CD4+ T cells from the infrapatellar fat pad (IFP), an adipose tissue located in the knee, secrete IL-6 directly *ex vivo* [17]. These findings were unexpected considering that there was no additional stimulus, indicating recent activation of the T cells. This could suggest that these cells recognize adipose tissue antigens and could play a role in adipose tissue inflammation. Therefore, the aim of this study was to further characterize the IL-6+ CD4+ T cells of IFP, to gain a better understanding of the possible role of these cells in adipose tissue.

Materials and methods

Human subjects

Patients with primary osteoarthritis undergoing knee joint replacement were included into the study (N=67: 62.7 % women, median age 71 years, median (IQR) BMI 29.7 kg/m2 (26.7-33.1). Preoperative blood, IFP, subcutaneous adipose tissue (SCAT) and synovium removed during surgery were obtained after informed consent. Due to limited numbers of cells not all experiments could be performed on each tissue sample. The study was approved by the local ethical committee. Buffy coats were from healthy donors and the study was approved by the local medical ethical committee.

Cell isolation

Stromal vascular fraction (SVF) was isolated as previously described [18] and was used for flow cytometric analysis or the isolation of IL-6+ and IL-6- CD4+ T cells. Adipocytes were isolated as previously described [18] and used for co-cultures with CD4+ T cells. PBMCs were isolated from buffy coats of healthy donors by standard ficoll plaque gradient. CD4+ T cells were purified using magnetic beads labelled with anti-CD4 (Invitrogen Dynal, Oslo, Norway), followed by removal of the magnetic beads, according to the manufacturer's instructions. The purity of the isolated CD4+ T cells was typically above 95%. CD4+ T cells were used for co-cultures with adipocytes.

Flow cytometric analysis

SVF was plated overnight in a 6-wells plate at a density of 5x10^6 cells/well maximum in DMEM 4.5 g/l glucose/F12/0.5% BSA/15 mM Hepes/glutamax/pen/strep (Invitrogen) (medium) supplemented with 50 IU/ml IL-2 (Peprotech). For polyclonal activation of T cells 20 ng/ml phorbol myristate acetate (PMA) and 200 ng/ml ionomycin was added after 16 h and incubated for 5 h. Brefeldin A 10 µg/ml was added after 1 h, where after cells were harvested and surface and intracellular stainings were performed. For *ex vivo* determination of cytokine production cells were incubated overnight in medium supplemented with 3 µg/ml Brefeldin A and 50 IU/ml IL-2. Cells were harvested and surface and intracellular stainings were performed. Exclusion of dead cells was performed in experiments when possible using the dead cell discrimination kit (Miltenyi Biotec), according to the manufacturer's specifications. Cells were first stained with antibody mixes containing the following surface markers: AF-700-conjugated CD3, Pacific blue-conjugated CD4, FITC-conjugated CD8, HLA-DR, TCRαβ, APC-conjugated CD8, CD28, CD45RO, CCR10, CXCR3, AF-647-conjugated CXCR5, PE-Cy-5-conjugated CD69, PE-Cy-7-conjugated CD14, CD25, CD27, CCR4, PercpCy5.5-conjugated CD38, CCR6, CCR7 and PE-Texas red-conjugated CD69 (all from BD Biosciences). Next, intracellular cytokines were detected using Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences) according to manufacturer's instructions. Antibody mixes for intracellular stainings contained the following cytokine antibodies: PE-conjugated Abs to interferon γ (IFNγ), IL10, tumour necrosis factor α (TNFα), IL-6, Pe-Cy-7-conjugated IL-4, FITC-conjugated IL-6 and the appropriate isotype controls (all BD Biosciences except the Ab to PE-conjugated IL-6 which was from eBioscience). Cells were fixed with paraformaldehyde and analysed with LSRII flow cytometer using Diva 6 software (BD Biosciences).

Isolation of IL-6$^+$ and IL-6$^-$ CD4$^+$ T cells from SVF

To isolate IL-6$^+$ cells an IL-6 capture complex was generated. The IL-6 capture complex consists of biotin labelled CD45 and biotin labelled IL-6 both complexed to avidin. First 1 µl of 200 µg/ml biotin labelled CD45 and 10 µl of 500 µg/ml biotin labelled IL-6 were combined and vortexed. Next, 1 µl of 5 mg/ml avidin was added and mixed again. The capture complex was incubated for 10 min at room temperature and vortexed before use. A total of 6 µl of the capture complex was added to 30 µl PBS/2%FCS containing 1x10^6 isolated SVF cells and incubated for 15 min. SVF was then incubated with complete medium with 50 IU/ml IL2

(Peprotech) overnight at 37°C under continuous rolling. Next day SVF was washed with PBS/2% FCS, where after dead cells were stained with Dead cell discriminator kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and with AF-700-conjugated CD3, Pacific Blue-conjugated CD4, APC-conjugated CD8, PE-Cy-7-conjugated CD14 (all from BD Biosciences, Breda, The Netherlands) and PE-conjugated IL-6 (eBiosciences, Vienna, Austria). Using a FACS Aria both CD3$^+$CD4$^+$CD14$^-$CD8$^-$IL-6$^+$ and CD3$^+$CD4$^+$CD14$^-$CD8$^-$IL-6$^-$ were sorted.

Preparation of cDNA for real-time quantitative polymerase chain reaction (qPCR)

Sorted cells were processed using the Smart-seq2 protocol [19] with minor changes. A total of 20 sorted cells were used per reaction. Reverse transcription was carried out with SMartscribe Reverse transcriptase (100 U/ml), without adding MgCl. ERCC RNA Spike-In Mix (ThermoFisher) controls were used to control for variability between samples. Furthermore, pre-amplification was carried out by denaturing at 95°C and using 19 PCR cycles. Purification of the resulting cDNA was performed with bead (Ampure XP beads) to sample ratio of 0.8:1 ratio. Real-time qPCR was performed with primers specific for IL-6, B2M and LMNA as well as ERCC-0074 and ERCC-0096 which were primers specific for the ERCC RNA Spike-In Mix to control for variability between samples. The cDNA was 1:10 diluted and qPCR was performed using SensiFast Sybr (Bio-line) and primers at a concentration of 250 nM in a total volume of 8 µl. The qPCR was performed on a real time PCR system (Bio-Rad CFX-384) with an activation step of 2 min on 95°C (hot start polymerase activation), a melting temperature of 95°C for 5 s and an annealing temperature of 58°C (ERCC-0074 and ERCC-0096), 58°C (B2M and LMNA) or 64°C (IL-6) for 10 s and an elongation step for 25 s on 72°C for 40 cycles. At the end of the protocol melting curves were performed from 65°C to 95°C to test specific binding of SensiFast Sybr.

High-throughput sequencing

RNA extraction, cDNA synthesis and linear amplification were performed as previously described [20]. Briefly, TCRβ repertoire was amplified in two steps. First, a linear amplification was performed using a mix of primers covering all the functional TCRβ variable genes. After purification, the amplification product was used in a normal PCR to obtain amplicons spacing from the TCRβ variable region to the TCRβ constant region. Amplicons were purified, quantified, prepared for

sequencing according to the sequencing platform manufacturer's manual and sequenced on a Roche Genome Sequencer FLX (titanium platform).

CDR3 sequence analysis

The bioinformatic pipeline used to extract TCRβ sequences was described previously [20]. In short, TCRβ reads obtained from the sequencing platform are "fingerprinted" based on the V-J-CDR3 identified in the sequence (V= Variable gene, J= Joining gene, CDR3= Complementary Determining Region 3). TCRβ sequences with unique fingerprint are regarded as clones. The frequency of each clone is calculated based on the total amount of reads. Clones with frequencies above 0.5% of the total repertoire are considered as Highly Expanded Clones (HECs).

IL-6$^+$ T cell tissue staining

IFP pieces were fixed in 4% formalin overnight followed by storage in EtOH, before embedding in paraffin. Four micrometre sections were deparaffinised and rehydrated. Antigen retrieval was performed with EDTA (pH 9) (DAKO, USA) at 96ºC for 30 min. After cooling, sections where blocked with blocking solution (10% Normal Donkey Serum (DS) in 1% BSA/PBS) for 30 min at RT. Sections were incubated overnight at 4°C with monoclonal mouse anti-CD3 (DAKO, Glostrub, Denmark) and polyclonal rabbit anti-IL-6 (Abcam, Cambridge, UK) in 1% DS/1% BSA/PBS. Sections were then washed with PBS and incubated with polyclonal donkey anti-mouse IgG Alexa Fluor 568 for CD3 and polyclonal donkey anti-rabbit IgG Alexa Fluor 488 for IL-6 for 1 hr at RT. Sections were washed with PBS, dried and covered with Vectashield Hard Set mounting medium with DAPI (Vector laboratories, Burlingame, USA) and analysed on a fluorescence imaging microscope.

Co-culture of adipocytes with CD4$^+$ T cells

Isolated CD4$^+$ T cells were cultured overnight at a density of 200.000 cells/well in 96-well plates in 200 µl medium. CD4$^+$ T cells were co-cultured with 20 µl of isolated adipocytes. Cultures were in absence or presence of 5 µg/mL plate-bound anti-CD3 antibody (clone OKT3, eBioscience, San Diego, USA) and 1 µg/ml soluble anti-CD28 antibody (Sanquin, Amsterdam, The Netherlands) as stimulus. As controls CD4$^+$ T cells or adipocytes alone were cultured in presence or absence of stimulus. After overnight culture Brefeldin A (10 µg/ml) was added for 5 hours after which CD4$^+$ T cells were harvested and intracellular staining for IL-6 was performed.

Detection of cytokines

Cytokines were measured in supernatant using the Milliplex Human Cytokine / Chemokine kit (Millipore), the Bio-Plex array reader and Bio-Plex software, according to manufactures' protocol.

Statistical analysis

Wilcoxon's signed rank test was used to compare differences between groups. A P value ≤ 0.05 was considered statistically significant.

Results

CD4+ T cells from IFP secrete IL-6 *ex vivo*

Secretion of IL-6 by CD4+ T cells present in the SVF was confirmed by multiplex ELISA using culture supernatant of sorted CD4+ T cells from IFP (Fig 1A). Next to IL-6, secretion of IFNγ, IL-8, FGF-2, fractalkine, eotaxin, MCP-1 and MIP1β was also evident in isolated CD4+ T cells (Fig. 1B). To determine whether the previously found IL-6+ CD4+ T cells population was the source of the IL-6 in the supernatant, we isolated IL-6+ CD4+ T cells from SVF using an in-house developed capture complex (Supporting Information Fig. 1A). The specificity of the isolation procedure was validated by ELISA and the results indicated that IL-6 could only be detected in the culture supernatant of IL-6+ CD4+ T cell population (Supporting Information Fig. 1B). However, the sensitivity of this procedure was lower than intracellular cytokine staining (data not shown). Moreover, q-PCR analyses indicated that mRNA expression of IL-6 could only be detected in the IL-6+ CD4+ T cells and not in IL-6- CD4+ T cells (Fig. 1C).

Intriguingly, a similar population was present in synovial tissue and IFP in paired samples, and only to a lower extend in peripheral blood, as analysed when available (Fig. 1D and E). This population could also be detected in SCAT and visceral adipose tissue (Supporting Information Fig. 3). Thus, our data indicate that the population of CD4+ T cells capable of producing IL-6 without additional stimulus *ex vivo* is not restricted to the IFP.

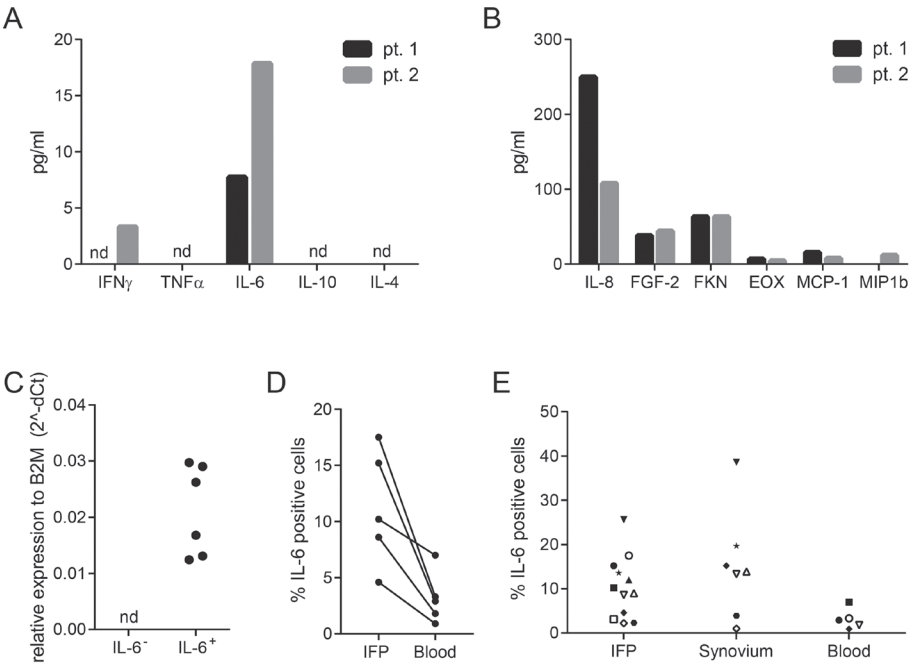

Figure 1. CD4+ T cells from IFP secrete IL-6 *ex vivo*

Spontaneous cytokine production by CD4+ T cells from SVF was confirmed and expanded by testing supernatants of sorted CD4+ T cells (12878 or 32347 cells/well) with luminex for 42 cytokines (A and B)(N=2). IL-6+ and IL-6- CD4+ T cells were isolated from SVF using an in-house generated capture complex (Supporting Information Fig. 1), cDNA was generated and IL-6 mRNA levels were determined (N=6)(C). The presence of IL-6+ CD4+ T cells was also determined in synovium and blood by flow cytometry (see gating strategy Supporting Information Fig 2) (N=2-12)(D and E). Each symbol represents a patient.

Phenotypic characterization of IL-6+ CD4+ T cells

To obtain insight into the possible function of this enigmatic T-cell population, we performed an extensive phenotypic characterization. All IL-6+ CD4+ T cells expressed TCRαβ and CD45RO (Fig. 2A) indicating that they are conventional αβ memory T cells. Furthermore, IL-6+ CD4+ T cells expressed both CD27 and CD28 (Fig. 2B). Since IL-6+ CD4+ T cells produced cytokines without additional stimulation, we hypothesized that these cells could be recently activated. Therefore, we assessed the activation states of these cells and found that IL-6+ CD4+ T cells expressed CD25 and CD69, and to a lesser extent CD38 and HLA-DR (Fig. 2C). Moreover, expression of CD69 appeared to be higher on IL-6+ CD4+ T cells than their IL-6- CD4+ T cells counterparts. Besides activation marker, CD69 is also expressed on tissue resident T cells [21, 22], we explored the possibility that these cells are tissue resident

by assessing the mRNA expression of Hobit, a transcription marker associated with tissue resident T cells [23]. Neither IL-6+ nor IL-6- CD4+ T cells expressed this transcription marker (data not shown). In conclusion, IL-6+ T cells from IFP are a novel population of memory CD4+ T cells, present in IFP in an activated state.

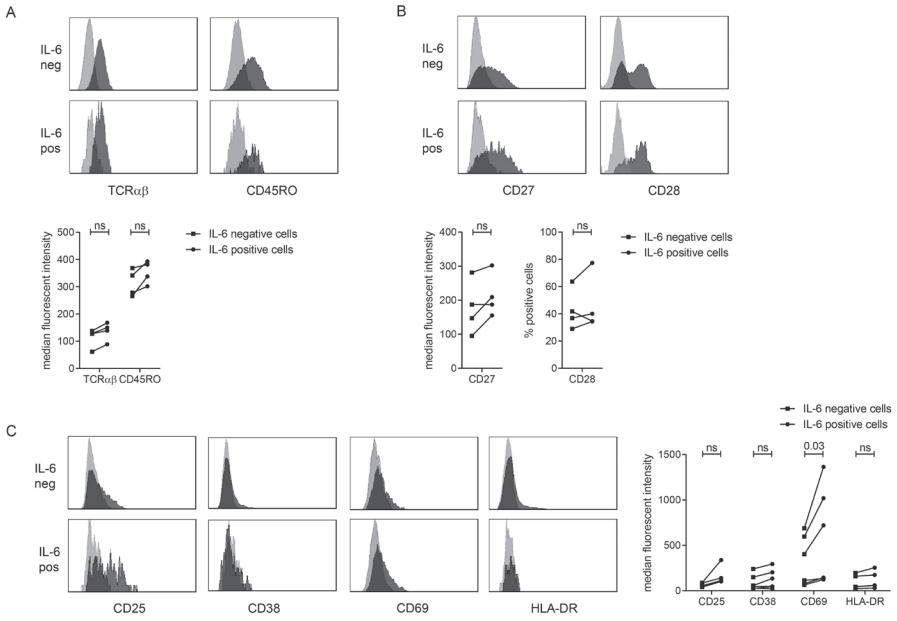

Figure 2. Phenotypic characterization of IL-6+ CD4+ T cells

Spontaneous IL-6+ CD4+ T cells and IL-6- CD4+ T cells from SVF were characterized by flow cytometry (see gating strategy Supporting Information Fig. 2) for general T cell markers (A)(N=4), co-stimulatory markers (B) (N=4) and activation markers (C)(N=4-6). Examples of stainings and summary graphs of all patients tested are given, Wilcoxon's singed rank test was used to compare differences between groups.

IL-6+ CD4+ T cells cannot be categorized as a conventional T helper subset

Next, we investigated whether IL-6+ CD4+ T cells also expressed other cytokines that are classically assigned to certain helper subsets. Intracellular cytokine staining revealed that IL-6 producing T cells do not secrete other cytokines such as IFNγ, TNFα, and IL-4 (Fig. 3A). Furthermore, chemokine receptor expression showed that IL-6 producing T cells expressed a variety of chemokine receptors (Fig. 3B), precluding their unambiguous assignment to a certain T helper subset defined by their chemokine receptor expression. These data suggest that IL-6+ CD4+ T cells are not a conventional T helper subset.

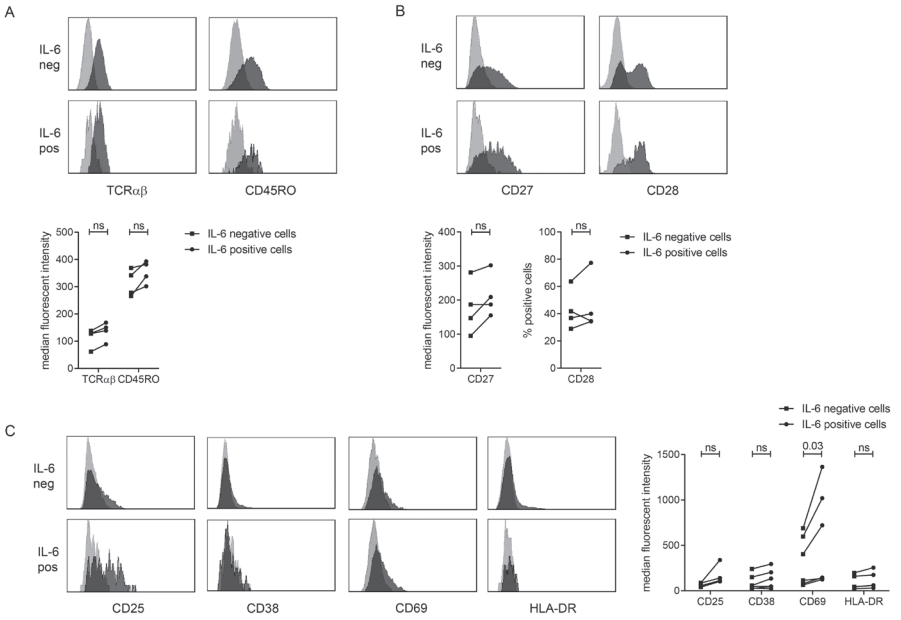

Figure 3. IL-6$^+$ CD4$^+$ T cells cannot be categorized as a conventional T helper subset

SVF cells were stimulated overnight with PMA/ionomycin and simultaneous cytokine production of CD4$^+$ T cells was assessed by flow cytometry (A)(N=12). Chemokine receptor expression was determined on spontaneous IL-6$^+$ CD4$^+$ T cells and IL-6$^-$ CD4$^+$ T cells from SVF (B)(N=3-5). Examples of all stainings and summary graphs of all patients tested are given, Wilcoxon's singed rank test was used to compare differences between groups.

TCRβ repertoire in IL-6$^+$ CD4$^+$ T cells and IL-6$^-$ CD4$^+$ T cells

The fact that IL-6$^+$ CD4$^+$ T cells from SVF display an activated state suggests that they have recently encountered antigen. Therefore, we determined the abundance and distribution of TCRβ rearrangements in the IL-6$^+$ and the IL-6$^-$ CD4$^+$ T cell populations. Comparing the total repertoire of the IL-6$^+$ CD4$^+$ T cells and the IL-6$^-$ CD4$^+$ T cells populations did not show major differences regarding the general clonal expansion (Fig. 4A). The distribution of the TCRβ rearrangements in the IL-6$^+$ and IL-6$^-$ CD4$^+$ T cell populations is presented in figure 4B for 1 patient, while a summary of all 3 patients is presented in figure 4C. In the pool of highly expanded clones (HECs), clones with read frequency above 0.5%, only a low number of TCRβ rearrangements were shared between the IL-6$^+$ and IL-6$^-$ CD4$^+$ T cell populations in all 3 patients (Fig. 4A, red dots and Fig. 4C). The percentage of HECs shared between the IL-6$^+$ and IL-6$^-$ CD4$^+$ T cell populations varied per patient and was between 5-15% (Fig. 4C) indicating that the IL-6$^+$ and IL-6$^-$ populations have a different TCRβ usage.

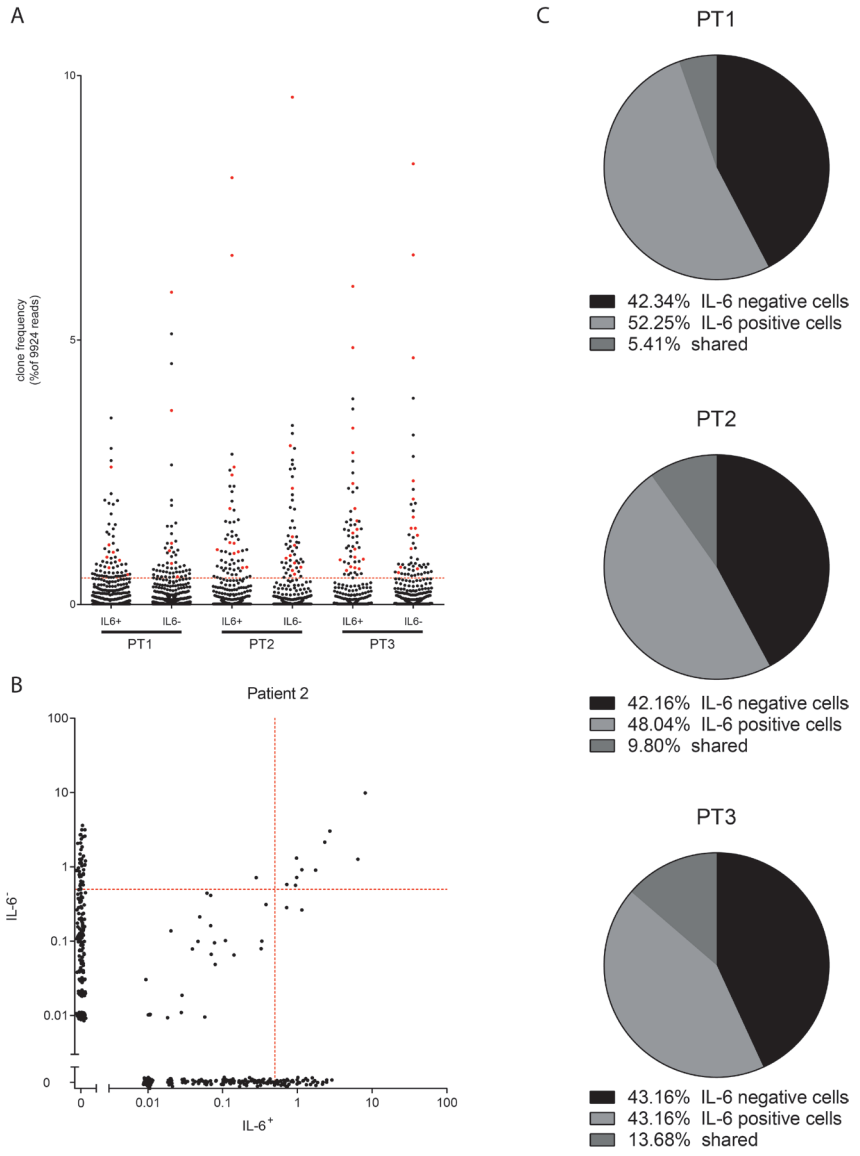

Figure 4. TCRβ repertoire in IL-6+ CD4+ T cells and IL-6- CD4+ T cells

Spontaneous IL-6+ CD4+ T cells and IL-6- CD4+ T cells were isolated from SVF and the abundance and distribution of the TCRβ repertoire was determined (N=3). Scatterplot representing the clonal repertoire of the IL-6+ CD4+ T cells and IL-6- CD4+ T cells (A). Each dot represents a single clone defined by its V-J-CDR3 combination (V= variable gene; J= joining gene; CDR3= complementary determining region 3), red dots represent clones shared between the IL-6+ CD4+ T cells and IL-6- CD4+ T cells populations within each patient. Scatter plots of overlapping clones between the IL-6+ (X-axis) and the IL-6- (Y-axis) population for one of the three patients (B). Each dot represents a single clone. Dots along the axes represent unshared clones. Grey dotted lines indicate the frequency limit of 0.5% for the definition of highly expanded clones (HECs). Pie charts showing the percentage of the HECs present only in the IL-6+ CD4+ T cells population (light grey), only in the IL-6- CD4+ T cells population (black), and shared between both populations (dark grey) (C).

Figure 5. IL-6$^+$ CD4$^+$ T cells are in close proximity with adipocytes and adipocytes can induce IL-6 in CD4+ T cells

IFP was stained for CD3 (in red) and IL-6 (in green) (A)(N=2). Adipocytes and CD4$^+$ T cells were co-cultured overnight after which intracellular staining for IL-6 was performed (B)(N=6). CD4$^+$ T cells were either unstimulated or αCD3/αCD28 stimulated. Examples of the staining is shown and a summary of all patients tested, each line represents a T-cell-adipocyte combination. Wilcoxon's singed rank test was used to compare differences between groups.

Adipocytes enhance IL-6 production in CD4$^+$ T cells

Immunofluorescence staining of IFP indicated that IL-6$^+$ CD4$^+$ T cells were usually scattered through adipocytes and did not form clusters with other immune cells within the tissue (Fig. 5A). Therefore, we hypothesized that adipocytes might stimulate IL-6 production by T cells. To address this hypothesis, we co-cultured adipocytes together with peripheral blood CD4$^+$ T cells overnight and assessed the percentage of IL-6 T positive cells. Co-culture of CD4$^+$ T cells with adipocytes led to a higher percentage of IL-6 positive cells compared to CD4$^+$ T cells cultured without adipocytes (Fig. 5B). This effect was independent of αCD3/αCD28 stimulation. These data indicate that IL-6$^+$ production by CD4$^+$ T cells can be enhanced by adipocytes.

Discussion

In this study, we investigated IL-6$^+$ CD4$^+$ T cells present in the SVF of the IFP in knee osteoarthritis patients. Phenotypic characterization indicated that they are conventional (TCRαβ) memory CD4$^+$ T cells with an activated phenotype. Their TCRβ repertoire is distinct from IL-6$^-$ CD4$^+$ T cells and they do not simultaneously secrete other cytokines. Furthermore, these IL-6$^+$ CD4$^+$ T cells are scattered through the adipose tissue and are in close proximity to adipocytes which are capable of enhancing production of IL-6 in CD4$^+$ T cells.

In order to determine whether these cells differ from the already defined T helper subsets [24], we determined cytokine production and chemokine receptor expression on IL-6-producing CD4$^+$ T cells. Both IL-6$^+$ and IL-6$^-$ CD4$^+$ T cells expressed a variety of chemokine receptors, however, no specific combinations were observed that could be used to assign IL-6$^+$ CD4$^+$ T cells to a specific T helper subset (*data not shown*). Together with the exclusively IL-6 positivity of these IL-6$^+$ CD4$^+$ T cells, the chemokine receptor expression profiles indicate that these IL-6$^+$ CD4$^+$ T do not belong to a previously defined T helper subset. However, transcription factors such as T-bet, GATA-3, FoxP3 and others have not been investigated in this study, which represents a limitation of our study.

Besides cytokine production and chemokine receptor expression, TCRβ analysis revealed that IL-6$^+$ CD4$^+$ T cells from SVF from IFP have a distinct TCRβ usages

compared to their IL-6$^-$ CD4$^+$ T cells counterparts. Although both IL-6$^+$ and IL-6$^-$ CD4$^+$ T cells underwent clonal expansion, this suggest that they recognize other epitopes / antigens. Moreover, the differential cytokine secretion could indicate that they were primed under different conditions than their IL-6$^-$ counterparts. Secretion of IL-6 *ex vivo* indicates that these cells have been recently activated. Whether these cells are activated in the adipose tissue or elsewhere and then attracted to adipose tissue is unknown. Both mechanisms have been previously described: CCL20 secreted by adipocytes can attract CD4$^+$ T cells [25], while the restricted repertoire of T cells in adipose tissue suggested their local expansion [11, 12].

As these IL-6$^+$ CD4$^+$ T cells express CD69, they could be tissue resident T cells [21, 22]. Although less is known about the phenotype of tissue resident CD4$^+$ T cells compared to their CD8$^+$ counterparts, it has been recently described that Hobit is a transcription factor upregulated in CD8$^+$ tissue resident T cells [23]. In our study, neither IL-6$^+$ CD4$^+$ T cells nor their IL-6$^-$ counterparts expressed this marker (*data not shown*). Alternatively, it is possible that CD69 indicates the recent activation of these cells.

Adipocytes have been shown to express MHCII [26], therefore it is conceivable that adipocytes initiate or sustain IL-6 production by CD4$^+$ T cells. Indeed, our data indicate that IL-6$^+$ T cells are in the proximity of adipocytes and adipocytes are capable of enhancing IL-6 secretion by CD4$^+$ T cells. However, it could also be possible that adipocytes influence the proliferation or survival of IL-6 producing T cells, this remains to be elucidated. Although the function and clinical relevance of these IL-6 producing T cells in adipose tissue is still unclear, it has been previously shown that IL-6 can affect adipocytes and enhance lipolysis [27, 28]. Our data suggest that T cells are situated in the vicinity of adipocytes and IL-6 production of T cells can be modulated by adipocytes. Therefore, it is conceivable that IL-6$^+$ T cells could in turn modulate adipocyte function. This is in line with previously published data indicating that a cross-talk between adipocytes and immune cells in adipose tissue exists [25]. It is therefore conceivable that the IL-6 secreted by IL-6$^+$ T cells could act as a feed-back mechanism on adipocytes by limiting their expansion through enhanced lipolysis.

In conclusion, we have found a novel population of CD4$^+$ T cells that secrete IL-6 directly *ex vivo* and are in an activated state. Phenotypic characterization of these cells suggested that they might recognize adipose tissue antigens and could affect adipose tissue function through interaction with adipocytes.

Acknowledgements

This work was financially supported by TI-Pharma, Foreum, The Dutch Arthritis Association, EU FP6 program Autocure, FP7 program Masterswitch, a grant from Centre for Medical Systems Biology (CMSB) within the framework of the Netherlands Genomics Initiative (NGI) and The Netherlands Organisation of Health Research and Development (ZonMw). The authors would like to acknowledge the support of the cooperating hospitals LUMC, Leiden, the Netherlands and Alrijne Ziekenhuis, Leiden, the Netherlands and orthopaedic surgeons and nurse practitioners. Furthermore, we thank Klaas van Gibsbergen and Natasja Kragten for their help with the Hobit PCR assay.

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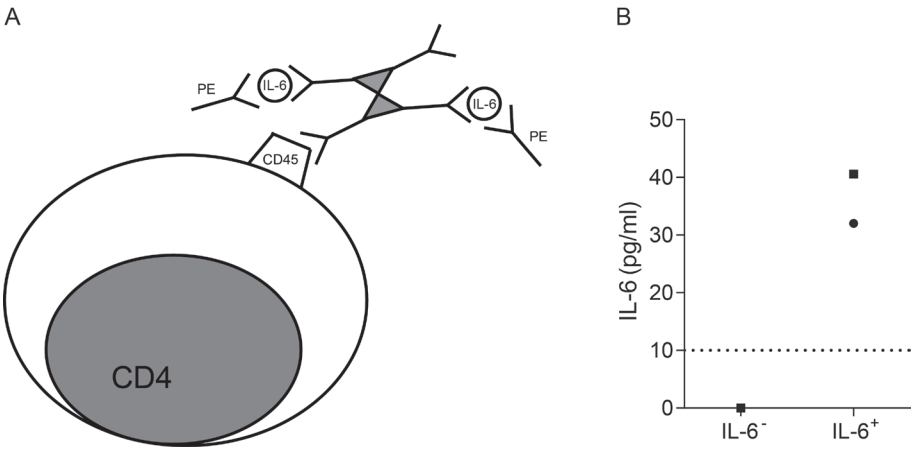
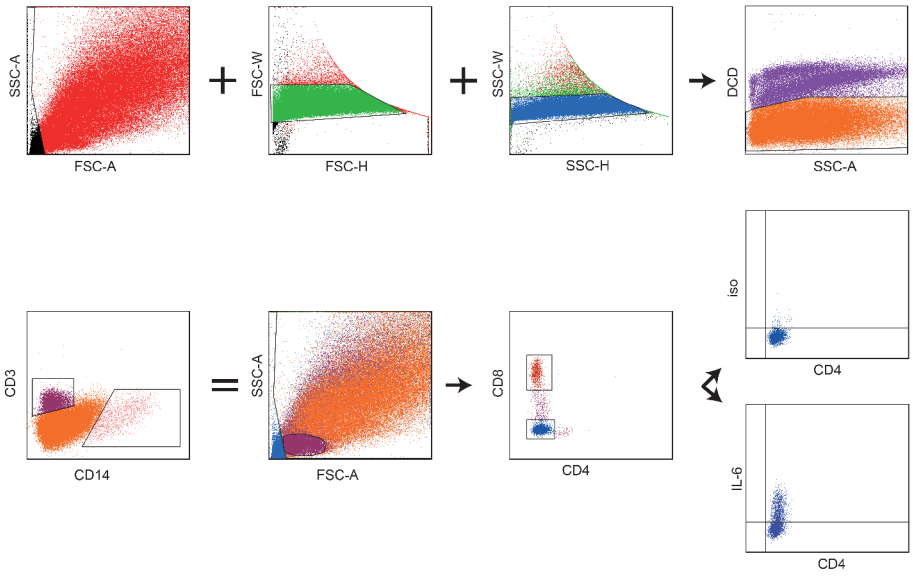
Supporting information



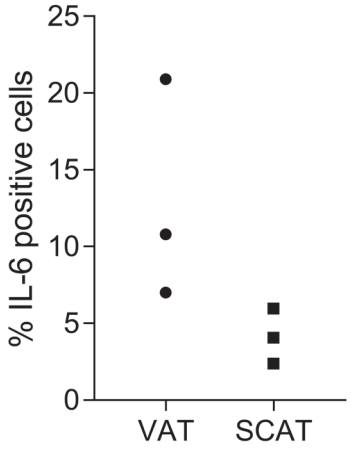
Supporting Figure 1. Capture complex

To isolate IL-6$^+$ cells from IFP a capture complex was designed (A). Isolated IL-6$^+$ CD4$^+$ T cells were able to produce IL-6 after isolation measured by ELISA (B).



Supporting Figure 2. Gating strategy

A live gate based on FSC-A/SSC-A was set on SVF cells, where after two gates were set to exclude doublets. These gates were joined and dead cells were excluded. Using a CD3 positive gate lymphocytes were more strictly gated, followed by a CD4 positive gate to analyse IL-6 positive CD4+ T cells.



Supporting Figure 3. IL-6 positive T cells in VAT and SCAT

The presence of IL-6^{+} CD4^{+} T cells was determined in VAT and SCAT by flow cytometry (see gating strategy Supporting Information Fig 2) (N=3).

Chapter 4

Lack of high BMI-related features in adipocytes and inflammatory cells in the infrapatellar fat pad (IFP)

Anja J. de Jong, Inge R. Klein-Wieringa, Stefan N. Andersen, Joanneke C. Kwekkeboom, Linda Herb-van Toorn, Badelog J.E. de Lange-Brokaar, Danny van Delft, John Garcia, Wu Wei, Huub J.L. van der Heide, Yvonne M. Bastiaansen-Jenniskens, Gerjo J.V.M. van Osch, Annemarie M. Zuurmond, Vedrana Stojanovic-Susulic, Rob G.H.H. Nelissen, René E.M. Toes, Margreet Kloppenburg, Andreea Ioan-Facsinay

Arthritis Research & Therapy. 2017; 19: 186.

Abstract

Background

Obesity is associated with the development and progression of osteoarthritis (OA). Although the infrapatellar fat pad (IFP) could be involved in this association, due to its intracapsular localization in the knee joint, there is currently little known about the effect of obesity on the IFP. Therefore, we investigated cellular and molecular body mass index (BMI)-related features in the IFP of OA patients.

Methods

Patients with knee OA (N=155, 68% women, mean age 65 years, mean (SD) BMI 29.9 kg/m2 (5.7)) were recruited: IFP volume was determined by magnetic resonance imaging in 79 patients with knee OA, while IFPs and subcutaneous adipose tissue (SCAT) were obtained from 106 patients undergoing arthroplasty. Crown-like structures (CLS) were determined using immunohistochemical analysis. Adipocyte size was determined by light microscopy and histological analysis. Stromal vascular fraction (SVF) cells were characterized by flow cytometry.

Results

IFP volume (mean (SD) 23.6 (5.4) mm^3) was associated with height, but not with BMI or other obesity-related features. Likewise, volume and size of IFP adipocytes (mean 271 pl, mean 1933 µm) was not correlated with BMI. Few CLS were observed in the IFP, with no differences between overweight/obese and lean individuals. Moreover, high BMI was not associated with higher SVF immune cell numbers in the IFP, nor with changes in their phenotype. No BMI-associated molecular differences were observed, besides an increase in TNFα expression with high BMI. Macrophages in the IFP were mostly pro-inflammatory, producing IL-6 and TNFα, but little IL-10. Interestingly however, CD206 and CD163 were associated with an anti-inflammatory phenotype, were the most abundantly expressed surface markers on macrophages (81% and 41%, respectively) and $CD163^+$ macrophages had a more activated and pro-inflammatory phenotype than their $CD163^-$ counterparts.

Conclusions

BMI-related features usually observed in SCAT and visceral adipose tissue could not be detected in IFP of OA patients, a fat depot implicated in OA pathogenesis.

Background

Obesity associates with the development and progression of osteoarthritis (OA). This association is not only observed in weight-bearing joints such as the knee, but also in hand OA[1, 2]. These observations suggest that in addition to mechanical factors, systemic factors associated with obesity play a role in the pathophysiology of OA.

Adipose tissue consists of adipocytes and the stromal vascular fraction (SVF), which contains a variety of cells, including immune cells. Obesity is usually accompanied by adipose tissue inflammation, characterized by changes in adipocytes and inflammatory cells, leading to a shift from an anti-inflammatory phenotype to a pro-inflammatory phenotype of the adipose tissue[3]. Adipocytes are generally enlarged in obesity and this growth causes expansion of the adipose tissue, but also adipocyte cell death and hypoxia[4-6]. This process is accompanied by macrophage infiltration and congregation around dead or necrotic adipocytes, resulting in formation of so-called crown like structures (CLS) where macrophages are thought to scavenge adipocyte remnants[7-9]. Furthermore, the polarization state of macrophages in the obese adipose tissue changes towards a more pro-inflammatory phenotype, thereby sustaining and promoting an inflammatory state of the adipose tissue[10-12].

The knee joint is characterized by the presence of an adipose tissue depot called the infrapatellar fat pad (IFP) also known as Hoffa's fat pad. The IFP is intracapsularly and extrasynovially located in close vicinity to the synovium, cartilage and bone. Due to its localization, it is conceivable that the IFP contributes to the pathophysiology of OA in the joint, through secretion of soluble mediators. Our group and others have shown that the IFP has a pro-inflammatory phenotype in patients with advanced knee OA [13-15] and could thereby contribute to inflammation in the joint (reviewed in[16]).

To date, little is known about the effect of obesity on the IFP. Two magnetic resonance (MR) studies suggested that, unlike subcutaneous tissue, the IFP does not enlarge with obesity[17, 18]. More recent studies addressing this question have shown contrasting results in humans [19, 20], while various body mass index (BMI)-related changes were observed in the IFP in mice [21]. Moreover, we have

previously reported differences in the IFP, such as enhanced TNFα secretion, in relation to high BMI[13]. Nonetheless, given the correlation between obesity and OA, it is important to fully comprehend the possible molecular and cellular BMI-related features in fat tissue located in close contact with the joints that are affected in OA, as is the case for the IFP.

Therefore, we extensively investigated the cellular and molecular features of adipose tissue typically associated with obesity to assess whether the IFP changes in OA patients with an increase in BMI and could thereby contribute to disease progression. We determined the association between high BMI and the volume of the IFP, the volume of adipocytes, their cytokine secretion profile, and the number and phenotype of adipose tissue immune cells.

Methods

Human subjects

In total, 155 patients with knee OA were recruited in the study. The patients were 68% women, with mean age 65 years and mean (SD) BMI 29.9 (5.7) kg/m^2. The distribution of the patients among BMI categories were: underweight (BMI below 18.5), none; normal (BMI 18.5-24.9), 28 patients; overweight (25.0-29.9), 61 patients; and obese (30.0 and above), 66 patients. Characteristics of the patients represented in Figs. 1, 2, 3 and 4 are shown in Additional file 1: Table S1. A total of 102 patients were part of the GEneration of Models, Mechanisms & Markers for STratification of OsteoArthritis patieNts (geMstoan) study, an observational study in patients with knee OA to find new biomarkers for OA progression. The patients were included between 2008 and 2014, had symptomatic knee OA, following the American College of Rheumatology (ACR) criteria[22], and attended the orthopaedic department of the Leiden University Medical Centre (LUMC) or the orthopaedic department of the Alrijne Ziekenhuis in Leiden. Written informed consent is available from all geMstoan patients. Of these 102 patients who participated in the geMstoan study, a total of 79 patients underwent magnetic resonance imaging (MRI), 30 of whom underwent joint-replacement surgery and 49 of whom underwent arthroscopy. IFPs and subcutaneous adipose tissue (SCAT) were obtained from 53 patients participating in geMstoan, who were undergoing joint replacement surgery after giving informed consent. Leftover IFP and SCAT

were obtained during arthroplasty from an additional 23 patients from the LUMC, and 30 patients from Erasmus Medical Centre (MC). SCAT was obtained from the thigh, next to the incision made for the total knee-replacement surgery. Diagnosis, age, gender and BMI were available for the latter patients. The study was approved by the local medical ethical committee. Consent was given in accordance with the guidelines of the Federation of Biomedical Scientific Societies (http://www.federa.org) after approval by the local ethical committee (MEC 2008-181 and MEC 2012-267). Not all experiments could be performed with each tissue sample as the sample size was limited.

MRI acquisition

We used a 3 T Philips Achieva MR system (Philips Healthcare, Best, The Netherlands) with an 8-channel dedicated knee coil. Sagittal proton density (PD) fast spin-echo (FSE)-driven equilibrium images were obtained with a field of view (FOV) of 150X150 mm, an acquisition matrix of 304X240, slice thickness of 3 mm., repetition time (TR) of 3000 ms and echo time (TE) of 34 ms. Subsequently, contrast enhanced (CE) MR images were obtained following injection of gadoterate meglumine (0.2 ml/kg) (Dotarem; Guerbet) into the cubital vein using a power injector (Medrad) with a rate of 2 ml/s followed by a 40-ml saline flush also at a rate of 2 ml/s. We subsequently obtained frequency selective fat-suppressed T1-weighted, FSE with TR of 655 ms, and TE of 20 ms, in both the axial and sagittal planes.

IFP volume determination by MRI

IFP volume was measured by manual segmentation of IFP boundaries on section-by-section sagittal PD FSE images, using the software program OsiriX. T1-weighted CE-images were used to distinguish and compare between IFP and non-IFP structures (Additional file 1: Figure S1). The software program OsiriX measured IFP volume by making a 3D-model of the drawn contours. Two independent observers measured IFP volume on all MRI scans. The intraclass correlation (ICC) was 0.957 for intra-observer reliability (measured in all images). The ICC was 0.909 for inter-observer reliability (measured in all images).

Measurement of adipocyte size

Adipocytes were isolated as previously described[13]. Briefly, adipose tissue was digested with collagenase type 1A (Sigma-Aldrich) for 1 h and the tissue was filtered through a 250-μm nylon mesh. Adipocytes were washed three times with

medium (DMEM/F12 supplemented with 0.5% free fatty acid (FFA) free bovine serum albumin (BSA), 15 mM Hepes, 2 mM glutamax and 100 U/ml penicillin/streptomycin) by allowing them to float to the surface, followed by removal of medium and addition of fresh medium. The diameter of 100 adipocytes was determined with light microscopy with an ocular micrometer and the mean volume was calculated, based on the formula $V = \pi d^3/6$, where d is the diameter of the adipocyte, and the mean volume of adipocytes was then calculated. Explants of the IFP and SCAT were also cryosectioned and stained with haematoxylin and eosin (H&E) and imaged using an Olympus SC30 camera (Olympus, Zoeterwoude, The Netherlands). The cross-sectional area of the imaged adipocytes was calculated using Fiji Is Just ImageJ software with the additional Adiposoft plugin. Three separate sections, with a minimum of 25 adipocytes in each section were measured per donor. The Adiposoft application was calibrated to identify cells with a diameter between 30 and 130 µm. A 0.33µm/pixel measuring scale was also used by the application to determine the cross-sectional area (size) of each adipocyte identified in the images. A manual inspection of output data was performed to confirm the consistency of the measurements.

Histological staining

Pieces of IFP were fixed in 4% formalin overnight followed by storage in EtOH, before embedding in paraffin. Sections of 4 µm at different depths in the tissue were deparaffinised in xylene (Merck, Germany) and EtOH. Endogenous antigens were peroxidised in a MetOH/H_2O_2 solution, and antigen retrieval was performed with EDTA (DAKO, USA) at 96 °C for 30 min. After cooling, two consecutive coupes of three different layers were stained for mouse anti-human CD68 (clone KP-1 1:800, DAKO, USA) or the isotype control (mIgG1 (DAKO, USA)) for 1 h at room temperature (RT). $CD68^+$ cells were then visualized using DAKO EnVision and DAB Ni kits, according to manufacturers' instructions (DAKO, USA; Vector Laboratories, Canada) before counterstaining with haematoxylin (Klinipath, The Netherlands). Slides were then embedded in pertex (Histolab Products, Sweden) and analysed using a Leica microscope and Leica software. Two to three slides per patient were used and a total of 11-37 high power field (HPF) pictures were taken depending on the size of the tissue. Two independent scorers (AJJ and SNA) quantified the amount of CLS (the ICC for inter-observer reliability was 0.985) in each HPF and an average of both scorers was generated for the amount of CLS per HPF.

SVF isolation

The stromal vascular fraction (SVF) was isolated from IFP and SCAT as previously described[13]. SVF cells were counted by light microscopy, surface staining was performed and the remaining cells were plated overnight in a 6-well plate at a density of maximal $5x10^6$ cells/well in medium (DMEM/F12 supplemented with 0.5% FFA free BSA, 15 mM Hepes, 2 mM glutamax and 100 U/ml penicillin/streptomycin) supplemented with 50 IU/ml IL-2 (Peprotech, USA) and 3 µg/ml Brefeldin A (Sigma Aldrich, Germany). Thereafter, cells were harvested using a cell scraper and surface and intracellular staining was performed. The SVF of the IFP from two patients was used to isolate $CD14^+$ cells using magnetic-labelled anti-CD14 beads (Miltenyi Biotec) according to the manufacturer's instructions. $CD14^+$ cells were plated in a 96-well plate and supernatant was harvested after 2 days of culture.

Flow cytometric analysis

Approximately 100,000 freshly isolated SVF cells were stained for 30 min at 4°C with surface antibody (Ab) solutions containing mixes of the following Abs: PE-conjugated CD1d, CD3, DC-SIGN, CD206 and CD16; FITC-conjugated HLA DR, CD11c and CD45; APC-conjugated CD8 and CD163; PE-Cy-7-conjugated CD14, PB-conjugated CD4 and Pe-Cy5-conjugated CD206 (all Abs from BD biosciences, except Ab to CD163 and CD206 which were from Biolegend). When specified, approximately 400,000 SVF cells harvested after overnight incubation with brefeldin A were stained for 30 min at 4°C for surface markers and intracellular cytokines using the BD intracellular cytokine fixation/permeabilization solution kit (BD Biosciences) according to the manufacturer's instructions. The following Abs were used: PE-Cy7-conjugated CD14; APC-conjugated CD163; PE-conjugated IL-10, TNFα and IL-6 (all Abs from BD biosciences, except Ab to IL-6 which was from eBioscience). Exclusion of dead cells was performed in all experiments using the Dead Cell discrimination kit (Miltenyi Biotec, Germany). Cells were fixed with 1% paraformaldehyde and analysed with a LSR II flow cytometer using Diva 6 software (BD biosciences).

Generation of fat-conditioned and adipocyte-conditioned medium

Fat-conditioned and adipocyte-conditioned medium (FCM and ACM, respectively) were obtained as previously described[13]. Briefly, FCM was obtained by culturing 100 mg/ml of small pieces of IFP or SCAT in 6-well plates in medium (DMEM/F12

supplemented with 0.5% FFA free BSA, 15 mM Hepes, 2 mM glutamax and 100 U/ml penicillin/streptomycin). Medium was refreshed after 2 h and supernatant was collected after 24 h. For ACM, 100 µl/ml of adipocytes were cultured in 6-well plates in medium for 24 h. Supernatants were collected and stored at -80°C until use.

Milliplex MAP analysis

Cytokines were measured in supernatants of $CD14^+$ cells isolated from SVF and in FCM and ACM using the Milliplex Human Cytokine / Chemokine kit (Millipore), the Bio-Plex array reader and Bio-Plex software in accordance with the manufacturer's instructions.

Statistical analysis

Associations between IFP volume measured by MRI and patient characteristics were determined by linear regression analysis after establishing that the assumptions underlying linear regression analysis were met. Both univariate and multivariate linear regression analyses were performed to determine which patient characteristics were associated with IFP volume. The unpaired or paired Student's *t* test (for normally distributed variables) or the Mann-Whitney test or Wilcoxon's matched-pairs signed rank test (for non-Gaussian distributions) was used to compare differences between groups. Correlation was tested by calculating the Pearson correlation coefficient (for normally distributed variables) or by using Spearman's ranked correlation test (for non-Gaussian distributions), as specified. For Pearson correlation, the trend line with 95% confidence interval was depicted. Correction for multiple testing was performed using Bonferroni's correction. A P value ≤ 0.05 was considered statistically significant.

Results

IFP volume is associated with gender and height but not with BMI

To obtain insight into whether the volume of the IFP is associated with obesity-related features or other patient characteristics, we made use of a clinically well-characterized population of patients participating in the geMstoan study (N=79). Patient characteristics are shown in Table 1. The mean (SD) IFP volume was 23.6 (5.4) mm^3. Univariate linear regression analysis indicated that BMI is not associated with

Table 1. Characteristics of patients (N=79) in whom infrapatellar fat pad volume was measured

Parameters	Value
Age, year, mean (SD)	62.1 (7.5)
Female, N (%)	54 (68.4)
BMI, kg/m^2, mean (SD)	29.4 (5.2)
Height, cm, mean (SD)	170 (9.0)
Weight, kg (SD)	85 (16.4)
Kellgren and Lawrence, N (%)[a]	
Grade 1	11 (14.1)
Grade 2	21 (26.9)
Grade 3	25 (32.1)
Grade 4	21 (26.9)

BMI body mass index

[a]N = 78 patients included in the analysis of Kellgren and Lawrence grade

Table 2. Association of patient characteristics with IFP size on MRI.

Variable	Univariate		Multivariate		Covariate height		Covariate gender	
	B (95% CI)	P value	*B* (95% CI)	P value	*B* (95% CI)	P value	*B* (95% CI)	P value
Age	-0.07 (-0.2; 0.1)	0.417			0.06 (-0.1; 0.2)	0.369	-0.08 (-0.2; 0.06)	0.258
Gender	6.93 (4.8; 9.0)	<0.001	4.24 (-0.8; 9.3)	0.098	3.33 (0.9; 5.7)	0.006		
BMI	-0.14 (-0.4; 0.1)	0.258			-0.05 (-0.2; 0.1)	0.560	-0.06 (-0.2; 0.1)	0.549
Waist-to-hip ratio	19.8 (5.9; 33.7)	0.006	-6.32 (-20.4; 7.7)	0.373	4.72 (-6.7; 16.1)	0.412	-9.5 (-24.7; 5.9)	0.222
Waist circumference	0.04 (-0.06; 0.1)	0.391			0.003 (-0.1; 0.1)	0.932	-0.02 (-0.1; 0.06)	0.682
Fat percentage	-0.29 (-0.4; -0.2)	<0.001	0.01 (-0.3; 0.3)	0.943	-0.13 (-0.3; -0.1)	0.024	0.02 (-0.2; 0.2)	0.824
Height	0.41 (0.3; 0.5)	<0.001	0.31 (0.2; 0.4)	<0.001			0.29 (0.2; 0.4)	<0.001
Weight	0.08 (0.01; 0.2)	0.023	-0.02 (-0.1; 0.1)	0.773	-0.02 (-0.1; 0.1)	0.475	0.04 (-0.02; 0.1)	0.207
KL-score	0.28 (-0.9; 1.5)	0.648			-0.29 (-1.2; 0.6)	0.523	-0.5 (-1.5; 0.5)	0.291

IFP infrapatellar fat pad, MRI magnetic resonance imaging, BMI body mass index, KL Kellgren and Lawrence

IFP volume (Table 2). Other obesity-related features such as waist-to-hip ratio (R^2 = 0.09) and fat percentage (R^2 = 0.19) were associated with IFP volume, while waist circumference was not. Moreover, IFP volume was associated with male gender (R^2 = 0.37), height (R^2 = 0.47) and weight (R^2 = 0.07), but not with age, nor with radiographic damage (Kellgren-Lawrence (KL) scores) (Table 2). Multivariate linear regression analysis in which all factors that were significantly associated with IFP volume were included indicated that only height remained independently associated with IFP volume (Table 2). The association with gender was still present, but was no longer significant. To understand how the factors included in the multivariate analysis vary with height and gender, we included height or gender as covariates in the linear regression analysis. These analyses indicate that gender is a confounder for most of the observed associations and that only height remained significantly associated with IFP volume upon correction for gender (Table 2). Stratification based on gender indicated that height is associated with IFP volume in women but not in men (data not shown). Of all patients, 17.9% had cardiovascular comorbidities, indicating the presence of metabolic complications. There was no association between IFP volume and BMI upon stratification for the presence of these comorbidities (data not shown).

Adipocyte volume, and the number of CLS and SVF cells in the IFP did not correlate with BMI

Next, we investigated adipocytes and SVF cells in the IFP. The average volume of adipocytes was 271 pl (Fig. 1a), and the average size was 1933 µm (Fig. 1b). Neither the volume nor the size of adipocytes correlated with BMI. Furthermore, the distribution of adipocyte sizes was not different between lean and obese individuals. Macrophage staining indicated that CLS (Fig. 1c) were present in small numbers in the IFP (mean = 0.18 CLS/HPF) and did not correlate with BMI (Fig. 1d). Likewise, the number of SVF cells in the IFP did not correlate with BMI (Fig. 1e). In contrast, we did observe BMI-related differences in adipocyte volume (Additional file 1: Figure S2a) and size (Additional file 1: Figure S2b-c), and the number of SVF cells (Additional file 1: Figure. S2d) in SCAT, a control adipose tissue. Direct comparison between the IFP and SCAT revealed that IFP adipocytes are smaller compared to SCAT adipocytes (Additional file 1: Figure S2e-f), but IFP has more SVF cells compared to SCAT (Additional file 1: Figure S2g).

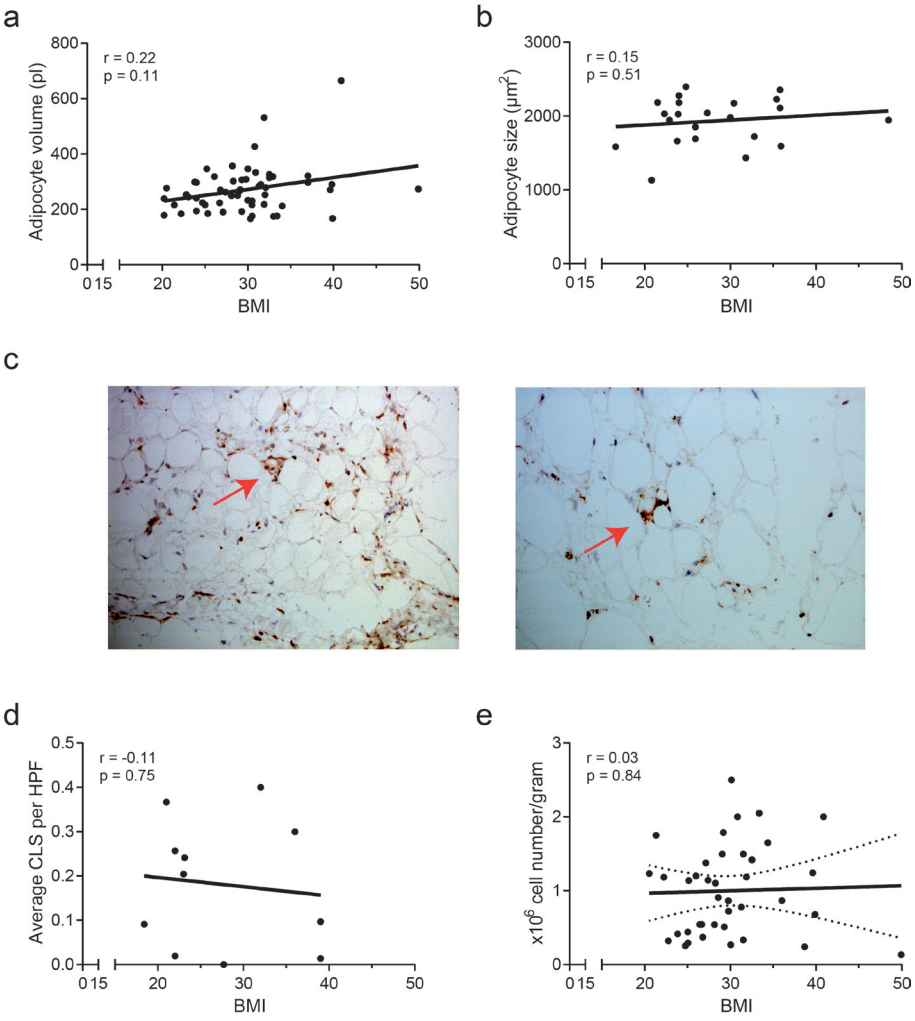


Figure 1. Adipocyte volume, and the number of crown-like structures (CLS) and stromal vascular fraction (SVF) cells in infrapatellar fat pad (IFP) did not correlate with body mass index (BMI)

Adipocytes were isolated from the IFP from patients with osteoarthritis (OA), who were undergoing total knee-replacement surgery, adipocyte volume was determined and the correlation with BMI was assessed (N=56) (a). Adipocyte size was determined upon haematoxylin and eosin (H&E) staining and the correlation with BMI was assessed (N=18) (b). IFP tissue was stained for CD68 and the number of CLS was quantified. A representative picture of the staining at x20 (left) and x40 (right) magnification (c) and the summary of all results is shown (N=11) (d). The number of SVF cells per gram adipose tissue was determined and the correlation with BMI was assessed (N=39) (e). Correlation was tested using Spearman's rank correlation (a, b) or Pearson correlation coefficient (e). Each dot represents one patient. HPF high power field

Cell infiltrate in adipose tissue

Next, we investigated the type of immune cells in the IFP. Therefore, we determined the percentage of macrophages and T cells in the SVF from the IFP, since these cell types are the most abundant immune cells in adipose tissue. Our data indicated that there was no correlation between $CD3^+$ T cells (Fig. 2a), $CD4^+$ T cells (Fig. 2b), $CD8^+$ T cells (Fig. 2c) or macrophages (Fig. 2d) and BMI, suggesting no BMI-related differences in the types of immune cells in the IFP.

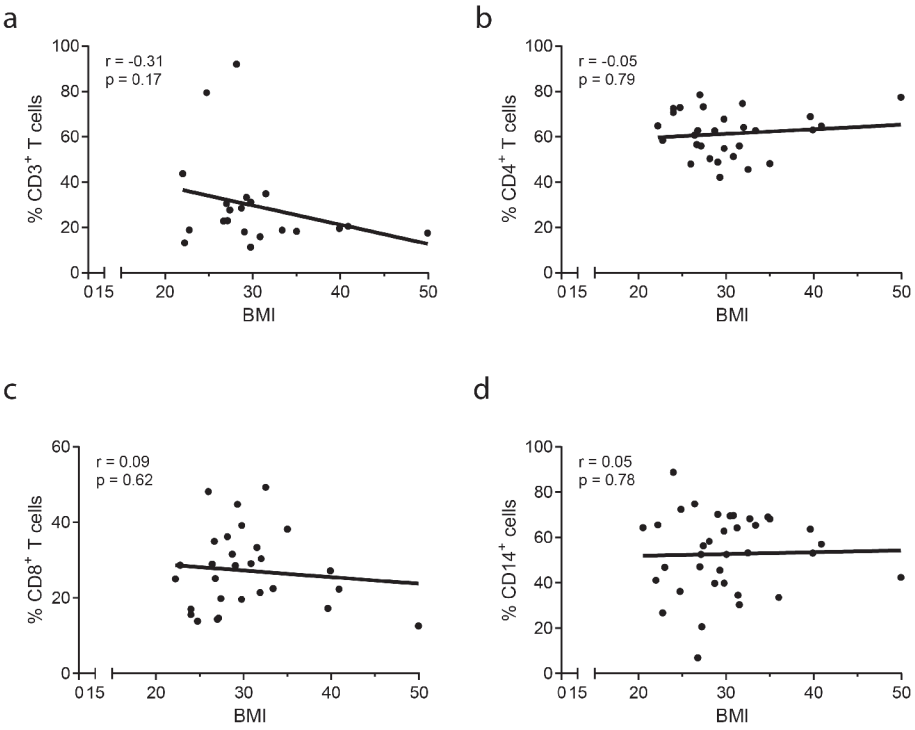


Figure 2. Cell infiltrate in adipose tissue.

The stromal vascular fraction (SVF) of the infrapatellar fat pad (IFP) was isolated and T cells and macrophages were characterized by flow cytometry (gating strategies were performed as described in Additional file 1: Figure S3). Percentages of $CD3^+$ T cells (N=21) (a), $CD4^+$ T cells (N=29) (b), $CD8^+$ T cells (N=29) (c) and macrophages (N=37) (d) and their correlation with body mass index (BMI) was determined using Spearman's rank correlation. A P value < 0.05 was considered significant. Each dot represents one patient.

Previously, we found that TNFα secretion by FCM but not ACM correlated with BMI[13]. Using a different cohort of patients, we now showed that FCM derived from the IFP of patients with a high BMI (BMI>30) had higher levels of TNFα compared to FCM derived from the IFP of patients with a low BMI (BMI $\leq$ 25), confirming

our previous data (Additional file 1: Table S2). In line with our previous findings, levels of TNFα in ACM did not differ between these high and low BMI groups (Additional file 1: Table S2). We further expanded our analyses to a broad range of cytokines. These studies indicated that several cytokines, such as interferon (IFN)α, IL-2, IL-3, IL-4, IL-5, IL-9, IL-13, IL-15, IL-17, platelet-derived growth factor (PDGF)-ABBB, sCD40L, transforming growth factor (TGF)α and TNFβ were undetectable in most FCM and ACM samples. Moreover, FCM had higher levels of granulocyte macrophage-colony stimulating factor (GM-CSF), IL-8 and monocyte chemotactic protein 3 (MCP3) in the group with BMI > 30 compared to the group with BMI $\leq$ 25 (Additional file 1: Table S3). Likewise, although ACM contained very low levels of TGFα, these seemed to be lower in the group with BMI > 30 compared to the group with BMI $\leq$ 25 (Additional file 1: Table S4). These differences, however, were no longer significant after correction for multiple testing.

Phenotypic characterization of macrophages in the IFP

Because macrophages are abundant in the adipose tissue and their phenotype has been shown to change with obesity, we investigated the phenotype of macrophages in the IFP. Virtually all macrophages expressed human leukocyte antigen (HLA)-DR, while CD1c, CD1d, CD11c and DC-SIGN were present on a low percentage of cells (Fig. 3a). Furthermore, among the surface molecules associated with a pro-inflammatory macrophage phenotype, CD16 was the most abundantly expressed, being present on approx. 20% of macrophages, while 80% of the macrophages expressed the mannose receptor CD206, a surface molecule associated with an anti-inflammatory phenotype. Furthermore, less than half of the macrophages expressed the scavenger receptor CD163. None of the surface markers correlated with BMI.

Intracellular cytokine staining revealed that macrophages from the IFP produced mainly IL-6 and TNFα, but little IL-10 directly ex vivo (Fig. 3b), indicating a predominantly pro-inflammatory phenotype. No correlation with BMI was observed for any of these cytokines (data not shown). The intracellular cytokine findings were confirmed in culture supernatants of isolated macrophages from two patients (Fig. 3d). Furthermore, these analyses revealed that $CD14^+$ cells from the IFP were also capable of secreting eotaxin, fibroblast growth factor-2 (FGF-2), Flt3-ligand, fractalkine, growth-regulated oncogene (GRO), granulocyte colony stimulating factor (G-CSF), GM-CSF, IFNα2, IFNγ, IL-1α, IL-1β, IL1-RA, IL-7, IL-8,

IL-12p40, IP-10, MCP-1, MCP-3, MDC, macrophage inflammatory protein (MIP)1α, MIP1β, RANTES, sCD40L, sIL-2Rα, TNFβ and vascular endothelial growth factor (VEGF) (data not shown).

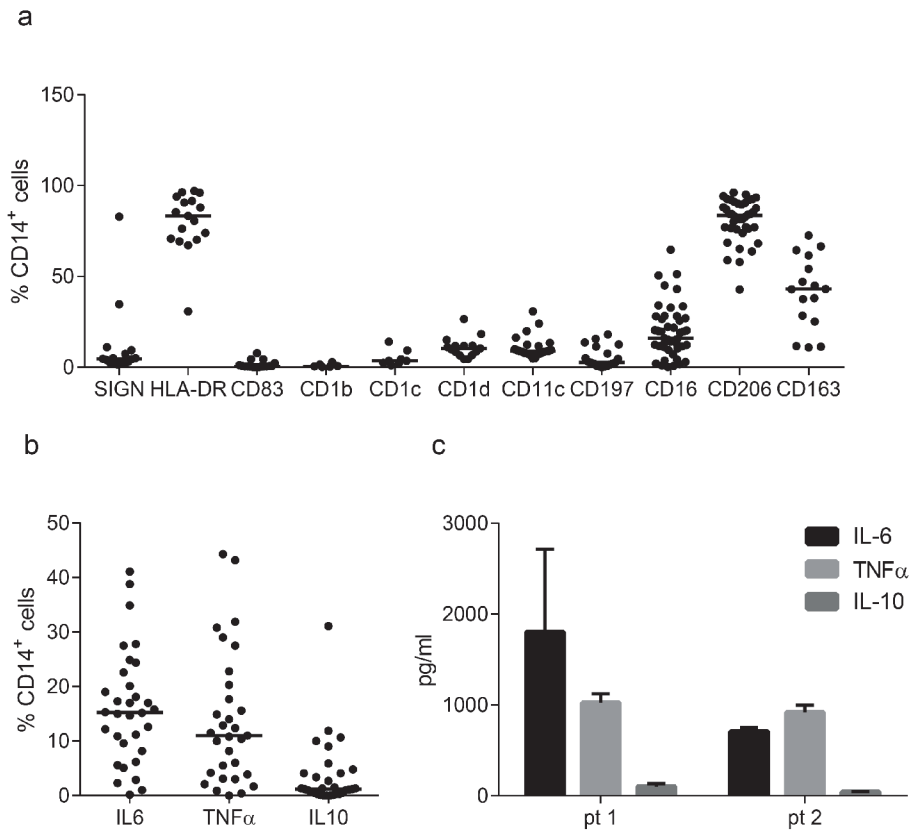


Figure 3. Phenotypic characterization of macrophages in the infrapatellar fat pad (IFP).

The stromal vascular fraction (SVF) was isolated from the IFP and macrophages were characterized by flow cytometry. Percentages of $CD14^+$ macrophages positive for each specified marker are depicted (N=6-45) (a). *Ex vivo* intracellular cytokine production by $CD14^+$ macrophages in the IFP is depicted (N=13) (b). Cytokines were measured in supernatant of unstimulated $CD14^+$ from the SVF (N=2) (c). Median (a and b) or mean (c) is indicated; each dot represents one patient. Pt patient

$CD163^+$ macrophages in the IFP are pro-inflammatory

Surface marker expression indicated an anti-inflammatory phenotype of IFP macrophages, while the cytokine production profile suggests that pro-inflammatory macrophages are predominant. However, a restricted percentage of macrophages secrete IL-10, indicating them as anti-inflammatory.

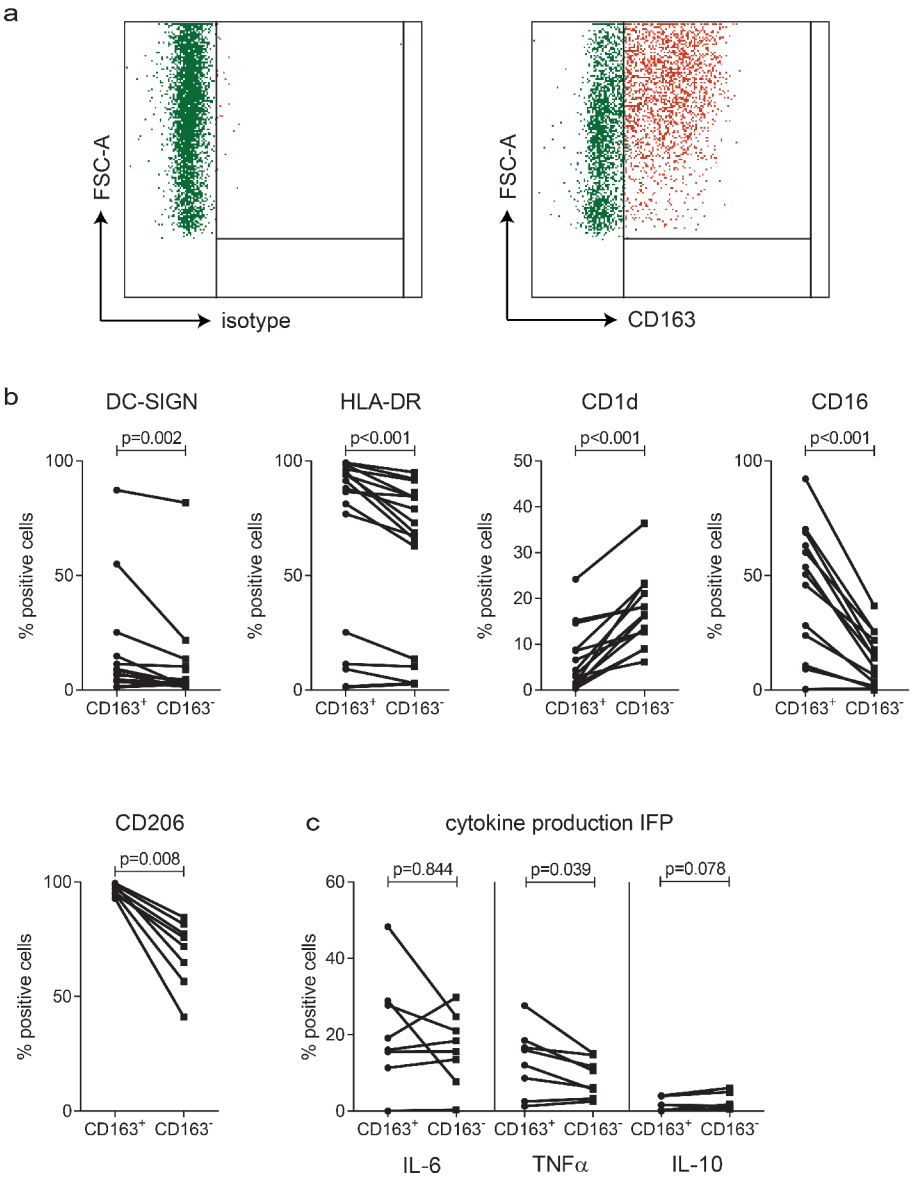


Figure 4. CD163$^+$ macrophages in the infrapatellar fat pad (IFP) are pro-inflammatory.

Total macrophage population (left), and CD163$^+$ (red) and CD163$^-$ CD14$^+$ (green) macrophages (right) are depicted against the forward scatter-area (FSC-a) (a). Differences between CD163$^+$ and CD163$^-$ CD14$^+$ macrophages in surface marker expression (N=7-12) (b) and *ex vivo* intracellular cytokine production (N=7-8) (c). Each line indicates one patient sample. A P value < 0.05, determined by the Wilcoxon signed rank test (b) or paired Student's *t* test (c) was considered significant.

The scavenger receptor CD163 has been associated with the resolution of inflammation and tissue regeneration[23-25], but also with inflammation[26-31]. This indicates that the phenotype of $CD163^+$ macrophages is unclear. Therefore, we set out to investigate whether the $CD163^+$ macrophages comprise the anti-inflammatory macrophage population and compared them to their $CD163^-$ counterparts. Flow cytometric characterization indicated that $CD163^+$ macrophages are bigger than their $CD163^-$ counterparts (Fig. 4a). Furthermore, we found that the percentages of cells positive for CD16, CD206, DC-SIGN and HLA-DR were higher, while the percentage of cells positive for CD1d was lower in $CD163^+$ macrophages compared to their $CD163^-$ counterparts (Fig. 4b). Additionally, $CD163^+$ macrophages were more often positive for IL-6 and TNFα than for IL-10 and seemed to be more positive for TNFα than $CD163^-$ macrophages (Fig. 4c). These data indicate that although CD163 is regarded as an anti-inflammatory macrophage marker, $CD163^+$ macrophages from the IFP display a pro-inflammatory phenotype.

Discussion

In this study, we investigated cellular and molecular BMI-related features in the IFPs from OA patients. Our data indicate no substantial differences in the volume of the IFP, adipocyte size or in immune cell numbers and types in relation according to BMI. These data are important as they indicate differential regulation of BMI-related features in different fat tissues. Molecularly, we confirmed the previously reported increase in TNFα secretion in the high-BMI group, but did not find additional significant differences in other cytokines tested. Extensive characterization of macrophages present in the IFP indicated that most cells bear surface markers associated with an anti-inflammatory phenotype (CD206 and CD163), while they secrete predominantly pro-inflammatory cytokines (TNFα and IL-6). Comparisons between $CD163^+$ and $CD163^-$ macrophages in the IFP indicated that $CD163^+$ are pro-inflammatory, larger and more activated than $CD163^-$ macrophages.

A high BMI does not necessarily reflect obesity, however, in our geMstoan population, waist circumference and fat percentage both correlated well with BMI (data not shown), indicating that high BMI does reflect obesity in this population of patients. Furthermore, the presence of cardiovascular comorbidities in the obese is higher as compared to lean individuals indicating a high BMI is accompanied

by metabolic complications in our population. Although we cannot formally assess this for the other patients included in this study, they are comparable to the geMstoan patients (age above 50 years, all diagnosed with OA), suggesting that a high BMI in these patients also reflects obesity rather than high muscle content.

Our findings that IFP volume determined by MRI is not associated with BMI is in line with previous published data[17, 18]. Other obesity-related factors such as waist-to-hip ratio, fat percentage, weight and the presence of cardiovascular comorbidities were not associated with IFP volume, were no longer significant in the multivariate analysis or when corrected only for gender (data not shown), in line with known gender differences in these obesity-related features[32]. Together, these data support our conclusion that IFP volume is not associated with obesity.

In contrast, we found that height and gender are associated with IFP volume, in agreement with previous studies[18, 33-35]. Multivariate analysis indicated, however, that only height is independently associated with IFP volume, while the association with gender is partially explained by height, leading to a strongly diminished effect size when compared to univariate analysis. Moreover, the association was no longer significant, possibly reflecting a lack of sufficient power. Furthermore, stratification based on gender indicated that height is only associated with IFP volume in women. Although the mechanisms underlying the association between gender and IFP volume remain unknown, it is conceivable that sex hormones could play a role in this as they have been previously described to direct fat storage to different anatomical locations[36].

In contrast to a previous study[17], we did not find an association between IFP volume and age, possibly due to differences in the investigated OA populations, and in the analyses performed.

Previously published data indicate that obesity is accompanied by enlargement of adipocytes[5, 6] and inflammatory cell infiltration in conventional adipose tissues[12, 37, 38]. However, these BMI-related features do not seem to be present in the IFP, although we observed these features in SCAT. Moreover, we did not find an association between any type of inflammatory cells and BMI. However, due to the small sample size available for $CD3^+$ T cells the power of this analysis is limited and the lack of association should be interpreted with care. Although

a recent study indicated that obesity-related changes such as in adipocyte size and cellular infiltrate occur in the IFP [19], our observations are in line with other studies indicating that the IFP is not enlarged in obesity[17] and does not have obesity-related features [20], supporting earlier findings that the IFP is different from SCAT[13, 20].

The lack of enlargement in the IFP with obesity could be due to its localization inside the knee capsule, which could strongly limit its growth. The question remains, however, as to how the IFP deals with the metabolic stress (i.e. nutrient overload) that accompanies obesity. One could speculate that IFP adipocytes are less metabolically active and are rather inefficient in removing free fatty acids from the circulation and storing them in lipid droplets. Therefore, IFP adipocytes, unlike adipocytes in other adipose tissues, are not enlarged with obesity. Moreover, this could explain the very small number of CLS and the lack of BMI-related increase in inflammatory cells in the IFP, as IFP adipocytes might undergo less apoptosis/cell death, which are the primary events in adipose tissue inflammation. However, this remains to be investigated. Moreover, our data are relevant as they indicate that understanding of the biology of fat tissue located in close contact with joints affected by OA is important to better comprehend the association between obesity and OA, but caution should be taken in translating findings observed in fat tissues to the IFP, a fat tissue implicated in OA pathogenesis.

Our data indicated that macrophages from the IFP have a dual phenotype, with surface markers associated with an anti-inflammatory phenotype and more pro-inflammatory cytokine profiles. Almost all IFP macrophages expressed CD206, a marker generally associated with M2-like macrophages and tissue-resident macrophages. The phenotype and function of $CD163^+$ macrophages is unclear, as $CD163^+$ macrophages have been associated with wound healing and the resolution of inflammation[23-25], but have also been implicated in inflammation, such as in spondyloarthritis[26-29], psoriasis[29, 30] and inflammatory bowel disease[27, 31]. Our data are in line with previous publications showing that macrophages from SC and omental adipose tissue express both CD163 and CD206 and display a pro-inflammatory cytokine profile[39].

Studies in mice suggest that obese adipose tissue becomes infiltrated with macrophages, which form CLS, take up lipids and acquire a more pro-inflammatory

phenotype[40, 41]. In the IFP, $CD163^+$ macrophages appeared to have a more activated state compared to $CD163^-$ macrophages and were larger than $CD163^-$ macrophages. This could indicate that $CD163^+$ macrophages are scavenging up dead adipocytes, thereby acquiring more lipids. Supporting our hypothesis, a recent study in mice showed that phagocytosis by macrophages leads to upregulation of both CD206 and CD163[42].

We did not observe obesity-related differences in TNFα production by macrophages (*data not shown*). This is surprising in view of the finding that the IFP in obese individuals secretes more TNFα[13] and that macrophages are the most abundant cell type in the SVF. Differences in the regulation of secretion vs production could explain the discrepancy. Alternatively, it is possible that other cells, albeit less numerous, would secrete more TNFα than macrophages[43] and this secretion could be modulated by obesity. Finally, it is possible that dissociation of cells from the tissue could influence their cytokine production.

Conclusions

In conclusion, no cellular BMI-related features previously reported in other adipose tissues were found in the IFPs of OA patients with regard to the IFP volume, adipocyte volume and size, CLS, immune cell numbers or type. These data confirm our previous findings that the IFP is different to SCAT and raise the intriguing possibility that the IFP might be a different type of fat than the conventional SCAT and visceral adipose tissues.

Acknowledgements

The authors would like to acknowledge the support of the cooperating hospitals LUMC, Leiden, The Netherlands, Alrijne Ziekenhuis, Leiden, The Netherlands, Erasmus MC, Rotterdam, The Netherlands, and orthopaedic surgeons and nurse practitioners.

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Supporting information

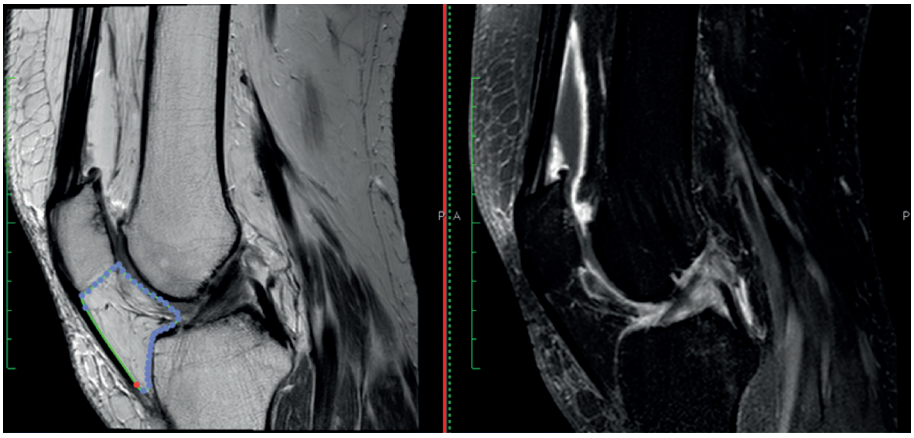


Figure S1. IFP volume determination

IFP volume was measured on section-by-section sagittal PD FSE images (left), T1-weighted CE-images were used to distinguish and compare between IFP and non-IFP structures (right).

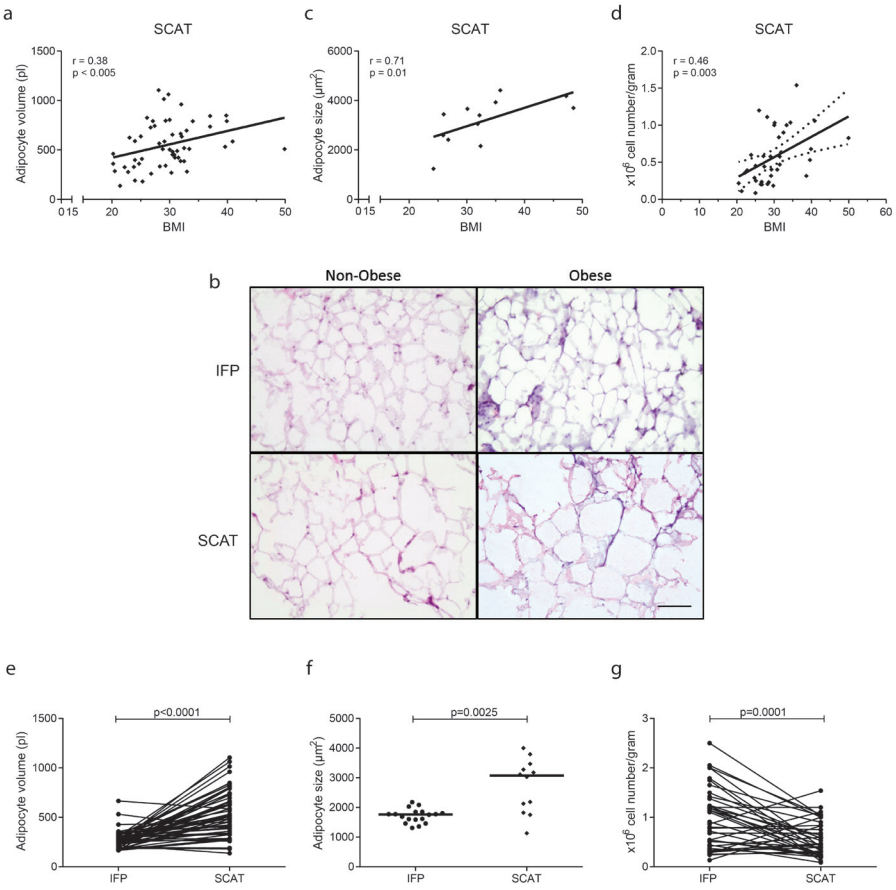


Figure S2. Adipocyte volume and size and number of SVF cells in SCAT correlate with BMI

Adipocytes were isolated from control SCAT of OA patients and adipocyte volume was determined and correlated to BMI, N=56 (a). SCAT tissue was H&E stained and adipocyte size was determined. Representative images of H&E stained cryosections of adipose tissue from both the IFP and SCAT tissue of non-obese and obese donors (b). Scale bar= 100µm. The size of adipocytes from SCAT were correlated with BMI, N=12 (c). Number of SVF cells per gram SCAT was determined and correlated with BMI, N=38 (d). A comparison between IFP and SCAT tissue for adipocyte volume (e), size (f) and number of SVF cells per gram (g) is given. Correlations were determined using Spearman's rank correlation (a, c) or Pearson correlation coefficient (d). P-values for differences between groups (as indicated) were tested by Wilcoxon signed rank test (e), unpaired student's t-test (f) or paired student's t-test (g). Medians are depicted and each dot represents one patient.

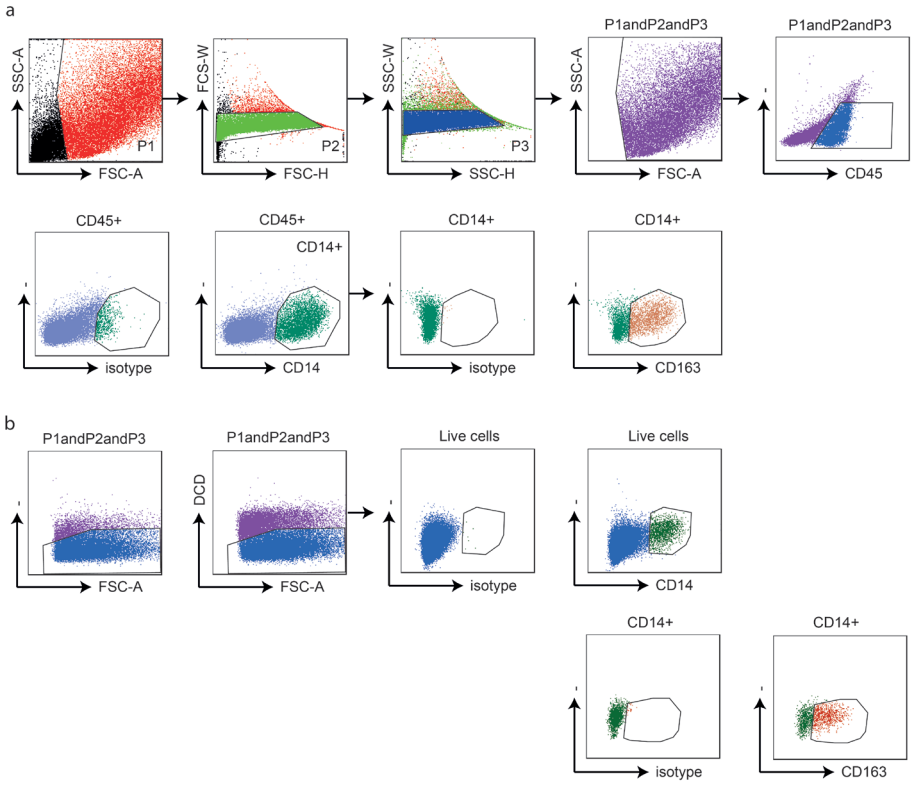


Figure S3. Gating strategy

Gating strategy used for phenotypical stainings (a), a live gate based on FSC-A/SSC-A was set on the stromal vascular cell fraction, followed by two gates to exclude double cells. The intersection of this gate was used for gating hematopoietic cells ($CD45^+$), after which $CD3^+$ T cells or $CD14^+$ macrophages were gated. Within the $CD14^+$ population phenotypic markers were further analyzed. Alternatively, $CD14^+CD163^+$ or $CD14^+CD163^-$ cells were selected and phenotypic markers were further analyzed. Within the $CD3^+$ T cell population the expression of CD4 and CD8 were analyzed. Gating strategy used for intracellular stainings (b), a live gate based on FSC-A/SSC-A was set, followed by two gates to exclude double cells (as depicted in a). Within the intersection of these gates, cells positive for dead cell discriminator were excluded. $CD14^+$ cells in this live gate were further analyzed for cytokine production or $CD14^+CD163^+$ or $CD14^+CD163^-$ were gated to analyze cytokine production.

Table S1. Patient characteristics for all figures.

Patient characteristics	Fig 1 and Fig S2 (N=82)	Fig 2 (N=44)	Fig 3 (N=62)	Fig 4 (N=20)
Age, year, mean (SD)	65 (9.8)	67 (9.3)	67 (10.1)	70 (7.1)
Female, N (%)	66	64	63	70
BMI, kg/m^2, mean (SD)	30.2 (6.4)	29.7 (5.6)	29.8 (5.3)	29.8 (4.0)

Table S2. Levels of TNFα in FCM and ACM.

Differences between BMI ≤ 25 group and BMI > 30 group determined with Mann-Whitney test are indicated with p-value.

	TNFα						
	BMI ≤ 25 (N=8-11)		BMI >25 and ≤ 30 (N=7-8)		BMI > 30 (N=9-11)		p-value
	median	(min – max)	median	(min – max)	median	(min – max)	
FCM	0.0	(0.0 – 15.8)	6.3	(0.0 – 2173. 9)	8.6	(0.0 – 165.1)	0.04
ACM	5.0	(1.4 – 40.1)	16.8	(3.6 – 35.9)	5.8	(1.3 – 168.3)	0.51

Table S3. Levels of cytokines/chemokines in FCM.

Differences between BMI ≤ 25 group and BMI > 30 group determined with Mann-Whitney test are indicated with p-value. The Bonferroni adjusted p-value indicating significance is < 0.001.

	FCM						
Cytokines (pg/ml)	BMI ≤ 25 (N=8)		BMI >25 and ≤ 30 (N=7)		BMI > 30 (N=9)		p-value
	median	(min – max)	median	(min – max)	median	(min – max)	
EGF	0.0	(0.0 – 5.7)	3.4	(0.0 – 10.3)	5.2	(0.0 – 10.3)	0.09
Eotaxin	26.9	(14.9 – 70.2)	51.3	(26.7 – 80.5)	58.2	(0.0 – 73.8)	0.07
FGF-2	184.9	(64.7 – 1313.9)	182.4	(55.8 – 505.4)	283.1	(106.4–1150.2)	0.54
FLT3L	12.5	(0.0 – 18.7)	15.9	(4.8 – 40.8)	17.4	(0.0 – 28.7)	0.19
Fractalkine	170.0	(58.5 – 472.1)	307.3	(147.1 – 873.6)	384.7	(2.4 – 432.3)	0.09
G-CSF	56.2	(0.0 – 467.4)	91.0	(0.0 – 523.8)	301.5	(0.0 – 3890.7)	0.15
GM-CSF	30.5	(17.8 – 50.0)	38.3	(27.1 – 407.0)	51.5	(0.0 – 83.4)	0.03
GRO	730.3	(100.3 – 1157.9)	1877.0	(656.3 – 8925.5)	2515.0	(0.0 – 9158.9)	0.23
IFNα	0.0	(0.0 – 1.3)	0.0	(0.0 – 19.1)	0.0	(0.0 – 6.5)	0.60
IFNγ	4.8	(0.0 – 22.8)	6.2	(4.0 – 47.5)	12.7	(0.0 – 23.8)	0.19
IL1α	0.0	(0.0 – 5.7)	9.9	(0.0 – 171.8)	2.8	(0.0 – 18.3)	0.26
IL1β	0.0	(0.0 – 7.9)	4.7	(0.0 – 1318.5)	6.2	(0.0 – 33.1)	0.07
IL1RA	19.3	(0.0 – 98.7)	35.0	(11.4 – 1705.4)	23.8	(0.0 – 566.4)	0.59
IL2	0.0	(0.0 – 0.0)	0.0	(0.0 – 5.2)	0.0	(0.0 – 0.0)	0.99
IL3	0.0	(0.0 – 0.0)	0.0	(0.0 – 0.0)	0.0	(0.0 – 0.0)	0.99
IL4	0.0	(0.0 – 0.0)	0.0	(0.0 – 8.9)	0.0	(0.0 – 11.1)	0.21
IL5	0.0	(0.0 – 0.6)	0.0	(0.0 – 4.3)	0.0	(0.0 – 8.0)	0.59
IL6	754.8	(189.3 – 2958.1)	981.4	(634.5 – 2935.8)	1306.2	(28.4 – 7628.7)	0.09
IL7	4.6	(1.0 – 12.1)	5.4	(2.7 – 34.3)	14.1	(0.0 – 31.0)	0.06
IL8	1131.0	(306.2 – 7708.9)	2433.7	(1126.1 – 9513.0)	1945.7	(20.5 – 4123.6)	0.04
IL9	0.0	(0.0 – 0.0)	0.0	(0.0 – 1.2)	0.0	(0.0 – 0.6)	0.99
IL10	4.5	(0.0 – 12.1)	7.9	(4.2 – 788.1)	13.3	(0.0 – 192.4)	0.06
IL12p40	4.1	(0.0 – 7.9)	5.4	(2.8 – 25.8)	2.8	(0.0 – 25.8)	0.83
IL12p70	4.8	(0.0 – 11.0)	5.1	(3.4 – 11.4)	5.5	(0.0 – 13.5)	0.31
IL13	0.0	(0.0 – 0.0)	0.0	(0.0 – 6.4)	0.0	(0.0 – 3.1)	0.99
IL15	0.0	(0.0 – 0.0)	0.0	(0.0 – 6.1)	0.0	(0.0 – 4.0)	0.99
IL17	0.0	(0.0 – 0.0)	0.0	(0.0 – 4.0)	0.0	(0.0 – 0.0)	0.99
IP10	42.0	(0.0 – 242.8)	316.2	(73.9 – 1483.6)	63.6	(0.0 – 709. 4)	0.81
MCP1	4468.0	(0.0 – 10067.9)	9023.6	(0.0 – 10086.7)	8929.9	(86.1 – 9799.0)	0.27
MCP3	2.2	(0.0 – 41.1)	18.3	(0.0 – 90.9)	33.1	(0.0 – 102.6)	0.03
MDC	37.2	(9.6 – 219.9)	54.5	(11.6 – 224.7)	47.6	(0.0 – 940.9)	0.69
MIP1α	17.0	(5.4 – 52.2)	77.9	(21.8 – 527.8)	82.7	(0.0 – 637.6)	0.20
MIP1β	20.7	(0.0 – 143.2)	103.1	(38.0 – 1031.0)	68.7	(0.0 – 248.9)	0.23
PDGF-AA	10.9	(3.4 – 16.5)	16.4	(8.7 – 35.0)	18.0	(1.0 – 25.0)	0.24
PDGF-ABBB	0.0	(0.0 – 0.0)	0.0	(0.0 – 0.0)	0.0	(0.0 – 15.5)	0.99
RANTES	6.6	(0.0 – 20.5)	18.1	(3.3 – 673.6)	10.5	(0.0 – 62.5)	0.47
sCD40L	0.0	(0.0 – 28.0)	0.0	(0.0 – 33.5)	0.0	(0.0 – 15.8)	0.64
sIL2-RA	3.3	(0.0 – 6.1)	2.8	(0.0 – 48.7)	0.0	(0.0 – 18.7)	0.74
TGFα	0.0	(0.0 – 0.5)	0.0	(0.0 – 3.3)	0.0	(0.0 – 0.0)	0.47
TNFβ	0.0	(0.0 – 3.5)	0.0	(0.0 – 3.5)	0.0	(0.0 – 3.5)	0.58
VEGF	45.0	(0.0 – 664.4)	81.0	(41.5 – 477.2)	88.7	(0.0 – 428.3)	0.12

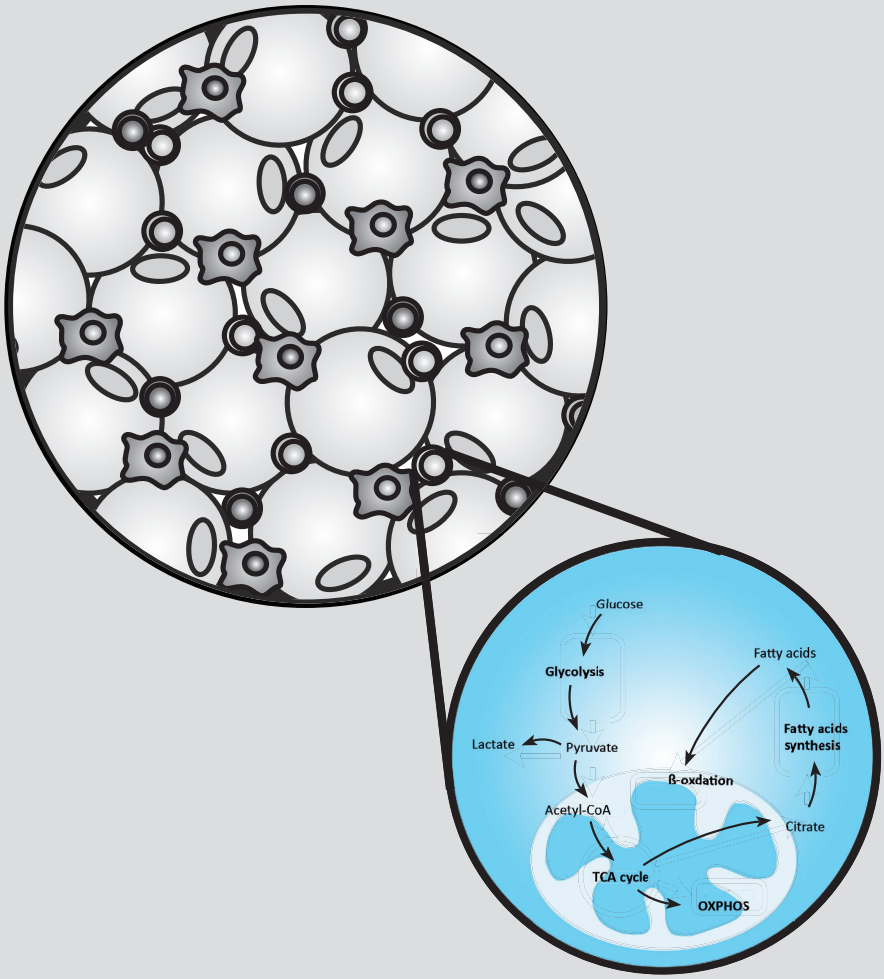
Table S4. Levels of cytokines/chemokines in ACM.

Differences between BMI ≤ 25 group and BMI > 30 group determined with Mann-Whitney test are indicated with p-value. The Bonferroni adjusted p-value indicating significance is < 0.001.

	ACM						
Cytokines (pg/ml)	BMI ≤ 25 (N=11)		BMI >25 and ≤ 30 (N=8)		BMI > 30 (N=11)		p-value
	median	(min – max)	median	(min – max)	median	(min – max)	
EGF	0.0	(0.0 – 6.4)	0.0	(0.0 – 12.5)	0.0	(0.0 – 0.0)	0.48
Eotaxin	9.7	(0.0 – 30.2)	20.8	(9.2 – 34.1)	8.9	(0.0 – 41.0)	0.99
FGF-2	50.8	(0.0 – 151.5)	58.2	(10.4 – 470.6)	49.8	(0.0 – 578.1)	0.57
FLT3L	4.0	(0.0 – 11.2)	4.0	(0.0 – 13.5)	0.0	(0.0 – 19.0)	0.40
Fractalkine	17.0	(0.0 – 174.3)	64.5	(32.6 – 370.8)	32.6	(0.0 – 229.9)	0.48
G-CSF	67.7	(3.9 – 556.1)	144.6	(17.5 – 1043.1)	100.6	(0.0 – 648.4)	0.85
GM-CSF	3.4	(0.5 – 40.1)	13.0	(0.0 – 59.9)	4.8	(0.7 – 36.0)	0.61
GRO	254.8	(118.6 – 2379.3)	1306.4	(117.2 – 2936.4)	880.3	(103.0 – 3663.5)	0.40
IFNα	2.0	(0.0 – 33.7)	5.3	(0.0 – 80.0)	2.5	(0.0 – 9.9)	0.73
IFNγ	2.5	(0.0 – 21.3)	4.6	(0.0 – 61.6)	0.0	(0.0 – 10.7)	0.32
IL1α	0.0	(0.0 – 3.2)	0.0	(0.0 – 4.6)	0.0	(0.0 – 6.7)	0.48
IL1β	0.4	(0.0 – 3.4)	2.6	(0.0 – 3.9)	0.8	(0.0 – 6.2)	0.29
IL1RA	0.0	(0.0 – 15.5)	8.9	(0.0 – 24.0)	2.5	(0.0 – 31.4)	0.77
IL2	0.0	(0.0 – 1.1)	0.3	(0.0 – 1.4)	0.0	(0.0 – 2.0)	0.68
IL3	0.0	(0.0 – 5.7)	0.0	(0.0 – 4.8)	0.0	(0.0 – 5.2)	0.99
IL4	0.0	(0.0 – 0.0)	0.0	(0.0 – 0.0)	0.0	(0.0 – 14.5)	0.04
IL5	0.0	(0.0 – 0.0)	0.0	(0.0 – 0.1)	0.0	(0.0 – 0.6)	0.21
IL6	142.7	(28.9 – 979.2)	760.2	(44.4 – 1737.7)	300.0	(37.2 – 2854.8)	0.52
IL7	0.0	(0.0 – 32.9)	4.1	(0.0 – 61.1)	0.0	(0.0 – 15.7)	0.99
IL8	670.5	(98.9 – 5468.8)	2022.5	(145.5 – 5221.9)	1543.8	(328.5 – 9676.4)	0.27
IL9	0.0	(0.0 – 0.0)	0.0	(0.0 – 0.0)	0.0	(0.0 – 0.0)	0.99
IL10	1.0	(0.0 – 21.9)	5.2	(0.0 – 57.6)	0.0	(0.0 – 10.2)	0.15
IL12p40	0.0	(0.0 – 12.5)	0.9	(0.0 – 26.1)	0.0	(0.0 – 2.9)	0.59
IL12p70	0.0	(0.0 – 5.5)	0.0	(0.0 – 8.1)	0.0	(0.0 – 4.8)	0.66
IL13	0.0	(0.0 – 1.2)	0.0	(0.0 – 1.3)	0.0	(0.0 – 1.8)	0.25
IL15	0.0	(0.0 – 1.9)	0.0	(0.0 – 0.0)	0.0	(0.0 – 1.9)	0.76
IL17	0.0	(0.0 – 0.0)	0.0	(0.0 – 0.0)	0.0	(0.0 – 2.1)	0.99
IP10	10.5	(0.0 – 294.1)	51.9	(0.0 – 122.5)	18.1	(0.0 – 3768.0)	0.50
MCP1	391.5	(138.7 – 7492.1)	825.5	(108.0 – 2380.8)	582.9	(51.2 – 9260.7)	0.75
MCP3	0.0	(0.0 – 20.6)	16.3	(0.0 – 38.0)	0.0	(0.0 – 65.1)	0.49
MDC	0.0	(0.0 – 6.4)	1.6	(0.0 – 61.1)	0.0	(0.0 – 9.3)	0.67
MIP1α	18.8	(0.0 – 67.2)	42.2	(11.6 – 71.5)	29.0	(0.0 – 293.4)	0.56
MIP1β	0.0	(0.0 – 15.4)	21.1	(0.0 – 36.1)	3.0	(0.0 – 61.8)	0.24
PDGF-AA	2.7	(0.0 – 7.8)	4.6	(0.0 – 13.3)	4.0	(0.0 – 9.6)	0.19
PDGF-ABBB	0.0	(0.0 – 7.5)	0.0	(0.0 – 7.5)	0.0	(0.0 – 5.7)	0.74
RANTES	0.5	(0.0 – 6.1)	3.9	(0.0 – 6.7)	1.5	(0.0 – 53.9)	0.63
sCD40L	0.0	(0.0 – 7.0)	0.0	(0.0 – 7.0)	0.0	(0.0 – 21.5)	0.99
sIL2-RA	0.0	(0.0 – 4.8)	0.0	(0.0 – 13.8)	0.0	(0.0 – 4.6)	0.74
TGFα	0.5	(0.0 – 1.4)	0.0	(0.0 – 1.4)	0.0	(0.0 – 2.0)	0.02
TNFβ	0.0	(0.0 – 13.6)	0.0	(0.0 – 39.5)	0.0	(0.0 – 0.0)	0.99
VEGF	17.5	(0.0 – 50.8)	53.7	(17.2 – 105.0)	14.9	(0.0 – 139.3)	0.59

Part 2

Cellular immunometabolism



Chapter 5

Fatty acids, lipid mediators and T cell function

Anja J. de Jong, Margreet Kloppenburg, René E.M. Toes and Andreea Ioan-Facsinay

Frontiers in Immunology. 2014; 5: 483.

Abstract

Research towards the mechanisms underlying obesity-linked complications has intensified during the last years. As a consequence, it has become clear that metabolism and immunity are intimately linked. Free fatty acids and other lipids acquired in excess by current feeding patterns have been proposed to mediate this link due to their immune modulatory capacity. The functional differences between saturated and unsaturated fatty acids, in combination with their dietary intake are believed to modulate the outcome of immune responses. Moreover, unsaturated fatty acids can be oxidized in a tightly regulated and specific manner to generate either potent pro-inflammatory or pro-resolving lipid mediators. These oxidative derivatives of fatty acids have received detailed attention during the last years, as they have proven to have strong immune modulatory capacity, even in pM ranges. Both fatty acids and oxidized fatty acids have been studied especially in relation to macrophage and T-cells functions. In this review, we propose to focus on the effect of fatty acids and their oxidative derivatives on T-cells, as it is an active area of research during the past 5 years. The effect of fatty acids and their derivatives on activation and proliferation of T-cells, as well as the delicate balance between stimulation and lipotoxicity will be discussed. Moreover, the receptors involved in the interaction between free fatty acids and their derivatives with T-cells will be summarized. Finally, the mechanisms involved in modulation of T-cells by fatty acids will be addressed, including cellular signalling and metabolism of T-cells. The *in vitro* results will be placed in context of *in vivo* studies both in humans and mice. In this review, we summarize the latest findings on the immune modulatory function of lipids on T-cells and will point out novel directions for future research.

Introduction

Obesity is increasing in the Western society, and obesity-linked complications are under intense scrutiny. Among these, not only metabolic disorders, such as diabetes mellitus and dyslipidaemia, but also cardiovascular disorders, such as hypertension and ischemic heart diseases, have been shown to be associated with obesity [1, 2]. More recently, also chronic diseases in which inflammation plays a role such as osteoarthritis, rheumatoid arthritis, inflammatory bowel disease, chronic obstructive pulmonary disease, and asthma have been associated with obesity [3-6]. Likewise, evidence exists indicating a relationship between obesity and increased susceptibility for infections [7], as well as a lower response to vaccination [8] indicating that obesity can impact immune responses. Although the mechanisms underlying these associations are unclear, adipose tissue-derived inflammation could play a role in the development and progression of these diseases. Indeed, adipose tissue serves not only as an energy depot but it is also a highly active metabolic and endocrine organ, affecting whole body metabolism [3, 9, 10].

The adipose tissue consists of adipocytes and the stromal vascular fraction, in which a variety of immune cells can be found. Among these, macrophages and T cells are the most abundant [11]. Expansion of the adipose tissue is accompanied by an increased infiltration of immune cells with a pro-inflammatory phenotype. The cross-talk between the infiltrating cells and the tissue-resident adipocytes leads to secretion of adipokines, cytokines, chemokines, and lipids with a predominant pro-inflammatory character [3, 10]. Moreover, the levels of various adipokines and cytokines are altered in obese individuals compared to lean ones (e.g. leptin, adiponectin, IL-6) [12].

This cross-talk has also been shown to affect the function of adipocytes, such as lipolysis, which will most likely result in an altered concentration of circulating free fatty acids. Indeed, obese persons have higher levels of free fatty acids in plasma compared to lean persons [13-15]. Whether and which of these soluble factors (adipokines, cytokines, lipids, etc) contribute to obesity-mediated inflammatory effects in diseases is still under investigation.

Several indirect lines of evidence suggest that fatty acids can modulate the immune response. One of these is that levels of several fatty acids are associated

with levels of inflammatory markers in healthy individuals [16]. More directly, the type of fatty acids contained in the diet has been suggested to influence the risk of development of inflammatory diseases in which the immune system plays an important role. Fatty acids from animal sources, such as meat and poultry, are mainly saturated fatty acids or omega-6 (ω6) unsaturated fatty acids, while fatty acids derived from plant-based foods, oils, and certain types of fatty fish consist mainly of ω3 unsaturated fatty acids. Diets rich in ω6 polyunsaturated fatty acids (PUFAs) increase the risk of development of inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, and asthma [17]. On the other hand, diets rich in ω3 PUFAs seem to have anti-inflammatory effects as indicated both by the decreased risk and the amelioration of these diseases [17-20]. Moreover, it has been shown that after *in vivo* challenge with a pathogen, the host survival and pathogen clearance is affected by diets enriched in fatty acids, however this is dependent on the infectious agent and the fatty acids used [21, 22].

Additionally, unsaturated fatty acids can be oxidized to generate either potent pro-inflammatory or pro-resolving lipid mediators. These lipid mediators have strong immune modulatory capacity and are generated in a timely and controlled manner during an inflammatory response [23]. Pro-inflammatory lipid mediators, such as prostaglandins and leukotrienes, are produced first. When the tissue-damaging or infectious agent is removed, the production of pro-resolving lipid mediators associates with restoration of normal tissue homeostasis [24].

In conclusion, several publications indicate that fatty acids and lipid mediators derived from fatty acids can potently influence the immune system. Although it is often unclear which cells are responsible for the immune modulatory effects of fatty acids, these lipids have been shown to affect the function of several immune cells, especially antigen-presenting cells and T-cells [25, 26]. In this review, we propose to summarize the knowledge regarding the effect of fatty acids and their oxidative derivatives on T-cells, both *in vitro* and *in vivo*, as it has been an active area of research during recent years.

Fatty acids

Fatty acids serve not only as fuel for cells but are also components of cell membrane phospholipids and glycolipids, and precursors of bioactive lipid mediators [27]. Fatty acids are hydrocarbon structures which can differ in the number of carbon atoms and the degree of saturation. Therefore, they are classified according to length (number of carbon residues in the lipid backbone), saturation, and number and position of the double bonds. Fatty acids can be either short-chain (4-10 carbons), medium-chain (12-14 carbons), long-chain (16-18 carbons) or very long-chain fatty acids (20 or more carbons). In addition, these lipids can be classified according to the presence or absence of a double bond in the backbone. Fatty acids that do not have a double bond are called saturated fatty acids and those having one or more double bonds are called monounsaturated fatty acids (MUFAs) or PUFAs, respectively. The position of the double bond determines whether PUFAs can be characterized as ω3, ω6 or ω9, whereby ω indicates the methyl end of the fatty acid and the number indicates the carbon counted from the methyl end which contains a double bond. Table 1 shows an overview of fatty acids most commonly used in literature. Some of the PUFAs, especially AA, DHA, and EPA and their influence on the immune system has been extensively reviewed elsewhere [27, 28], therefore, they will be excluded from the present review.

Table 1. Examples of saturated, monounsaturated and polyunsaturated fatty acids that are most commonly used in literature.

Type	Isomer	Name
Saturated	4:0	butyric acid
	12:0	lauric acid
	14:0	myristic acid
	16:0	palmitic acid (PA)
	18:0	stearic acid (SA)
Monounsaturated	18:1 (ω9)	oleic acid (OA)
Polyunsaturated	18:2 (ω6)	linoleic acid (LA)
	18:3 (ω3)	alpha-linolenic acid
	18:3 (ω6)	gamma-linolenic acid
	20:4 (ω6)	arachidonic acid
	20:5 (ω3)	eicosapentaenoic acid (EPA)
	22:5 (ω3)	docosapentaenoic acid (DPA)
	22:6 (ω3)	docosahexaenoic acid (DHA)

Effect of fatty acids on T-cells

Several studies have focused on the effect of fatty acids on T-cells. Although these studies differ in experimental setting, especially regarding the concentration of the fatty used, the type of T-cell studied, and the T-cell stimulus, the overall data suggest that low concentrations of fatty acids can influence the proliferation of T-cells, whereas higher concentrations induce apoptosis in a dose-dependent manner, through the induction of several pathways [29-34]. In addition, the concentration at which apoptosis occurs is also determined by the degree of saturation and the length of the fatty acid. While short-chain fatty acids (SCFA) are not toxic even at a concentration of 800 μM or higher [30], longer and more unsaturated fatty acids can already be toxic in low concentrations [e.g. linoleic acid (LA)] is toxic at 100 μM [30-32]).

In contrast to the apoptotic effects, the modulatory effects of low, non-toxic concentrations of fatty acids on proliferation do not seem to correlate with length of the fatty acids tested. There are only a few studies studying the proliferation of T-cells after incubation with fatty acids and they use different stimuli [29, 33, 35]. While proliferation of anti-CD3/anti-CD4 stimulated T-cells was not influenced by palmitic acid (PA) [29], concanavalin A (ConA) stimulated T-cells proliferated less in the presence of PA [33], indicating that fatty acids can have different effects depending on the stimulus used for T-cell activation. This is supported by the fact that while proliferation of T-cells stimulated with ConA was differentially affected by the saturated fatty acids stearic acid (SA), and the unsaturated fatty acids oleic acid (OA) and LA [33], stimulation with anti-CD3/anti-CD28 led to increased proliferation with all tested fatty acids [e.g. LA, SA, γ-linoleic acid (GLA), PA, OA and palmitoleic acid] independently of saturation degree [35]. Overall the data indicate that different fatty acids can modulate proliferation of T-cells, although this is likely dependent on the stimuli used.

Only a handful of studies have investigated cytokine production by T-cells treated with fatty acids and they generally indicate that fatty acids can modulate secretion of several cytokines, such as TNFα, IL-6, IL8, IL1β, IL-2, IL10 and IFNγ [36-38]. This modulation has been shown primarily for unsaturated fatty acids such as OA and LA using T-cells activated by a polyclonal stimulus, such as ionomycin [38] or ConA [37]. The effect of saturated fatty acids on cytokine secretion by activated T-cells is unclear. However, saturated fatty acids induced cytokine secretion in T-cells in the

absence of T-cell activation in a dose-dependent manner [36], while unsaturated fatty acids were unable to do so.

In conclusion, fatty acids are toxic to T-cells when administered in high concentrations and can induce proliferation and cytokine production when administered in non-toxic concentrations. The effects of fatty acids on T cell function seem to be dependent not only on the saturation grade and length of fatty acids, but also on the T-cell stimulus used.

Mechanisms by which fatty acid exert their effects

How fatty acids exert their effects on different immune cells is incompletely understood, despite the fact that several possible mechanisms have been proposed. One possibility is that fatty acids can diffuse through the membrane of T-cells in a passive process, as some studies have shown that T-cells incorporate fatty acids in their membrane upon exposure [38, 39].

In addition to this passive mechanism, there are several active pathways possible, in general mediated by cellular receptors. Among the receptors involved in the recognition of free fatty acids, fatty acid transport proteins (FATPs) form a family of six transmembrane proteins, with distinct tissue expression patterns [40]. FATPs are generally involved in the uptake of fatty acids, although there are no studies regarding their expression in T-cells. In addition to FATP, the fatty acid binding proteins (FABPs) are a family of homologous cytoplasmic proteins with high affinity for fatty acids. Even though FABPs are cytoplasmic proteins, it has been suggested that these FABPs play a role in uptake, transport, storage, and metabolism of fatty acids, with distinct patterns of tissue expression [41, 42]. Interestingly, one of these receptors, the epidermal-FABP (FABP5) is also expressed on $CD4^+$ T-cells [43].

Furthermore, a series of G protein-coupled receptors have been identified recently, which recognize fatty acids with different affinities and expression patterns. Five different receptors have been identified that recognize extracellular fatty acids with different lengths: free fatty acid receptor 1 (FFA1; GPR40) and GPR120 recognize long-chain fatty acids, GPR84 recognizes medium-chain fatty acids, while SCFA are recognized by FFA2 (GPR43) and FFA3 (GPR41) [44-46]. The expression pattern of these receptors on T-cells has been only partly investigated. These studies indicated

that FFA1 and FFA3 are not expressed on T-cells [47, 48], while the medium-chain fatty acid receptor, GPR84, is expressed by $CD4^+$ and $CD8^+$ cells although less abundantly than by monocytes and neutrophils [49]. Smith et al., recently showed that FFA2 is expressed on colonic regulatory T-cells (cTreg) [50].

In conclusion, several mechanisms could be involved in the modulation of T-cells function by fatty acids; a passive mechanism, in which the fatty acids diffuse through the membrane or active mechanisms, in which FATPs, FABPs or other receptors could be involved. Their relative contribution to fatty acids uptake awaits further investigation.

Downstream effects of fatty acids

There are several studies regarding downstream effects of fatty acids. These studies focus mainly on the effects of OA, LA or PA on resting human T-cells [36] or Jurkat cells (T-cell leukaemia cell line) [31, 34, 37, 51, 52]. As previously mentioned, fatty acids can influence T-cells in a dose-dependent manner, with low doses modulating proliferation and higher doses inducing apoptosis. Treatment of T-cells with fatty acids induces neutral lipid accumulation, such as triacylglycerol and cholesterol esters, and incorporation of the fatty acids in phospholipids [31, 34, 37]. The induction of neutral lipid accumulation is mediated by activation of insulin signalling pathways and glucose utilization, indicated by elevated expression of insulin receptor and GLUT4 and by increased glucose consumption and lactate production [34, 36].

It is believed that this pathway enables T-cells to evade the toxic effects of fatty acids at both low and high concentrations. However, when T-cells are exposed to too high concentrations, these mechanisms might not be sufficient to protect the cells from apoptosis and these results in the induction of intrinsic apoptotic pathways [31, 34, 37].

Several apoptotic characteristics are induced by fatty acids in T-cells, such as mitochondrial depolarization [31, 34], caspase activation [34, 37], DNA fragmentation [31, 34], chromatin condensation [31], cytochrome c [34] and phosphatidylserine externalization [31], indicating that T-cells treated with high concentrations fatty acids die due to apoptosis. In addition, T-cells incubated with fatty acids also induce oxidative and nitrosative stress leading to apoptosis,

indicated by higher levels of reactive oxygen species (ROS), reactive nitrogen species, and lower levels of catalase activity [34, 51, 52].

How these mechanisms would affect proliferation and cytokine production of T-cells is unclear and awaits further investigation. Likewise, it is unclear whether these mechanisms are still utilized by T-cells that have been activated by their cognate antigen on antigen-presenting cells and which display an increased energy demand in order to proliferate, differentiate and execute their effector functions.

In conclusion, free fatty acids induce proliferation of resting T-cells in low concentration, while higher concentrations induce apoptosis. In figure 1, we present possible mechanisms involved in the modulatory effects of fatty acids in T-cells.

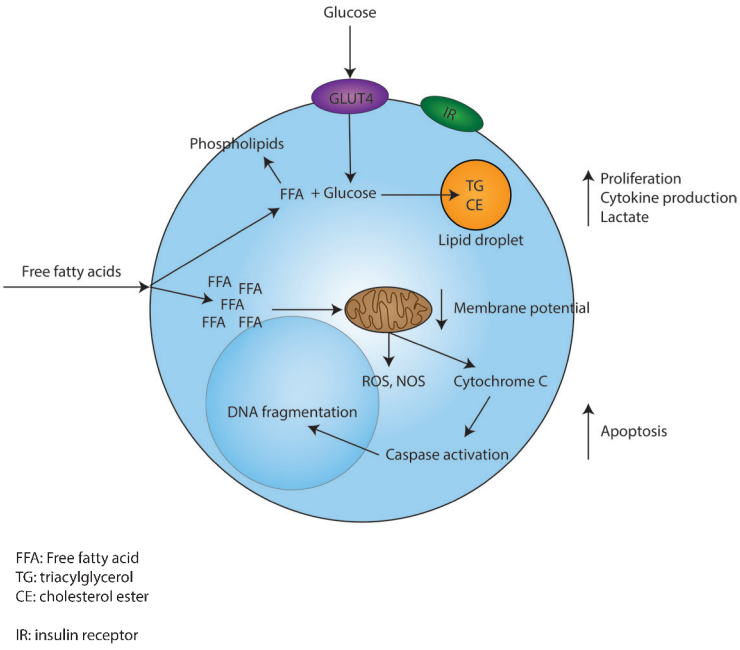


Figure 1. Proposed mechanism through which free fatty acids exert their effects on T cells.

Free fatty acids enter T cells through currently unknown mechanisms. Low concentrations of free fatty acids are incorporated in phospholipids, while uptake of glucose sustains the formation of triacylglycerol and cholesterol esters. In addition, low concentrations of free fatty acids induce proliferation, cytokine production and lactate production by T cells. However, when a T cell encounters high concentrations of free fatty acids this will lead to depolarization of the mitochondrial membrane and the induction of intrinsic apoptotic pathways, which eventually lead to apoptosis.

In vivo relevance

As mentioned above, obese people express generally higher plasma levels of free fatty acids after fasting when compared to healthy controls. Likewise, the turnover rate of free fatty acids is higher after fasting [14, 15]. Furthermore, some studies indicated T-cell numbers are decreased in obese persons [53-55] and T-cell proliferation and T-cell subset composition are altered compared to lean persons [54-57]. The mechanisms underlying these differences between obese and lean individuals are unclear. However, some studies addressing the effect of dietary fatty acid content on the function of T-cells, both in rodent models [58-62] and in humans [63, 64] suggest that fatty acids can be modulators of T-cell function and phenotype also *in vivo*.

Rodent studies show that different high-fat diets can decrease PHA [58] and conA [60] induced proliferation of T-cells when compared to control diets. Furthermore, rodent models showed that diets rich in fatty acids can have an effect on IL-2 production and signalling, since IL-2 production [58] and the expression of IL-2 receptor α-chain (CD25) [59] were increased when mice or rats are fed fatty acid rich diets. More recently, some studies investigated the effects of SCFAs on the immune system. SCFAs are metabolites of microbiota in the gut and are believed to play an important role in the balance between pro-inflammatory and anti-inflammatory effects in the gut. Indeed, recent studies show that adding SCFAs, especially butyrate and propionate, to the diet leads to induction of T-regulatory cells in the gut [61, 62]. In line with this, butyrate was shown to ameliorate T-cell-dependent experimental colitis [61]. In summary, these studies indicate that fatty acids can affect T-cell proliferation, cytokine production and skewing *in vivo*.

There are only two studies regarding the effect of dietary change of fatty acid content on the function of T-cells in humans [63, 64]. A diet containing MUFAs did not affect conA stimulated T-cell proliferation [63], while another study using several diets with different fatty acids composition described a higher PHA and conA induced proliferation of T-cells compared to baseline for most diets investigated [64]. A direct comparison of these studies is, however, difficult due to differences in diet composition.

Overall, the data suggests that fatty acids can influence both rodent and human T cells *in vivo*. A firm conclusion is, however, difficult to formulate, as rodent studies

show decrease in proliferation, while human studies either show unaffected proliferation or induction of proliferation. Furthermore, it remains uncertain whether the effects of saturated and unsaturated fatty acids are different.

Lipid mediators

Lipid mediators are produced through conserved biosynthetic pathways involving specific enzymes, which exert their function on lipid precursors that are released from membranes. There are several families of lipid mediators, which can be divided into pro-inflammatory lipid mediators and the more recently described specialized pro-resolving lipid mediators (SPM). Pro-inflammatory lipid mediators include prostaglandins and leukotrienes, while SPM include lipoxins, resolvins, maresins and protectins [23].

Pro-inflammatory lipid mediators and T-cells

Prostaglandins and their influence on T-cells are widely studied and these studies have been reviewed elsewhere [65, 66]. In short, PGE2 is the most studied prostaglandin and it has been shown to impair proliferative responses of T-cells [67-69]. In addition, PGE2 can affect T-cell cytokine production, whereby Th17 associated cytokines, such as IL17, are upregulated [70-73]. Moreover, PGE2 can change the Th1/Th2 balance by favoring Th2 skewing *in vitro* and in a mice model [69, 74]. The effect of PGE2 on skewing of naïve T-cells towards Th17 cells is uncertain since there are contradicting studies showing either an induction of Th17 cells [75] or a reduction of Th17 cells [76], depending on the combination of cytokines used to induce Th17 differentiation. PGE2 can exert these effects on T-cells through E-prostanoid (EP) 2 receptor and EP4, which are present on T-cells [72, 73, 75]. So, prostaglandins can affect T-cell proliferation and T-cell skewing through the EP2 and EP4 receptors.

Another family of pro-inflammatory lipid mediators are the leukotrienes, but the effects of leukotrienes on T-cells are not completely known. A human study revealed that both Th1 and Th2 express cysteinyl leukotriene receptor 1 (CYSLTR1) mRNA, although expression of CYSLTR1 was higher in Th2 cells compared to Th1 cells. In addition, activation of the CYSTLR1 with LTD4, LTC4 or LTE4 led to the induction of calcium signalling in Th2 cells, but a much weaker response in Th1

cells. LTD4 can also act as chemo attractant for Th2 cells [77]. This indicates that Th2 cells are more susceptible than Th1 cells to respond to leukotrienes.

Different *in vivo* mice models have indicated that mice deficient in 5-lipoxygenase (5-LO), which is one of the key enzymes in leukotriene production, have less T-cells infiltrating the inflamed area [78-81], diminished numbers of $CD4^+CD25^+$ regulatory T-cells, higher levels of IFNy, and increased T-bet (Th1 lineage commitment) [80]. In addition, LTB4 dose-dependently down-regulates the differentiation of naïve T-cells into Tregs, while it enhances the differentiation of Th17 cells, with more IL17 secretion and higher levels of RORγt mRNA expression [82].

In summary, pro-inflammatory lipid mediators seem to stimulate T-cell migration (either directly or indirectly) and to favor skewing toward Th2 or Th17 instead of Th1 or Tregs.

Pro-resolving lipid mediators and T-cells

Pro-resolving lipid mediators were recently described and have been shown to be involved in the resolution phase of inflammation. There are only a few studies investigating the effects of pro-resolving lipid mediators on T-cells; however, these studies indicate that these lipid mediators can influence T-cells.

Lipoxins, especially lipoxin A4 (LXA4) and lipoxin B4 (LXB4), have been shown to inhibit TNFα secretion from both PBMCs and peripheral T cells stimulated with anti-CD3 Abs [83], and the effects observed with LXA4 were mediated by the LXA4 receptor (FPR2/ALX), expressed on T-cells [83, 84]. FPR2/ALX expression is higher on activated $CD25^+$ and memory $CD45RO^+$ $CD4^+$ T-cells when compared to $CD25^-$ and naïve $CD45RA^+$ $CD4^+$ T cells [84], indicating that activated T cells can respond better to the agonist of this receptor.

Administration of resolving D1 (RvD1) led to reduced infiltration of $CD4^+$ and $CD8^+$ T-cells in the perivascular tissue of the eye of LPS induced uveitis in rats, resulting in the amelioration of the disease. This suggest that RvD1 prevents migration of T-cells into the eye, either directly or indirectly, thereby preventing disease in a rat model [85].

Moreover, protectin 1 (PD1) could reduce T-cell infiltration in zymosan A induced peritonitis [86] and into the lungs of OVA-sensitized and challenged mice [87]. Furthermore, *in vitro* studies show that PD1 inhibits cytokine secretion, such as TNFα and IFNγ by T-cells and can induce apoptosis of T-cells [86]. Therefore, PD1 could influence the inflammatory response by preventing the migration of T-cells and induction of apoptosis of T-cells.

In contrast to their pro-inflammatory counterparts, pro-resolving lipid mediators appear to inhibit cytokine production and migration of T-cells and to induce their apoptosis, thereby promoting resolution of inflammation.

Future research plans

In this review, we have summarized the current knowledge on the influence of fatty acids and lipid mediators on T-cells. These data indicate that fatty acids and their derivatives do affect the function and phenotype of T-cells and that the type and concentration of free fatty acids determines the outcome of this modulation and most likely the outcome of T-cell-dependent immune reactions. However, the presented data indicate that substantial further research is needed as there are still several remaining questions that need to be elucidated. These include a more detailed insight into the mechanisms involved in fatty acid uptake and usage by the cells, as well as into the possible i*n vivo* impact of these changes.

Although some data exist on the effect of fatty acids on T-cell proliferation and cytokine production, these are inconsistent due to usage of different stimuli for T-cells. Future research should probably focus on more physiological stimuli, including antigen-specific stimulation using antigen-presenting cells. Moreover, the molecular events linking fatty acid uptake to modification of T-cell function and phenotype need to be identified, as they could constitute drug targets for future therapies of obesity-linked complications. Likewise, it is of interest whether stimulated cells use the same mechanisms as unstimulated cells to evade the toxic effects and the induction of proliferation and cytokines. Stimulated cells have a different energy demand than unstimulated cells, and therefore, could respond differently to fatty acids. This would be directly relevant for understanding modulation of ongoing immune responses by free fatty acids and consequently

for development of efficient vaccination strategies or efficient drugs in T-cell-mediated diseases.

Additionally, *in vivo* studies systematically investigating the effect of various dietary fatty acids on T-cells would be needed as the current data are scarce and inconsistent. The latter is most likely due to a lack of standardization in the diets and the outcome measures used in the published studies. Also, current research is mainly focused on ω3 and ω6-rich diets, while relatively little is known about other fatty acids, which are also abundant in diet, such as OA, palmitic and palmitoleic acid and others. Future research about the effect of these fatty acids is needed.

Current knowledge regarding the effects of lipid mediators on T-cells is mainly focussed on pro-inflammatory lipid mediators. Therefore, research both *in vitro* and *in vivo* addressing the effects of pro-resolving lipid mediators on T-cell proliferation, differentiation, and function would be of interest. This would not only provide novel insights into the link between inflammation resolution and the adaptive immunity, which is a yet unexplored area of research, but could also lead to identification of new drug targets for modulation of T-cell responses.

Concluding remarks

The Western world experiences an epidemic of obesity due to excess intake of food. Among the many nutrients acquired in excess, free fatty acids have an important impact on various bodily functions, as recent research has made clear. In this review, we have provided an overview of the current knowledge about the effects of fatty acids and their derivatives on T-cells. The summarized data indicate that the effect of fatty acids on T-cells is either stimulatory or lipotoxic, depending on the capacity of the T-cells to evade the toxic effects of the fatty acids. Both fatty acids and their oxidative derivatives can influence T-cell proliferation, skewing, and differentiation and this is usually dependent on the type of fatty acid. This suggests that the type of fatty acid in the diet, rather than its quantity, determine the outcome of an immune reaction in a certain individual. Future research will teach us whether and how fatty acids or lipid mediators can be used as drug targets in treating obesity-related or T-cell-driven diseases.

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Chapter 6

The fate of oleic acid in CD4$^+$ T cells

Anja J. de Jong, Anna T. Hoekstra, Marieke Heijink, Bart Everts, Joanneke C. Kwekkeboom, Celia R. Berkers, Martin Giera, Tom W.J. Huizinga, Margreet Kloppenburg, René E.M. Toes, Andreea Ioan-Facsinay

manuscript in preparation

Abstract

The metabolism of immune cells is intimately linked to their function. We have previously shown that exposure to fatty acids enhances proliferation of CD4+ T cells. In this study, we analysed the contribution and fate of oleic acid (OA) to T cell proliferation to get insight into the underlying mechanisms.

Purified CD4+ T cells from healthy volunteers were activated by αCD3/CD28 in the presence of 0.1, 1 or 10 μg/ml OA and proliferation was measured on day 4. Glycolysis and mitochondrial respiration of CD4+ T cells were assessed using Seahorse technology. Metabolomics studies were performed using 13C-oleic acid (13C-OA) and liquid chromatography/mass spectrometry (LC-MS). Moreover, 13C-containing lipid species were determined by gas chromatography or LC-MS. Furthermore, TCR signalling was assessed by measuring calcium flux and ZAP70 phosphorylation.

OA enhanced αCD3/CD28-induced proliferation, even when OA was removed after 24 hours, indicating that early OA-induced changes can prime T cells for enhanced proliferation. Functional metabolic analyses indicated an increase in glycolysis and in some donors in mitochondrial respiration upon activation of T cells, although both were unaffected by OA treatment. In line with these data, metabolomics analysis indicated an increase in overall metabolic parameters upon activation of T cells for 24 hours but only a modest effect of OA on glycolysis and TCA cycle intermediates. Similarly, inhibition of fatty acid transport into mitochondria using etomoxir did not affect OA-mediated enhanced proliferation, indicating that changes in cellular metabolism do not contribute substantially to the observed effects of OA. Interestingly, OA enhanced pyrimidine synthesis after 8 hours and 24 hours. By using 13C-OA we found OA is taken up by cells and incorporated predominantly into phosphatidylcholines. Furthermore, we found that OA enhanced Ca-flux responses without affecting phosphorylation of ZAP70.

Our study indicates that rather than used as energy source, OA is used as membrane building block influencing calcium signalling in the T cell which may allow for stronger cellular activation in response to TCR triggering.

Introduction

The metabolism of $CD4^+$ T cells and their function are intimately linked. Naïve resting $CD4^+$ T cells have a quiescent state and generally depend on the breakdown of glucose, fatty acids and amino acids to fuel oxidative phosphorylation (OXPHOS) to maintain their homeostasis [1]. However, in order to proliferate, $CD4^+$ T cells need to adjust their metabolism and enhance growth-promoting pathways. To this end, aerobic glycolysis is greatly enhanced, as well as OXPHOS [1, 2]. Aerobic glycolysis is fuelled by the enhanced uptake of glucose through the upregulation of glucose transporter 1 (GLUT1) and will generate ATP and lactate from glucose [1, 3, 4]. Although this pathway is relatively inefficient in producing ATP, it has the benefit of generating metabolic intermediates that can be used to produce macromolecules used for cell growth and proliferation, such as nucleic acids, amino acids or lipids. In addition, uptake of amino acids, such as glutamine is increased and can either directly serve as building block for protein synthesis or serve as fuel for the TCA cycle, which also will generate intermediates to produce macromolecules [2, 5-7].

Fatty acids have been shown to affect both proliferation and function of $CD4^+$ T cells [8]. However, little is known about the dependence of $CD4^+$ T cells on fatty acids for their proliferation. Fatty acid oxidation (FAO) has been shown to be crucial for the development of T regulatory (Treg) cells. In contrast, FAO suppresses T effector cells which are dependent on fatty acid synthesis [3, 9, 10]. Furthermore, FAO has been shown to be important for memory T cells, as FAO promotes effector memory $CD4^+$ T cells [11]. Moreover, $CD8^+$ T cells are reliant on FAO to persist in skin tissue [12]. Some studies suggested that exogenous fatty acids fuel FAO [9, 12], however, recently it has been shown that $CD8^+$ memory T cells use extracellular glucose and endogenous lipids to support FAO rather than using exogenous fatty acids directly [13, 14]. In addition, it has been shown that both fatty acid biosynthesis and uptake are needed for activation and proliferation of $CD4^+$ T cells [15]. Overall, the exact mechanism by which exogenous fatty acids contribute to FAO and influence $CD4^+$ T cells is poorly understood.

In obese persons the levels of free fatty acids in plasma are elevated [16, 17]. The effect of free fatty acids on T cells appears to be concentration-dependent: low concentrations leads to enhanced proliferation, while high concentrations lead to apoptosis [8]. The underlying mechanisms leading to enhanced proliferation in the

presence of exogenous fatty acids are yet unknown. Possible mechanisms include oxidation of fatty acids to generate energy or incorporation of fatty acids into complex macromolecules used as signalling molecules or building blocks for the daughter cells. The aim of this study was to gain more insight into the mechanisms underlying the enhanced proliferation of CD4+ T cells upon exogenous fatty acid uptake. To this end we used oleic acid (OA) as a model free fatty acid, as it was previously shown to enhance CD4+ T cell proliferation [18].

Materials and methods

Peripheral blood CD4+ T cell isolation

PBMCs were isolated from heparinized blood or buffy coats of healthy blood bank donors by standard ficoll plaque gradient. Purification for CD4+ T cells was performed using Dynabeads FlowComp Human CD4 isolation kit (Invitrogen Dynal, Oslo, Norway) according to manufacturer's protocol. In short, PBMCs were incubated with an anti-CD4 antibody which binds to the target cells. CD4+ T cells which have bound the specific antibodies were captured by magnetic beads followed by removal of magnetic beads. The purity of CD4+ T cells was typically above 95%. Written informed consent was obtained from all donors and the study was approved by the local medical ethical committee.

T cell proliferation assays

Purified CD4+ T cells were plated in a density of 100.000 cells/well in 200 µl in a 96-well plate in DMEM high glucose (4.5 g/l) with no sodium pyruvate (Sigma Aldrich, Germany), supplemented with 0.5% fatty acid free BSA, 100 U/ml penicillin/streptomycin and 2 mM glutamax (medium). Cells were activated with 5 µg/ml plate-bound (pb.) αCD3 (clone OKT3, eBioscience, San Diego, USA) and 1 µg/ml soluble (sol.) αCD28 (Sanquin, Amsterdam, The Netherlands). Cells were treated with 0.1, 1 or 10 µg/ml oleic acid (Sigma Aldrich, Germany) or 0.1, 1 or 10 µg/ml U-13C18 OA (U13C OA) (Cambridge Isotope Laboratories, United Kingdom) or ethanol control (EtOH) (solvent control). OA diluted in EtOH was conjugated to fatty acid free BSA by addition of DMEM high glucose (4.5 g/l) with no sodium pyruvate, supplemented with 2% fatty acid free BSA, 100 U/ml penicillin/streptomycin and 2 mM glutamax, followed by 2 rounds of vortexing and sonication (30 sec). For inhibition experiments, etomoxir (ETO, 50 µM, 100 µM or 200 µM), C75 (20 µM and

40 µM), TOFA (2 µM and 20 µM) or DMSO control were added to the cultures for the indicated periods of time. For short-term treatment, OA and inhibitors were removed after 24 hours by 3 rounds of washing and fresh medium with 1 µg/ml soluble αCD28 was added to the cells. Proliferation during the last 18-20 hours of a 4 day culture was measured upon addition 0.5 µCi/well ^{3}H-Thymidine.

CTV staining

$CD4^+$ T cells were labelled with 5 µM cell trace violet (CTV) (Life Technologies) in 1 ml of PBS/0.1%BSA at 37°C. After 15 minutes cells were washed three times with cold 10% FCS/medium after which cells were taken up in DMEM high glucose (4.5 g/l) with no sodium pyruvate, supplemented with 0.5% fatty acid free BSA, 100 U/ml penicillin/streptomycin and 2 mM glutamax and plated in a density of 100.000 cells/well in 200 µl medium in a 96-well plate. Cells were stimulated with 5 µg/ml pb. αCD3 and 1 µg/ml sol. αCD28 and treated with 0.1, 1 or 10 µg/ml OA or solvent control. After 24 hours cells were harvested and CTV dilution was measured on LSR-II (BD Bioscience) or OA was washed away and cells were harvested on day 4 to measure CTV dilution on LSR-II.

T cell metabolism assays

Purified blood $CD4^+$ T cells were plated in 50 µg/ml poly-D-lysine (Millipore) coated XF-plates (Seahorse) in a density of 300.000 cells/well in 160 µl XF media (nonbuffered DMEM high glucose (4.5 g/l) with no sodium pyruvate (Sigma Aldrich, Germany), supplemented with 0.5% fatty acid free BSA, 100 U/ml penicillin/streptomycin and 2 mM glutamax). Cells were incubated for 1 hours in a non-CO2 incubator. Prior to measuring of the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) 40 µl of 0.1 µg/ml, 1 µg/ml or 10 µg/ml OA or solvent control was added. Basal OCR and ECAR levels were measured as well as levels after injection of 300.000 Dynabeads® Human T-Activator CD3/CD28 (Invitrogen) using an XF-96 Flux analyser (Seahorse Bioscience). In addition, unstimulated or 5 µg/ml pb. αCD3 and 1 µg/ml sol. αCD28 stimulated $CD4^+$ T cells were pre-treated for 24 hours with OA after which cells were transferred to poly-D-lysine coated XF-plates (Seahorse) and incubated for 1 hours in a non-CO2 incubator after which basal OCR and ECAR levels were measured.

T cell metabolomics

Unstimulated or 5 µg/ml pb. αCD3 and 1 µg/ml sol. αCD28 stimulated cells were plated in 200 µl DMEM high glucose (4.5 g/l) with no sodium pyruvate, supplemented with 0.5% fatty acid free BSA, 100 U/ml penicillin/streptomycin and 2 mM glutamax in a density of 300.000 cells/well in a 96-well plate and treated with 0.1, 1 or 10 µg/ml OA or 0.1, 1 or 10 µg/ml U13C OA. After 8 and 24 hours, 4 wells per condition were pooled and cells ($1.2x10^6$ cells) were washed with ice cold PBS and metabolites were extracted in 50 µl lysis buffer containing methanol/acetonitrile/dH2O (2:2:1). Samples of medium cultured for 8 and 24 hours were also collected. One ml of lysis buffer was added to 10 µl of medium to extract metabolites. Samples were spun at 16.000g for 15 minutes at 4 °C. Supernatants were collected for LC-MS analysis. LC-MS analysis was performed on an Exactive mass spectrometer (Thermo Scientific) coupled to a Dionex Ultimate 3000 autosampler and pump (Thermo Scientific). The MS operated in polarity-switching mode with spray voltages of 4.5 kV and -3.5 kV. Metabolites were separated using a Sequant ZIC-pHILIC column (2.1 x 150 mm, 5 µm, guard column 2.1 x 20 mm, 5 µm; Merck) using a linear gradient of acetonitrile and eluent A (20 mM (NH4)2CO3, 0.1% NH4OH in ULC/MS grade water (Biosolve)). Flow rate was set at 150 µl/min. Metabolites were identified and quantified using LCquan software (Thermo Scientific) on the basis of exact mass within 10 ppm and further validated by concordance with retention times of standards. Peak intensities were normalized based on total ion count (TIC).

Uptake of OA

Purified CD4+ T cells were treated as specified under T-cell metabolomics. After 24 hours, cells were washed with 180 µl of PBS (pH 7.4 [-] CaCl2 and [-] MgCl2) (Gibco). For GC-MS analysis cell pellet was resuspended in 20 µl PBS (pH 7.4 [-] CaCl2 and [-] MgCl2) and transferred into 250 µl acetone in an Agilent glass vial. Remaining cell pellet was resuspended in 10 µl EtOH and transferred to the same vial. Lipids were hydrolyzed at 60 ˚C for 30 min. using 10 µl 10 M sodium hydroxide if the total fatty acid content was planned to be analyzed. Thereafter 10 µl 1 µg/ml palmitic acid-d31 (Cambridge Isotope Laboratories) in ethanol was added and samples were homogenized. Fatty acids were derivatized by adding 100 µl 172 mM PFBBr in acetone and the reaction was allowed to proceed for 30 min. at 60 ˚C. Then a liquid-liquid extraction was performed using 500 µl n-hexane and 250 µl water, the upper, n-hexane, layer was transferred into an Agilent auto sampler vial with

glass insert and analyzed using GC-MS. For analysis of calibration range samples 10 µl U13C OA in EtOH and for medium samples 10 µl medium was used instead of 30 µl PBS/EtOH containing T-cells. Fatty acid content was analyzed using a Bruker Scion 436 GC coupled to Bruker Scion TQ MS. Injection was performed using an Agilent CTC PAL autosampler (G6501-CTC): 1 µl sample was injected splitless at 280 ˚C. The GC was equipped with an Agilent VF-5ms (25 m × 0.25 mm i.d.,0.25 µm film thickness) column, helium was used as carrier gas at a flow of 1.20 ml/min. The following temperature gradient was used to separate the compounds: 1 min. 50 ˚C, linear increase at 40 ˚C/min. to 60 ˚C, 3 min. at 60 ˚C, linear increase at 25 ˚C/min to 237 ˚C, linear increase at 3 ˚C/min to 250 ˚C, linear increase at 25 ˚C/min to 315 ˚C and 1.55 min. at 315 ˚C. The total analysis time per sample was 19.81 min. The transfer line and ionization source temperature were 280 ˚C. The pressure of the chemical ionization gas, methane, was set at 15 psi. Negatively charged ions were detected in the Selected Ion Monitoring mode. U13C OA was quantified using GC-MS data. An external calibration of U13C OA was used, palmitic acid-d31 served as internal standard for this calibration. The calibration curve was weighted using a weight factor of two on the concentration.

Incorporation of OA

Purified $CD4^+$ T cells were treated as specified under T -cell metabolomics. After 24 hours, cells were washed with 180 µl of PBS (pH 7.4 [-] CaCl2 and [-] MgCl2). For LC-FT/MS analysis, 3 wells were pooled and resuspended in 20 µl PBS (pH 7.4 [-] CaCl2, [-] MgCl2) and transferred into 295 µl of isopropanol in an 1.5 ml eppendorf tube, then the remainder of cells was resuspended in 10 µl PBS (pH 7.4 [-] CaCl2, [-] MgCl2) and transferred into the isopropanol. For calibration range samples 30 µl PBS (pH 7.4 [-] CaCl2, [-] MgCl2) and for medium samples 30 µl of medium was added to 290 µl (calibration range samples) or 295 µl (medium samples) isopropanol. Then 5 µl of an internal standard containing ergosterol acetate (gift from prof. Bracher), LPC (19:0) (Avanti Polar Lipids), PC (11:0/11:0) (Avanti Polar Lipids), PE (15:0/15:0) (Avanti Polar Lipids) and TG (17:0/17:0/17:0) (Sigma Aldrich) in isopropanol was added. The final concentration, the concentration to be analyzed on the LC-FT/MS, of these lipids was 0.1 µg/ml except for ergosterol acetate which final concentration was 1 µg/ml. The samples were sonicated for 5 min. and spun at 16.100g for 5 min. Finally, 100 µl of supernatant was complemented with 100 µl water and put into an Agilent auto sampler vial with glass insert. Samples were measured immediately or stored at -80 ˚C until analysis. Lipids were analysed using a Dionex Ultimate 3000

LC-system coupled to a 12T Bruker solariX XR mass spectrometer. 10 µl of sample was injected and separated on a phenomenex kinetex 1.7 µm, c18 (50 x 2.1 mm; 100 Å) column, which was held at 50 ˚C. The mobile phase flow rate was 250 µl /ml and a gradient of eluent A (80% water, 20% acetonitrile, 5 mM ammonium formate and 0.05% formic acid) and B (90% isopropanol, 9% acetonitrile, 1% water, 5 mM ammonium formate and 0.05% formic acid) was used to elute the lipids from the column. The gradient was programmed as follows: 0-1 min. 50% B, 1-8 min. linear increase to 95% B, 8-10 min. stable at 95% B, 10-10.5 min linear decrease to 50% B and 10.5-15 min stable at 50% B. A post column flow splitter was used. Compounds were ionized using electron spray ionization. N_2 was used as nebulizer (1.3 bar) and drying gas (4.0 L/min.), the dry temperature of the source was set at 200 ˚C. The capillary voltage was 4300 V. Compounds were detected in the positive mode, m/z-values between 200 and 1200 were detected. Masses in the spectra of LC-FT/MS data were internally calibrated using the monoisotopic mass of LPC (19:0), PC (11:0/11:0), PE (15:0/15:0) and TG (17:0/17:0/17:0). Extracted Ion Chromatograms of the compounds to be quantified were formed. PCs and TGs were quantified using an external calibration curve of PC (19:0/19:0) (Avanti Polar Lipids) and TG (15:0/15:0/15:0) (Sigma Aldrich), respectively. As internal standard for the quantitation of PCs and TGs, PC (11:0/11:0) and TG (17:0/17:0/17:0) were used respectively. Linear calibration curves were weighted using a weight factor of two on the concentration [19].

Ca flux assay

Purified CD4+ T cells were incubated with 10 µg/ml OA or EtOH in a density of $3x10^6$ cells/well in 2.5 ml DMEM high glucose (4.5 g/l) with no sodium pyruvate, supplemented with 0.5% fatty acid free BSA, 100 U/ml penicillin/streptomycin and 2 mM glutamax in a 6-well plate. After 24 hours cells were harvested and $2x10^6$ were stained for 35 minutes at 37°C with 2µM Indo-1 AM (Thermo Scientific) in 0.2 ml DMEM high glucose (4.5 g/l) with no sodium pyruvate, supplemented with 2% fatty acid free BSA in the presence of 0.02% pluronic acid and 5 µg/ml αCD3 and 1 µg/ml αCD28. Cells were washed and taken up in 1 ml DMEM high glucose (4.5 g/l) with no sodium pyruvate, supplemented with 2% fatty acid free BSA and 1 mM $CaCl_2$. Before measuring cells were warmed up to 37°C in a waterbath and baseline was measured on LSR-II for 2 minutes before addition of 3 µg/ml goat-anti-mouse antibody (Dako) to cross-link αCD3 and αCD28 after which calcium flux was measured for an additional 5 minutes.

pZAP-70 WB

Purified $CD4^+$ T cells were incubated with 10 µg/ml OA or EtOH in a density of $2.5x10^6$ cells/well in 2.5 ml DMEM high glucose (4.5 g/l) with no sodium pyruvate, supplemented with 0.5% fatty acid free BSA, 100 U/ml penicillin/streptomycin and 2 mM glutamax in a 6-well plate. Cells were harvested after 24 hours and $4x10^6$ cells were incubated with 25 µg/ml αCD3 and 5 µg/ml αCD28 or medium as control in 500 µl for 15 min at 37°C in tubes. Cells were washed and cross-linking of αCD3 and αCD28 was achieved by incubating cells with 5 µg/ml goat-anti-mouse antibody in 500 µl medium at 37°C for 30 sec, 2 min or 5 min. Stimulation of T cells by cross-linking was stopped by addition of 1 ml of ice-cold PBS. Cells were washed with ice-cold PBS and taken up in 30 µl NP-40 lysis buffer (Invitrogen) in presence of 1:100 phosphatase/protease inhibitors (Cell Signaling). Cells were lyzed on ice for at least 1 hours while vortexing every 15 min and centrifuged for 15 min at max speed. Supernatants were collected and stored until use. Protein content of cell lysates was measured using the BCA assay (Thermo Scientific). Ten µg protein was diluted 4x with Laemmli sample buffer (Bio-Rad)/5% β-mercaptoethanol (Merck) and boiled for 5 min at 95°C. Proteins were separated by SDS/PAGE on a mini-protean precast gel (Bio-Rad) followed by protein transfer on a Trans-Blot Turbo Transfer pack (Bio-Rad). The membrane was washed and blocked with TBS/0.1% Tween/5% skim milk powder (Fluka) for 1 hour at RT under continuous rolling. Next, the membrane was incubated with zeta chain of TCR associated protein kinase (Zap70) rabbit antibody or pZap70 (Y319) Rabbit antibody (all from Cell Signaling) overnight at 4°C. The membrane was washed and incubated with anti-Rabbit HRP antibody for 1 hours at room temperature after which the membrane was washed with TBS/0.1% Tween and milliQ water. Zap70 or phosph-Zap70 was visualized using ECL solution.

Results

Oleic acid enhances proliferation of $CD4^+$ T cells during the first 24 hours

Oleic acid (OA) was used as a model fatty acid to study the effects of free fatty acids on $CD4^+$ T cells. A dose-dependent response in proliferation was observed, 4 days after αCD3/CD28 stimulation, when OA was added to the cells (Fig 1A), even when OA was removed after 1 day (Fig 1B). To get more insight into the kinetics of the proliferative response, we have also measured T cell proliferation at 24 hours.

A dose-dependent increase in tritium Thymidine incorporation was observed in response to OA treatment also at this early timepoint (Fig 1C). Enhanced cell proliferation in the presence of OA was also observed using CTV staining (Fig 1E), although this was less evident on day 1 (Fig 1D). These data indicate that the presence of OA during the first 24 hours of T cell stimulation enhances cell division.

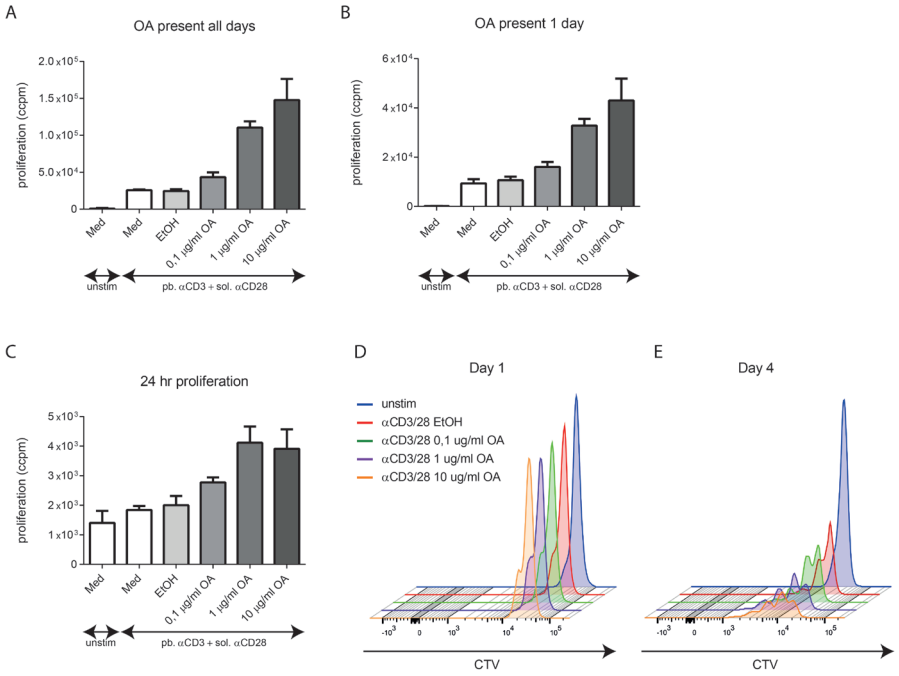

Figure 1. Oleic acid enhances proliferation of CD4+ T cells during the first 24 hours

Isolated CD4+ T cells were incubated with 0.1, 1 or 10 µg/ml OA or controls in the presence of pb. αCD3 and sol. αCD28. T cell proliferation was determined after 4 days with OA present the whole period of time (A) or OA washed away after 24 hours (B) or proliferation was determined after 24 hours of incubation with OA (C). Data are shown as mean + SD of triplicates and are from one representative experiments out of 3-6 performed. Isolated CD4+ T cells were labeled with CTV after which cells were incubated with 0.1, 1 or 10 µg/ml OA or controls in the presence of pb. αCD3 and sol. αCD28. After 24 hours (D) or after 4 days (E) dilution of CTV was determined. Representative plot out of 4 performed is shown.

Oleic acid does not affect glycolysis or mitochondrial respiration of CD4+ T cells

To study the effect of OA on CD4+ T cell metabolism, glycolysis and mitochondrial respiration was measured using Seahorse technology. Stimulation of CD4+ T cells enhanced glycolysis (Fig 2A), without affecting mitochondrial respiration (Fig 2C). Both metabolic pathways were unaffected by the OA treatment (Fig 2B and D). Stimulation of CD4+ T cells for 24 hours resulted in enhanced glycolysis (Fig 2E) and

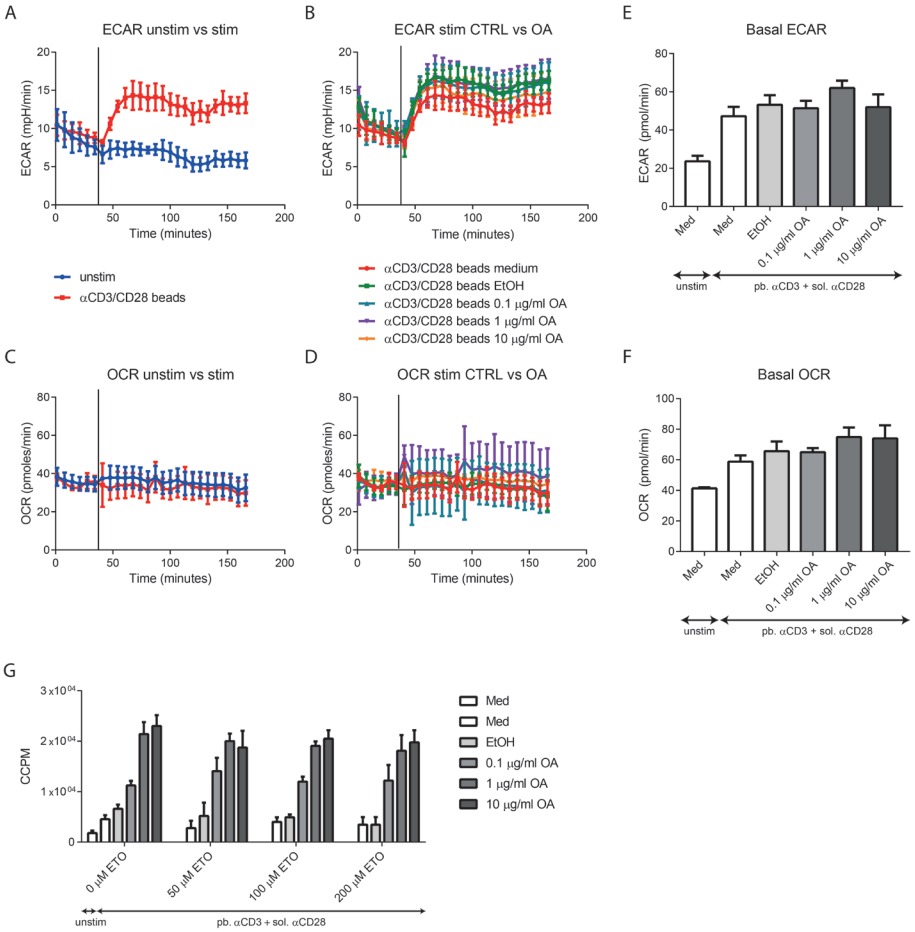

Figure 2. Oleic acid does not affect glycolysis or mitochondrial respiration of $CD4^+$ T cells

Isolated $CD4^+$ T cells were incubated with 0.1, 1, 10 μg/ml OA prior to measuring ECAR and OCR. ECAR (A and B) and OCR (C and D) was measured for 200 minutes upon activation with αCD3/αCD28 coated beads (indicated with line). Data are shown as mean + SD of quadruplicates and are from one representative experiments out of 6 performed. Basal ECAR (E) and OCR (F) of $CD4^+$ T cells treated with 0.1, 1, 10 μg/ml OA in presence of pb. αCD3 and sol. αCD28 for 24 hours was measured. Data are shown as mean + SD of quadruplicates and are from one representative experiments out of 8 performed. Proliferation of pb. αCD3 and sol. αCD28 stimulated $CD4^+$ T cells treated with 0.1, 1, 10 μg/ml OA in presence of 50 μM, 100 μM or 200 μM ETO or control for 24 hours after which OA and ETO was washed away and proliferation was measured on day 4. Data are shown as mean + SD of triplicates and are from one representative experiments out of 4 performed.

enhanced mitochondrial respiration, although the latter was donor-dependent (Fig 2F). Again, OA treatment did not affect any of these pathways (Fig 2E and F). In line with these findings, blocking of OA import into mitochondria using etomoxir did not influence the enhanced proliferation induced by OA (Fig 2G),

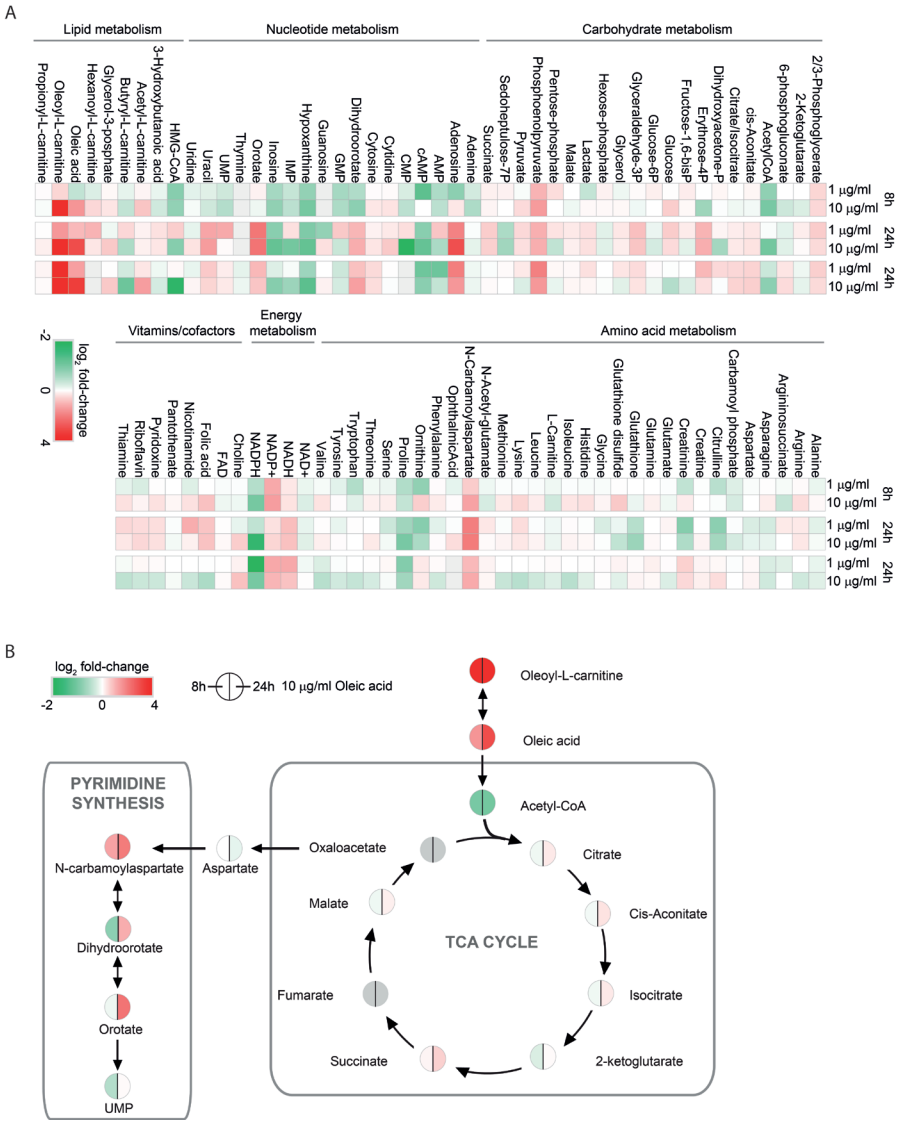

Figure 3. Oleic acid enhances TCA cycle and pyrimidine synthesis

T cells were incubated with 1 or 10 µg/ml OA in presence of pb. αCD3 and sol. αCD28 for 8 or 24 hours after which LC-MS analysis was performed and metabolites were determined. Metabolites decreased in OA treated samples compared to EtOH treated samples were displayed in green, while metabolites increased in OA treated samples compared to EtOH were displayed in red. Experiment was performed twice with time point 24 hours (A). TCA cycle and pyrimidine synthesis showed differences between EtOH and OA and is shown in detail (B).

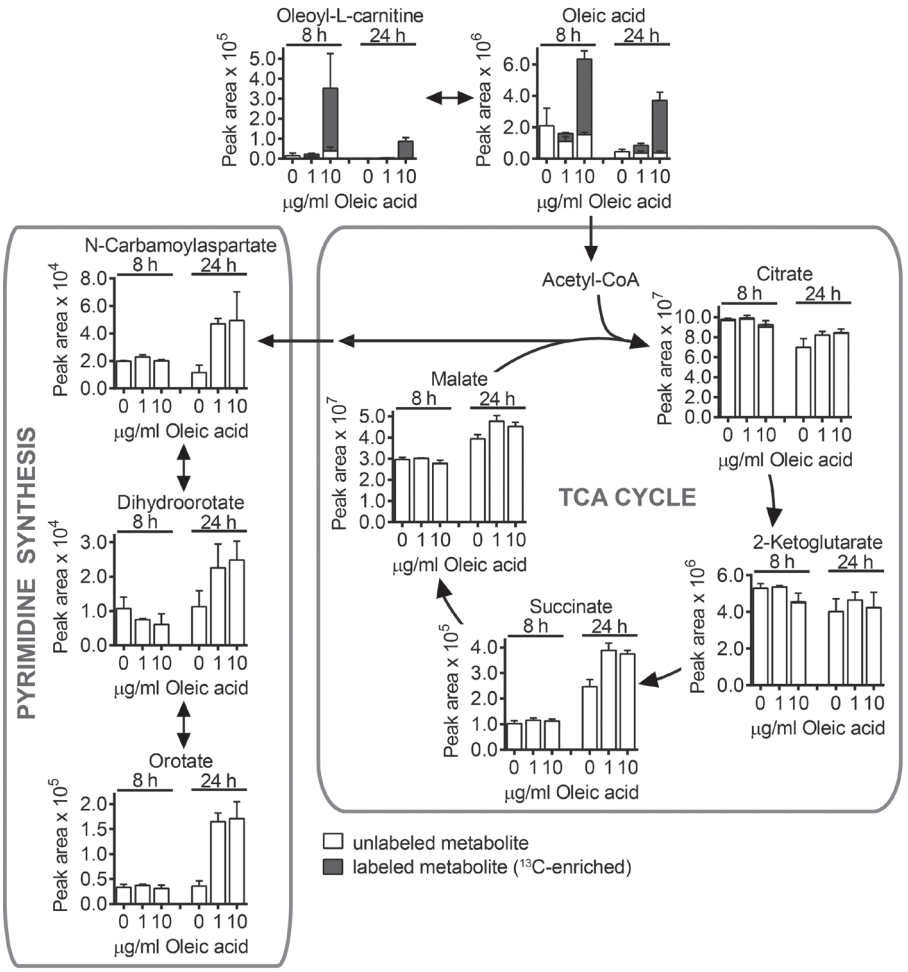

Figure 4. Oleic acid is not used as energetic substrate

CD4+ T cells were incubated with 1 or 10 µg/ml 13C-OA in presence of pb. αCD3 and sol. αCD28 for 8 or 24 hours after which LC-MS analysis was performed and unlabeled and labelled metabolites were determined. White bars represent unlabeled metabolites, grey bars represent labelled metabolites.

suggesting that OA treatment does not affect glycolysis and mitochondrial respiration.

Oleic acid enhances TCA cycle and pyrimidine synthesis

To determine whether OA does not affect any other metabolic pathway, a complete metabolomics analysis was performed after incubating CD4+ T cells for 8 hours or 24 hours in the presence of OA. These analyses demonstrated that stimulation

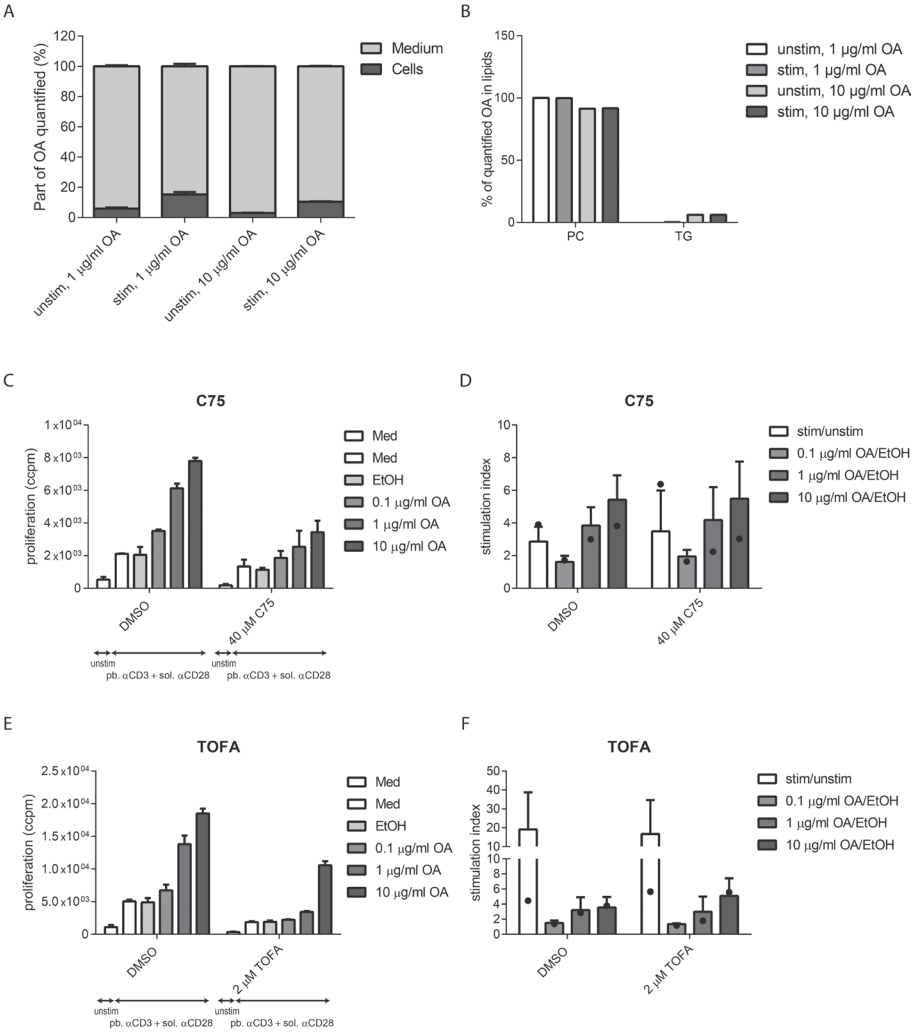

Figure 5. Oleic acid is incorporated in phosphatidylcholines

Isolated CD4+ T cells were treated with 1 or 10 µg/ml 13C-OA in presence or absence of pb. αCD3 and sol. αCD28 for 24 hours and GC-MS was used to determine the percentage of OA taken up by cells (A). LC-FT/MS was used to determine in which higher order lipids 13C-OA was incorporated (B). Proliferation of pb. αCD3 and sol. αCD28 stimulated CD4+ T cells treated with 0.1, 1, 10 µg/ml OA in presence of 40 µM C75 (C) or 2 µM TOFA (D) or control for 24 hours after which OA and inhibitors was washed away and proliferation was measured on day 4. Data are shown as mean + SD of triplicates and are from one representative experiments out of 3-4 performed. Summary of stimulation indexes for all experiments performed are shown for both C75 (E) and TOFA (F), dots in graphs shows stimulation indexes of representative experiments shown in C or D.

leads to an overall increase in all metabolic parameters (data not shown). OA only had a modest effect on glycolysis (phosphoenolpyruvate), early TCA cycle intermediates (cis-aconitate and citrate) and metabolites involved in pyrimidine synthesis (n-carbamoylaspartate, dihydroorotate and orotate) (Fig 3A and 3B). The effects induced by OA seemed to increase at 24 hours compared to 8 hours, however, these effects were independent on the concentration of OA.

Oleic acid is not used as energetic substrate

To determine whether OA would fuel the TCA cycle and pyrimidine synthesis, ^{13}C-OA was used. Although ^{13}C-OA was taken up by the cells and partly bound to L-carnitine, no trace of ^{13}C-OA could be detected in the TCA cycle or pyrimidine synthesis (Fig 4). In combination with the fact that etomoxir did not affect the effect of OA, we conclude that beta-oxidation of OA in not involved in the ability of OA to enhance T cell proliferation.

Oleic acid is incorporated in phosphatidylcholines

We additionally used ^{13}C-OA to estimate the amount of OA that was taken up and to map the fate of OA CD4$^+$ T cells. Around 15-40% of the added OA was taken up by the CD4$^+$ T cells (Fig 5A) and was mainly incorporated into phosphatidylcholines (PCs) (Fig 5B and Suppl. Fig 1). However, treatment of cells with a higher dose of OA also led to incorporation of OA into triglycerides. Blocking the formation of newly synthesized fatty acids at two different stages, with C75 (inhibitor of fatty acid synthase (FAS)) and TOFA (inhibitor of acetyl-CoA carboxylase (ACC)) revealed that both inhibited proliferation of CD4$^+$ T cells (Fig 5C and E, respectively). Enhanced proliferation with OA treatment was still observed as demonstrated by the stimulation indexes (Fig 5D and F). These findings indicate that newly synthesized fatty acids are important for αCD3/αCD28 induced proliferation, but not for the enhanced proliferation induced by OA.

Oleic acid enhances TCR signalling in CD4$^+$ T cells

As OA could also influence the signalling and thereby enhancing the proliferation of CD4$^+$ T cells, we next determined whether TCR signalling was affected. Therefore, we determined both phosphorylation of ZAP70 and calcium fluxes in the cell, both downstream of TCR signalling. Calcium flux analyses demonstrated that baseline calcium flux is unaffected by treatment with OA. However, stimulation of CD4$^+$ T cells treated with OA leads to enhanced calcium flux responses (Fig 6A), indicated

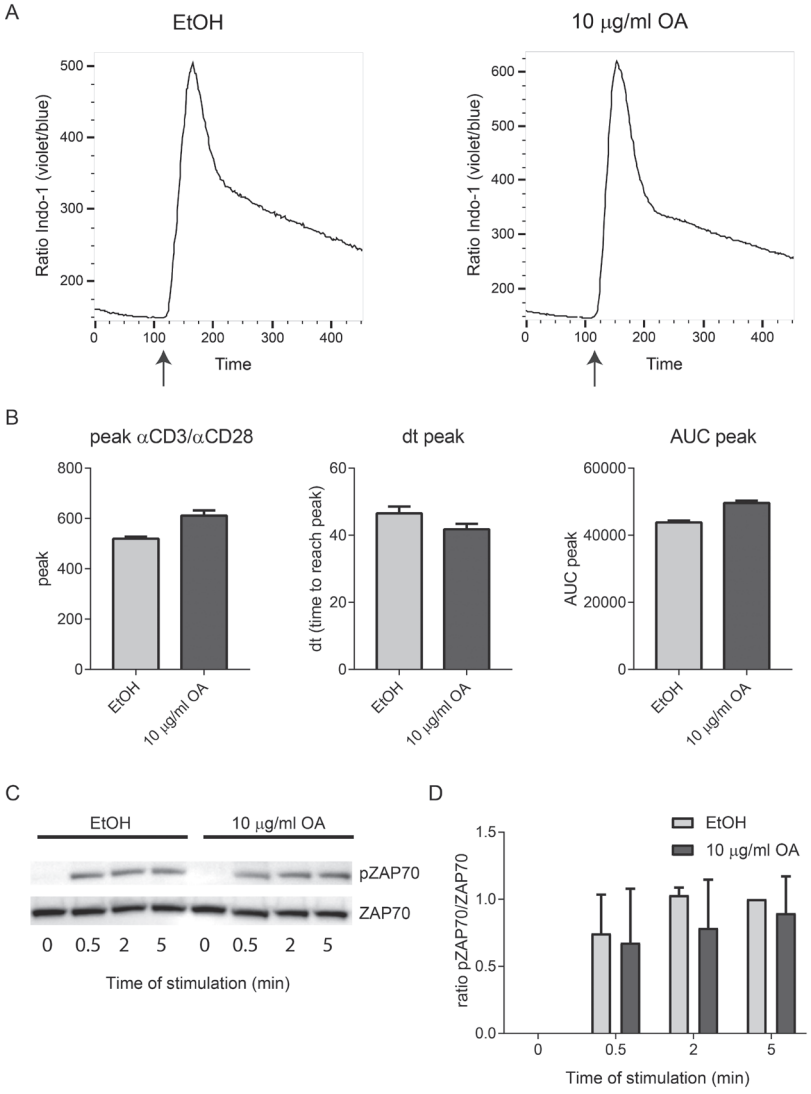

Figure 6. Oleic acid enhances TCR signalling in CD4+ T cells

Isolated CD4+ T cells were treated with 10 μg/ml OA or EtOH and after 24 hours cells were harvested, stained with indo-1 AM in the presence of 5 μg/ml αCD3 and 1 μg/ml αCD28. Basal levels of calcium flux was measured for 2 minutes after which 1 μg/ml goat-anti-mouse antibody was added to cross-link αCD3 and αCD28 (indicated with arrow) and calcium flux was measured for 5 minutes. Representative calcium flux plot is shown (A) and summary of 5 samples is shown to indicate highest point of peak in response to αCD3/αCD28, time to reach the peak and area under the curve of the peak (B). One representative experiments is shown out of 2 performed. Isolated CD4+ T cells were treated with 10 μg/ml OA or EtOH and after 24 hours cells were harvested, and incubated with 25 μg/ml αCD3 and 5 μg/ml αCD28 for 15 min at 37°C. Cross-linking was achieved by incubating cells with 5 μg/ml goat-anti-mouse antibody at 37°C for 30 sec, 2 min or 5 min. Stimulation was stopped by ice-cold PBS. Lysates were made and expression of pZAP70 (Y319) and ZAP70 was determined by western blot analysis. One representative experiment (A) and a summary of both experiments performed is shown (B).

by the calcium flux peak, time to reach this peak and the area under the curve of the calcium flux peak (Fig 6B). Stimulation of $CD4^+$ T cells induced phosphorylation of ZAP70, however, this was not affected by the treatment with OA (Fig 6C and D). These observations may suggest that although ZAP70 is unaffected, OA influences TCR triggering through enhanced calcium fluxes.

Discussion

In this study, we investigated the underlying mechanisms for enhanced proliferation of $CD4^+$ T cells in the presence of free fatty acids. Our study indicates that rather than used as a source of energy, OA is used as building block as it is incorporated into phosphatidylcholines. Moreover, it appears to influence TCR signalling indicated by enhanced calcium fluxes.

TCR triggering of $CD4^+$ T cells resulted in enhanced glycolysis and OXPHOS, as also previously described both in mice [1, 2] and humans [20]. However, treatment with OA did not influence either pathway, suggesting that OA is not used as energy source. This is in contrast to previous studies showing that affecting FAO, fatty acid uptake or biosynthesis does influence the metabolic state of T cells. Inhibition of FAO reduces OCR in memory $CD8^+$ T cells, while OCR of $CD8^+$ effector T cells was unaffected [21]. Furthermore, inhibition of fatty acid uptake or synthesis in naïve stimulated $CD4^+$ T cells decreased both OCR and ECAR, while OCR of Th2 cells was unaffected by these treatments [15]. This suggests that the effect of fatty acids on the metabolism of $CD4^+$ T cells is dependent on the differentiation state of the cells.

In line with recent findings [15], we showed that OA is incorporated in PCs, which are main components of the cell membrane. It remains unknown, whether these OA-containing PCs are used to generate new membranes, replacing the PCs already present in the membranes or in which membranes these OA-containing PCs are incorporated. However, as we have observed that blocking the synthesis of new fatty acids on two different stages (TOFA and C75) did not influence the stimulation index of OA this suggests that OA is incorporated in existing membranes.

Incorporation of OA-containing PCs in the membrane could influence membrane fluidity and subsequently could influence TCR signalling [11, 22]. We have also

observed that calcium flux is enhanced with treatment with OA, suggesting that TCR signalling of the cells was influenced, however, phosphorylation of ZAP70 was not influenced. Together with lymphocyte-specific protein tyrosine kinase (Lck), ZAP70 is one of the first steps of TCR signalling, leading to phosphorylation of linker for the activation of T cells (LAT), which recruit many other signalling molecules, such as phospholipase C gamma1 (PLCγ1) [23]. PLCγ1 signals induces intracellular calcium release, which serves as the primary trigger for the opening of calcium channels in the plasma membrane [24]. Although phosphorylation of ZAP70 was not influenced by OA treatment, other TCR signalling molecules such as LAT or PLCγ1 could still be affected. Therefore, future studies should investigate whether other molecules downstream of TCR signalling, such as LAT and PLCγ1, are affected by OA treatment and therefore contribute to higher calcium fluxes.

Furthermore, our metabolomics study showed that OA-treated CD4+ T cells have enhanced pyrimidine synthesis, which is supported by the observed elevated levels of incorporated ^{3}H-Thymidine. Pyrimidines not only support DNA synthesis, but pyrimidines also control the progression through the S phase of the cell cycle [25], suggesting that treatment with OA could influence this, resulting in enhanced proliferation of CD4+ T cells.

The increased proliferation of T cells in the presence of oleic acid could be relevant for inflammatory conditions associated with obesity, which is usually accompanied by increased free fatty acid levels. One example is the joint of osteoarthritis patients with a high BMI, in which there is a higher release of free fatty acids from the infrapatellar fat pad [26], likely resulting in high levels of free fatty acids in the joint. The enhanced proliferation of the joint T cells in a high free fatty acid environment could underlie the observed correlation between the frequency of CD4+ T cells in synovium and BMI [27].

On the other hand, increased free fatty acid levels in the microenvironment could also lead to accumulation of intracellular lipid droplets. These have been recently associated with increased tissue invasiveness of T cells in rheumatoid arthritis, resulting in increased inflammation [28]. Although a full understanding of the effects of fatty acid uptake on T cell function is still lacking, these data indicate that interfering with free fatty acid synthesis / uptake could constitute an effective therapeutic approach in obesity-related diseases.

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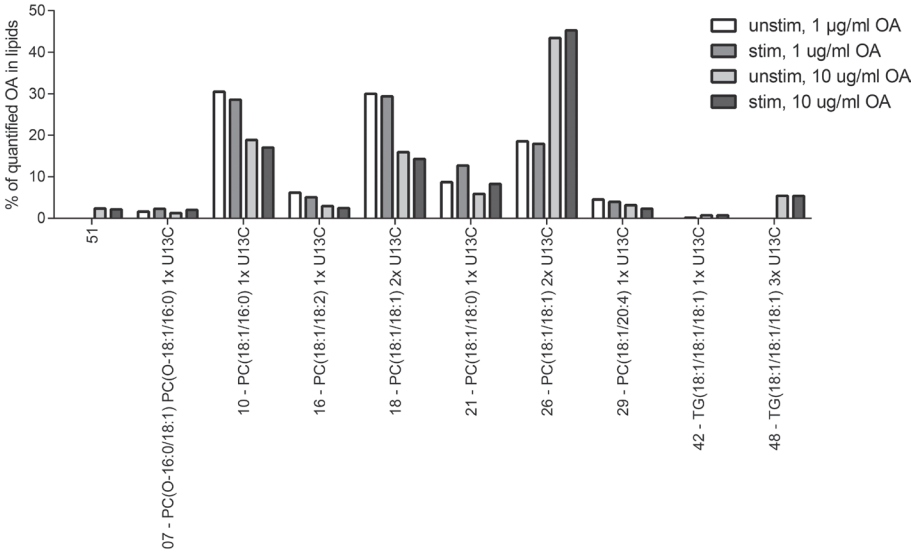
Supporting information



Supplementary figure 1. Oleic acid is incorporated in different phosphatidylcholines

Isolated $CD4^+$ T cells were treated with 1 or 10 µg/ml 13C-OA in presence or absence of pb. αCD3 and sol. αCD28 for 24 hours and LC-FT/MS was used to determine in which higher order lipids 13C-OA was incorporated. After we selected the top ten of lipids which contained the highest intensity of 13C-OA, we determined the relative amount of OA incorporated in these ten lipids. The sum of 13C-OA in these ten quantified lipids was used as total amount (100%).

Chapter 7

Summary and discussion

The association between osteoarthritis (OA) and obesity is not only present in weight-bearing joints [1-3], but also in non-weight-bearing joints [3-5]. This indicates that not only mechanical factors play a role in this association, but also systemic factors, such as low-grade inflammation, disturbed lipid metabolism and adipokines [2]. It is suggested that adipose tissue secreted factors could play an important role in OA. Although long thought to serve only as energy depot, adipose tissue is a highly active metabolic and endocrine organ, which can affect whole body metabolism [6-8]. Immunometabolism describes the intersection of the fields of immunology and metabolism and not only focusses on whole-body metabolism but also on cellular bioenergetics.

Part 1 Systemic immunometabolism

In the first part of this thesis, the potential role of the infrapatellar fat pad (IFP) in the pathophysiology of OA has been investigated. Therefore, the IFP has been characterized both on the cellular as well as the molecular level.

As first step to determine the potential role of the IFP in the pathophysiology of OA the IFP and synovium of OA and RA patients were compared in **chapter 2**. In both OA and RA inflammation is implicated, however, RA is generally associated with more inflammation in the synovium [9] and synovial fluid (SF) compared to OA [10-12]. We determined the levels of adipocytokines secreted by the fat tissue (fat-conditioned medium) and adipocytes (adipocyte-conditioned medium) and found that both the fat tissue and adipocytes are capable of secreting various adipocytokines. However, no significant differences were observed between RA and OA patients. The inter-donor variation was relatively high in both groups, as we only included 20 RA patients of with a limited amount of tissue. This could have limited the power of our study. Therefore, future research should elaborate on these findings with a larger cohort.

Furthermore, characterizing the IFP on the cellular level revealed that although the IFP of RA patients contained a higher number of cells than the IFP of OA patients, the percentages of different cell subsets, such as T cells, macrophages and endothelial cells were comparable between RA and OA patients. The only observed difference was the percentage of mast cells, which was lower in OA

patients compared to RA patients. These findings are in contrast to synovium as described in **chapter 2** and [9].

Surprisingly, we could not find any differences in the adipocytokine profile of the IFP of RA patients compared to OA patients, while the number of cells in the IFP are higher and RA is generally associated with a higher cytokine load than OA [12, 13]. Besides the power of the study being a limitation, RA patients investigated in this study had secondary OA which could also be influencing our results. However, RA patients included in this study did have a higher synovitis score as compared to the OA patients included in this study, indicating that inflammation in the joint was higher in RA patients. This suggests that the IFP might have a negligible role in inflammation of other joint tissues such as synovium and SF of RA patients, however, the increased cellular infiltrate could represent the higher cellular inflammatory load present in the joint of RA patients.

Among the immune cells present in the adipose tissue, T cells have been implicated to play a role in adipose tissue inflammation [14-17]. Several studies have shown that T cells from adipose tissue have a limited T cell receptor (TCR repertoire [14, 16-18], suggesting that they might have undergone clonal expansion, possibly recognizing adipose tissue antigens. Surprisingly, T cells from the IFP of OA patients have been shown to be capable of secreting IL-6 *ex vivo* [19], suggesting recent activation, which could indicate that these cells have been activated in the adipose tissue. Therefore, in **chapter 3** we aimed to extensively characterize this IL-6 secreting T cell population. We confirmed that $CD4^+$ T cells from the IFP are capable of secreting IL-6 *ex vivo* and could also demonstrate the presence of these cells in synovium, blood and subcutaneous adipose tissue.

Phenotypic characterization revealed that these IL-6^+ $CD4^+$ T cells are conventional (TCRαβ) T cells with an activated memory phenotype, supporting the hypothesis that these cells have recently been activated. Determining cytokine production and chemokine receptor expression by IL-6^+ $CD4^+$ T cells revealed that these cells could not be assigned to a specific T cell helper subset. However, transcription factors, such as T-bet, GATA-3, FoxP3 and others have not been investigated, which therefore limits our definitive conclusion. Furthermore, TCRβ gene analysis revealed that IL-6^+ $CD4^+$ T cells have a distinct TCRβ usage compared to their IL-

6^- counterparts, indicating that these cells might also recognize other antigens than their IL-6^- counterparts.

Since a cross-talk between adipocytes and T cells have been suggested [20, 21], we investigated the location of these IL-6^+ $CD4^+$ T cells. We observed that IL-6^+ $CD4^+$ T cells are scattered throughout the adipose tissue. Furthermore, adipocytes were capable of enhancing IL-6 production by $CD4^+$ T cells. Although the function of the IL-6^+ $CD4^+$ T cells remains unclear, it is possible that these T cells in turn can modulate the function of adipocytes as IL-6 has been shown to affect adipocytes and enhance lipolysis [22, 23], limiting the expansion of adipocytes. This would suggest a cross-talk between adipocytes and T cells, where IL-6^+ $CD4^+$ T cells would have a regulatory function in adipose tissue inflammation.

Obesity is known to be a major risk factor for OA [1-5]. In addition, obesity is usually accompanied by adipose tissue inflammation and therefore, in **chapter 4**, we aimed to investigate the influence of obesity on the IFP of OA patients as such data is lacking. Surprisingly, we could not observe any obesity-related changes in the IFP regarding IFP volume, adipocyte volume and size, crown-like structures, immune cell infiltrate and secretion profile.

It remains unknown why these obesity-related features in the IFP are lacking. It could be hypothesised that the space in the knee joint is limited and that the IFP is therefore limited in its growth. However, it could also be that the adipocytes are metabolically less active and therefore limited in their uptake of free fatty acids and capacity to store them in lipid droplets, subsequently preventing their growth with obesity. The lack of growth of the adipocytes could prevent the whole cascade of their death, infiltration of macrophages and other immune cells normally present in adipose tissue inflammation. Whether or not adipocytes in the IFP are metabolic less active remains to be elucidated.

Although we did not observe obesity-related features in the IFP, we did identify macrophages expressing markers associated with an anti-inflammatory phenotype (CD206 and CD163), while secreting predominantly pro-inflammatory cytokines (TNFα and IL-6). Since $CD163^+$ has been implicated to be involved in wound healing [24-26] and inflammation[27-31], we further investigated this population and revealed that $CD163^+$ macrophages are pro-inflammatory,

larger in size and have a more activated phenotype compared to their $CD163^-$ counterparts.

Part 2 Cellular immunometabolism

In the second part of this thesis we aimed to determine how fatty acids exert their effect on $CD4^+$ T cells. In **chapter 5** we started by reviewing existing literature regarding the effects of fatty acids and lipid mediators, oxygenized fatty acids, on T cells and their function. In this review, we proposed a mechanism by which free fatty acids exert their effects on T cells. Although the mechanism remains unknown, free fatty acids enter the cell and are incorporated into neutral lipids such as phospholipids, triacylglycerol and cholesterol esters. Low concentrations of fatty acids induce proliferation and cytokine production. However, high concentrations of fatty acids induce depolarization of the mitochondrial membrane and intrinsic apoptotic pathways, which eventually leads to apoptosis.

Fatty acids have been shown to be capable of enhancing proliferation of $CD4^+$ T cells [32], but the mechanisms underlying the enhanced proliferation are unknown. Several mechanisms can, however, be hypothesized: 1) fatty acids can be used as building blocks for their daughter cells, 2) degraded through fatty acid oxidation and serve as energy, or 3) can influence the signalling of the T cell. Therefore, in **chapter 6**, we aimed to gain insight into the underlying mechanisms of the enhanced proliferation of $CD4^+$ T cells in the presence of fatty acids. We used oleic acid, a fatty acid, which is known to be capable of enhancing proliferation of $CD4^+$ T cells. We observed that oleic acid is capable of inducing proliferation even when removed after 24 hr, indicating that early-induced changes occur. This is supported by the finding that after 24 hr we could find enhanced incorporation of ^{3}H-Thymidine, suggesting that cells are preparing for cell division. To study the effect of oleic acid on the metabolism of the cells, we performed functional metabolic analysis and found that both glycolysis and oxidative phosphorylation (OXPHOS) were unaffected by the supplementation with oleic acid. These findings suggest that oleic acid is not used as energy source. Metabolomics analyses revealed that oleic acid induced a modest increase in glycolysis (phosphoenolpyruvate), TCA cycle intermediates

(citrate) and pyrimidine synthesis, however, oleic acid was not used as substrate to fuel this. Furthermore, metabolomics analyses revealed that, rather than been broken down, oleic acid is incorporated in phosphatidylcholines and when added in higher amounts also in triglycerides. Oleic acid supplementation did not affect baseline calcium flux, although calcium flux responses after TCR stimulation resulted in a higher response. Even though calcium flux was influenced by oleic acid, phosphorylation of ZAP70 was not influenced.

In our study, we found that oleic acid induces pyrimidine syntheses, both observed in the metabolomics studies and the incorporation of ^{3}H-Thymidine. As pyrimidines are key components of DNA this suggests that CD4^{+} T cells are preparing for division. However, it has been shown that pyrimidines are key regulators of the cell cycle as well, since they control the progression through the S phase of the cell cycle [33]. This suggests that oleic acid supplementation can influence progression through the cell cycle by the induction of pyrimidine synthesis. In addition, pyrimidines are important for membrane lipid synthesis [34], and oleic acid is known to be incorporated into phosphatidylcholines which could suggest that the enhanced pyrimidine synthesis is important for generating phosphatidylcholines with oleic acid. However, it remains unknown whether the enhanced pyrimidine synthesis by oleic acid supplementation enhances progression through the cell cycle or the pyrimidines are used for DNA synthesis or membrane lipid synthesis. Therefore, further research is needed.

Furthermore, in our study we observed that oleic acid is incorporated in phosphatidylcholines, which are key components of membranes. Our study indicated that oleic acid containing phosphatidylcholines are incorporated into existing membranes as blocking the formation of newly synthesized fatty acids did not influence the stimulation index of oleic acid. However, it remains to be elucidated into which membranes these oleic acid containing phosphatidylcholines are incorporated. Incorporation of phosphatidylcholines into the cell membrane could influence the membrane fluidity and subsequently influence the TCR signalling. However, although we did find enhanced calcium fluxes with the supplementation of oleic acid, we could not find any differences in the phosphorylation of ZAP70. This could suggest that although ZAP70 is not influenced other down-stream molecules such as LAT or PLCγ1 could

be influenced by supplementation with oleic acid. Future research should focus on the effect of oleic acid supplementation on different aspects of TCR signalling, such as membrane fluidity, the formation of lipid rafts, and other TCR downstream molecules. This could indicate as to how oleic acid incorporation into phosphatidylcholines could enhance the proliferation of T cells treated with oleic acid.

Final conclusions

Our studies indicate that the inflammatory state of the joint does affect the cellular load of the IFP as the IFP of RA patients had a higher cellular infiltrate compared to IFP of OA patients. However, despite the higher cellular load, the secretory profile did not seem to be affected by the inflammatory state, indicating that the IFP has little contribution to a higher cytokine load in the SF (**chapter 2**). The lack of difference in secretory profile of the IFP could indeed be due to the limited contribution of immune cells to the secretion of adipocytokines to the SF in both RA and OA, therefore, it would have been of interest to determine the SF cytokine load of the patients included in this study, to confirm this hypothesis. However, as both RA and OA patient were end stage patients, this could also have affected the secretion profile of the IFP. Given the fact that these patients were end stage patients, the results obtained in this study cannot be extrapolated to earlier stages as inflammation in all joint tissues could be different at earlier disease stages.

The higher cellular load of the IFP of RA patients compared to OA patients is as expected, as RA patients are known to have a higher inflammatory load [12, 13]. However, as RA is an autoimmune disease, whereas OA is not considered to be an autoimmune disease it is surprisingly that the nature of inflammation in IFP was comparable between RA and OA IFP. Only the number of mast cells were higher in RA IFP compared to OA IFP, which is in contrast to mast cells present in synovium. This suggests that there might be differences between OA and RA that control the number of mast cells present in tissues. However, the signal controlling the mast cell presence and the origin of this signal remains to be elucidated. Furthermore, B cells were virtually absent in both RA and OA patients, while B cells have been implicated to play an important role in the

pathogenesis of RA. These data suggest little contribution of the IFP to the inflammatory processes in the joint.

Furthermore, our study indicated that obesity has little to no effect on the IFP (**chapter 4**), which is supported by recent findings [35]. However, another recent study demonstrated that some obesity-related features, such as adipocyte size and cellular infiltrate in the IFP were influenced by obesity [36]. This discrepancy is possibly due to the BMI of the patients studied, which were higher in the latter study. Further research is therefore needed. In addition, as obesity has little to no effect on the IFP this suggest that IFP does not behave as other adipose tissues with obesity. This is supported by the findings that IFP is metabolically more active than other adipose tissues such as subcutaneous adipose tissue [35, 37-39]. Although the cellular source is unknown, we did observe that TNFα secretion by IFP was BMI dependent (**chapter 4** and Klein-Wieringa et al.[37]). Since TNFα is implicated in the pathophysiology of OA this could be one of the underlying mechanisms for the association between obesity and OA. In addition, free fatty acids are known to be secreted in a BMI dependent manner by adipocytes, and are capable of modulating the immune response. Therefore, IFP could still contribute to the inflammatory processes in the joint through the secretion of soluble factors.

When characterizing the IFP we found two interesting cell population, the first being a population of T cells, secreting IL-6 directly *ex vivo* (**chapter 3**) and the second being a population of macrophages with an anti-inflammatory phenotype secreting pro-inflammatory cytokines (**chapter 4**). Both populations could be involved in the pathophysiology of the osteoarthritic joint.

It could be hypothesised that IL-6$^+$ CD4$^+$ T cells in the IFP are involved in the pathophysiology of the osteoarthritic joint through a cross-talk with adipocytes, as we demonstrated that adipocytes are capable of enhancing IL-6 in CD4$^+$ T cells (**chapter 3**) and it is known that IL-6 can modulate adipocytes [22, 23]. Overall, this would imply that IL-6$^+$ CD4$^+$ T cells would have a regulatory role as IL-6 enhances lipolysis and thereby limiting expansion of adipocytes. Although we could not observe a correlation between the number of IL-6$^+$ T cells in the IFP with BMI, this cross-talk between IL-6$^+$ CD4$^+$ T cells and adipocytes could be underlying the lack of obesity-related features in the IFP (**chapter 4**). However,

the signals mediating the cross-talk between adipocytes and IL-6$^+$ T cells besides the IL-6 secreted by the CD4$^+$ T cells still remain unknown. As adipocytes are capable of secreting various factors which are capable of influencing CD4$^+$ T cells [21, 32], future studies should elaborate on the factors mediating the cross-talk between adipocytes and IL-6$^+$ CD4$^+$ T cells. Among the factors secreted by adipocytes are free fatty acids, which are known to be secreted by adipocytes in a BMI-dependent manner [40]. Free fatty acids are capable of inducing proliferation of T cells and can influence cytokine production by T cells. Whether IL-6 production by T cells is affected by free fatty acids remains to be elucidated.

The population of macrophages in the IFP which has an anti-inflammatory phenotype, while secreting pro-inflammatory cytokines (**chapter 4**) could also play an important role in the pathophysiology of the osteoarthritic joint. As these CD163$^+$ macrophages display a more activated state and are larger than their CD163$^-$ counterparts, this could imply that they have been scavenging up dead adipocytes, thereby acquiring more lipids. This is supported by the expression of CD206 by these CD163$^+$ macrophages, as recently a study in mice showed that phagocytosis by macrophages leads to upregulation of both CD206 and CD163 [41]. Thus CD163$^+$ macrophages could play an important role in adipose tissue inflammation.

In OA patients, the percentage of CD4$^+$ T cells in the synovium is associated with VAS pain [19], suggesting that CD4$^+$ T cells could play a role in pain perception in knee OA patients. Therefore, CD4$^+$ T cells might represent the cellular basis for the association between synovitis and pain in OA patients. Furthermore, the percentage of CD4$^+$ T cells in the synovium also correlates with BMI [19]. This association might be mediated by fatty acids, as fatty acids greatly enhance the proliferation of CD4$^+$ T cells (chapter 5 and 6). Moreover, adipocytes from the IFP are capable of secreting free fatty acids in a BMI dependent manner [40], and the levels of free fatty acids in the serum of obese persons is elevated [42-44].

Future perspectives

Overall, our data indicate that obesity-related features normally observed in adipose tissue are not present in the IFP. However, this does not imply that the

IFP is not involved in the pathophysiology of OA. IFP could still play an important role through the secretion of fatty acids and possibly other mechanisms. To further elucidate the role of the IFP in the pathophysiology of OA several lines of investigations could be initiated.

As the IL-6$^+$ T cells could play a role in the pathophysiology of OA, the suggested cross-talk between IL-6$^+$ T cells and adipocytes in the IFP should be studied in further detail. For example, the signals mediating the cross-talk between IL-6$^+$ T cells and adipocytes should be investigated, in particular whether and which fatty acids are capable of enhancing IL-6 by T cells. Conversely, the effect of IL-6$^+$ T cells on adipocytes and other cells should be investigated, to evaluate both the function of these cells in adipose tissue, and whether IL-6 is indeed the main effector molecule engaged by IL-6$^+$ T cells to exert their function.

Obesity-associated changes in IFP deserve further attention, especially in the light of a recent study in which changes within IFP were detected in a group of patients with very high BMI. Future studies should include not only an in-depth characterization of different macrophage subsets and their functional role in IFP, but also a better characterization of adipocytes in IFP, including the possibility that IFP adipocytes are metabolically less active than their SCAT counterparts, as this is largely unexplored area of research. Furthermore, the cellular source of TNFα has been implicated in the pathophysiology of OA.

Our previous findings indicated that CD4$^+$ T cells could represent the cellular basis for the association between synovitis and pain in knee OA patients. Because BMI-related increases in free fatty acid concentrations could explain the correlation between the percentage of CD4$^+$ T cells in the OA synovium and BMI, it could be of importance to further elucidate the mechanisms by which fatty acids exert their effects on CD4$^+$ T cells. Although with our studies we excluded the possibility that oleic acid is used as energy source, it is still possible that oleic is incorporated in building blocks for daughter cells or influences signalling of the T cells or a combination of both. Therefore, future research should focus on understanding in which cellular compartments oleic acid is incorporated in phosphatidylcholines and how this can influence cell signalling and proliferation.

Moreover, the effect of oleic acid on the signalling of T cells should be investigated as we have observed that calcium signalling is enhanced in the presence of oleic acid. Different aspects of TCR signalling, such as membrane fluidity, the formation of lipid rafts, and other TCR downstream molecules should be investigated. These studies combined could suggest whether the enhanced proliferation in the presence of oleic acid is due to the fact that oleic acid is used as building block for the daughter cells or is influencing the signalling of the T cells.

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Chapter 8

Nederlandse samenvatting

Osteoartrose (OA) is een heterogene aandoening die alle gewrichten van het lichaam kan treffen, maar het komt vooral voor in heup-, knie- en handgewrichten. Lang dacht men dat OA enkel een ziekte was die veroorzaakt wordt door slijtage, maar tegenwoordig wordt het gezien als een complexere ziekte waarbij ontstekingsfactoren en alle gewrichtscompartimenten, zoals kraakbeen, bot en het gewrichtsvlies (synovium) een rol spelen [1, 2]. Overgewicht is een belangrijke factor die geassocieerd is met de ontwikkeling en progressie van OA. Oorspronkelijk werd gedacht dat de toegenomen druk op de gewrichten de oorzaak is, maar de associatie tussen OA en obesitas is niet alleen voor gewicht dragende gewrichten [3-5], maar ook voor niet-gewicht dragende gewrichten zoals de handen [5-7]. Dit suggereert dat niet alleen mechanische factoren een rol spelen bij de associatie tussen OA en obesitas, maar ook systemische factoren. Deze kunnen onder andere bestaan uit milde ontstekingsreacties, een verstoord lipide metabolisme, glucose metabolisme en/of adipokines [4].

Immunometabolisme is een nieuw veld dat immunologie met metabolisme verbind. Hierbij wordt niet alleen gekeken naar de interactie tussen de stofwisseling van het lichaam en het immuunsysteem, maar ook naar de interactie tussen de stofwisseling op cellulair niveau en de immuuncel functies.

Van vetweefsel werd lang aangenomen dat het fungeert als energieopslag van het lichaam, maar inmiddels is aangetoond dat vetweefsel een metabool en endocrien actief orgaan is dat het hele lichaam kan beïnvloeden [8-10]. Met overgewicht zijn er allerlei veranderingen waar te nemen in het vetweefsel: de adipocyten (ofwel vetcellen) vergroten, immuuncellen infiltreren het vetweefsel en pro-inflammatoire adipocytokines worden uitgescheiden. Dit zorgt ervoor dat het vetweefsel een algehele pro-inflammatoire staat krijg in plaats van de anti-inflammatoire staat bij een normaal gewicht.

In het kniegewricht zit een vetlichaam, genaamd Hoffa's vetlichaam, waarvan gedacht wordt dat het een belangrijke rol kan spelen in OA. Echter, de contributie van Hoffa's vetlichaam bij de ontstekingsprocessen in het gewricht en het effect van obesitas op het vetlichaam zijn onbekend. Daarom hebben we in het eerste deel van het proefschrift Hoffa's vetlichaam gekarakteriseerd zowel op cellulair als moleculair niveau om de mogelijke rol van Hoffa's vetlichaam in de pathofysiologie van OA te bepalen.

Als eerste stap hebben we in de studies beschreven in **hoofdstuk 2** Hoffa's vetlichaam van reumatoïde artritis (RA) patiënten met Hoffa's vetlichaam van OA patiënten vergeleken. In beide ziekten speelt ontsteking een rol, maar RA heeft over het algemeen gesproken meer ontsteking in het synovium [11] en het synoviale vocht [12-14] dan OA. We hebben de hoeveelheid van adipocytokines uitgescheiden door het vetlichaam (vet-geconditioneerd medium) en adipocyten (adipocyten-geconditioneerd medium) bepaald en vonden dat zowel vetlichaam als adipocyten in staat zijn om verschillende adipocytokines uit te scheiden. Echter, er bleek geen verschil te zijn tussen RA en OA patiënten.

Het karakteriseren van Hoffa's vetlichaam op cellulair niveau liet zien dat Hoffa's vetlichaam van RA-patiënten wel een hoger cel aantal heeft dan Hoffa's vetlichaam van OA patiënten, maar de percentages van de verschillende cel subsets, zoals T cellen, macrofagen en endotheelcellen waren vergelijkbaar tussen RA en OA patiënten. Het enige waargenomen verschil was het percentage mestcellen, dit percentage was lager in OA patiënten ten opzichte van RA patiënten. Deze bevindingen zijn in tegenstelling met de bevindingen van het synovium zoals beschreven in **hoofdstuk 2** en [11]. Onze bevindingen suggereren dat Hoffa's vetlichaam een verwaarloosbare rol speelt in de ontsteking van andere weefsels in het gewricht zoals het synovium of synoviaalvocht van RA patiënten aangezien er geen verschil is in het profiel van uitgescheiden adipocytokines tussen RA patiënten en OA patiënten. Echter, het aantal cellen in Hoffa's vetlichaam zou wel de hogere cellulaire ontsteking in het gewricht van RA patiënten kunnen vertegenwoordigen. Zoals verwacht is er een verhoogd aantal cellen in Hoffa's vetlichaam van RA patiënten ten opzichte van Hoffa's vetlichaam in OA patiënten. RA patiënten hebben namelijk vaak een hogere ontsteking dan OA patiënten. Echter, het is verrassend dat de aard van de ontsteking in Hoffa's vetlichaam vergelijkbaar was tussen het Hoffa's vetlichaam van RA en OA patiënten, aangezien RA gezien wordt als een auto-immuunziekten in tegenstelling tot OA.

In het vetlichaam zijn verschillende immuuncellen aanwezig. Van T cellen wordt gedacht dat ze een grote rol spelen bij de milde ontsteking in vetweefsel [15-18]. Meerdere studies hebben aangetoond dat T cellen in het vetweefsel een beperkt T cel receptor (TCR) repertoire hebben [15, 17-19]. Dit suggereert dat ze clonale uitbreiding hebben ondergaan en waarschijnlijk vetweefsel antigenen herkennen. Verrassend genoeg zijn T cellen uit Hoffa's vetlichaam in staat om direct *ex vivo*

IL-6 uit te scheiden [20]. Dit suggereert dat deze T cellen recent geactiveerd zijn, misschien wel in het vetlichaam zelf. Daarom hebben we in de studies beschreven in **hoofdstuk 3** deze IL-6 positieve populatie verder onderzocht en gekarakteriseerd. We bevestigden de bevinding dat $CD4^+$ T cellen uit Hoffa's vetlichaam in staat zijn om IL-6 direct *ex vivo* uit te scheiden en konden deze IL-6 positieve T cellen ook in synovium, bloed en subcutaan vetweefsel aantonen.

Fenotypische karakterisering liet zien dat deze $IL\text{-}6^+$ $CD4^+$ T cellen conventionele (TCRαβ) T cellen zijn met een specifiek geactiveerd geheugen, wat onze hypothese ondersteunt dat ze recent zijn geactiveerd. Daarnaast hebben we cytokine-productie en chemokine-receptor expressie levels bepaald waaruit bleek dat we deze populatie niet aan een bepaalde T helper subset kunnen toewijzen. Verder onthulde TCRβ gen-analyse dat $IL\text{-}6^+$ $CD4^+$ T cellen een andere TCRβ gebruik hebben dan $IL6^-$ $CD4^+$ T cellen, dit zou kunnen betekenen dat $IL\text{-}6^+$ $CD4^+$ T cellen andere antigenen herkennen dan hun $IL6^-$ tegenhangers.

Aangezien eerdere studies hebben gesuggereerd dat er een samenspraak is tussen adipocyten en T cellen [21, 22], hebben we de locatie van de $IL\text{-}6^+$ $CD4^+$ T cellen in het vetlichaam bepaald. We vonden dat de $IL\text{-}6^+$ $CD4^+$ T cellen verspreid liggen door het vetlichaam. Verder vonden we dat adipocyten in staat waren om IL-6 productie door $CD4^+$ T cellen te verhogen. Ook al blijft de functie van de $IL\text{-}6^+$ $CD4^+$ T cellen onduidelijk, het is heel goed mogelijk dat deze cellen op hun beurt adipocyten kunnen beïnvloeden, temeer omdat aangezien van IL-6 bekend is dat het de lipolyse van adipocyten kan verhogen. Dit beperkt de toename in grootte van adipocyten beperkt [23, 24], en suggereert dat er inderdaad een samenspraak is tussen adiopcyten en T cellen, waar $IL\text{-}6^+$ $CD4^+$ T cellen een regulatoire functie hebben bij de ontsteking in het vetlichaam en daardoor een rol kunnen spelen in de pathofysiologie van OA.

Obesitas staat bekend als een belangrijke risicofactor voor de ontwikkeling en progressie van OA [3-7]. Daarnaast gaat obesitas vaak gepaard met ontsteking van het vetweefsel. In de studies beschreven in **hoofdstuk 4** hebben we de mogelijke invloed van obesitas op Hoffa's vetlichaam bij OA patiënten beschreven, omdat kennis hierover ontbreekt. Verrassend genoeg konden we geen obesitas gerelateerde veranderingen in Hoffa's vetlichaam aan tonen; we onderzochten het volume van Hoffa's vetlichaam, het volume en formaat van de adipocyten,

de zogenaamde "crown-like structures", de infiltrerende immuuncellen en de cytokines die door het vetlichaam, de adipocyten en de immuuncellen uitgescheiden worden.

Het blijft onduidelijk waarom obesitas gerelateerde kenmerken niet aanwezig zijn in Hoffa's vetlichaam. Een verklaring hiervoor zou kunnen zijn dat er simpelweg te weinig ruimte is in het gewricht waardoor Hoffa's vetlichaam beperkt is in zijn groei. Maar het zou ook kunnen dat de adipocyten in Hoffa's vetlichaam metabool minder actief zijn en daardoor beperkt zijn in de opname van vetzuren en de opslag in vetdruppels, waardoor ze gehinderd zijn in hun vermogen om te groeien met obesitas. De onmogelijkheid van de adipocyten om te groeien zou dan het hele proces van hun dood, infiltratie van macrofagen en andere immuuncellen, dat normaal gesproken plaats vindt in vetweefsel, kunnen voorkomen en daardoor ook het optreden van ontsteking. Of adipocyten in Hoffa's vetlichaam echt minder metabolisch zijn moet verder onderzoek uitwijzen.

In onze studie konden we geen obesitas gerelateerde veranderingen in Hoffa's vetlichaam vinden. Hieruit blijkt dat dit vetlichaam zich anders dan vetweefsel zoals bijvoorbeeld subcutaan vetweefsel gedraagt. Deze bevindingen worden ondersteund door de bevinden dat Hoffa's vetlichaam metabool actiever is dan ander vetweefsel zoals subcutaan vetweefsel [25-28]. Ook al weten we niet wat de cellulaire bron is, we hebben wel gevonden dat de secretie van TNFα door Hoffa's vetlichaam BMI-afhankelijk is (hoofdstuk 4 en Klein-Wieringa et al. [26]). Aangezien TNFα is betrokken bij de pathofysiologie van OA, zou het Hoffa's vetlichaam op deze manier een rol kunnen spelen.

Ook al hebben we geen obesitas gerelateerde kenmerken gevonden in Hoffa's vetlichaam, we hebben wel macrofagen geïdentificeerd die geassocieerd zijn met een anti-inflammatoir fenotype (CD206 en CD163) terwijl ze pro-inflammatoire cytokines (TNFα en IL-6) uitscheiden. Aangezien $CD163^+$ is betrokken bij wondgenezing [29-31] en ontsteking [32-36] hebben we deze macrofaag subpopulatie verder onderzocht. Daarbij hebben we gevonden dat $CD163^+$ macrofagen in Hoffa's vetlichaam pro-inflammatoir, groter en meer geactiveerd zijn vergeleken met hun $CD163^-$ tegenhangers. Aangezien deze $CD163^+$ macrofagen groter en actiever zijn, zou dit kunnen betekenen dat deze macrofagen dode adipocyten hebben opgeruimd en daarbij vetten hebben opgenomen. Dit zou

kunnen betekenen dat deze CD163$^+$ macrofagen een belangrijke rol spelen in vetweefsel ontsteking en daarbij betrokken zijn bij de pathofysiologie van OA.

Eerdere bevindingen laten zien dat CD4$^+$ T cellen de cellulaire basis kunnen zijn voor de associatie tussen synovitis en pijn in de knie van OA patiënten [20]. Aangezien de uitscheiding van vetzuren door Hoffa's vetlichaam is geassocieerd met BMI en vetzuren CD4$^+$ T cellen kunnen beïnvloeden zou dit de correlatie tussen het percentage CD4$^+$ T cellen in het OA synovium en met BMI kunnen verklaren. Daarom is het van belang dat de mechanismen waarmee vetzuren T cellen kunnen beïnvloeden bestudeerd worden. Daarvoor hebben we in het tweede deel van dit proefschrift onderzocht hoe vrije vetzuren hun effect uitoefenen op CD4$^+$ T cellen. Hiervoor hebben we eerst in **hoofdstuk 5** een overzicht gemaakt van de bestaande literatuur met betrekking tot de effecten van vrije vetzuren en lipide mediatoren (geoxideerde vetzuren) op T cellen en hun functie. In dit overzicht hebben we een model voorgesteld hoe vrije vetzuren hun effect op T cellen uitoefenen. Ook al is het mechanisme nog onbekend, het is wel duidelijk dat vrije vetzuren de cel in gaan. In de cel kunnen ze geïncorporeerd worden in neutrale vetzuren zoals fosfolipiden, triglyceriden en cholesterolesters. Lage concentraties van vetzuren kunnen proliferatie en cytokineproductie opwekken, terwijl hoge concentraties van vetzuren kunnen leiden tot depolarisatie van het mitochondriale membraan. Dit kan geprogrammeerde celdood en apoptose tot gevolg hebben.

Eerder is beschreven dat vetzuren in staat zijn om de proliferatie van CD4$^+$ T cellen op te wekken [37], maar het onderliggende mechanisme voor de verhoogde proliferatie is onbekend. Hypothetisch zijn meerdere mechanismes mogelijk: vetzuren kunnen 1) gebruikt worden als bouwsteen voor dochter cellen, 2) afgebroken worden door vetzuur oxidatie en als energiebron dienen, of 3) de signalering van de T cel beïnvloeden. Daarom was ons doel in de studies beschreven in **hoofdstuk 6** om meer inzicht te verkrijgen in de onderliggende mechanismes van de verhoogde proliferatie van CD4$^+$ T cellen in de aanwezigheid van vetzuren. In ons onderzoek hebben we oliezuur gebruikt, want hiervan is bekend dat het de proliferatie van CD4$^+$ T cellen kan verhogen. We vonden dat oliezuur in staat is om proliferatie te induceren, zelfs als het verwijderd werd na 24 uur. Dit suggereert dat de geïnduceerde veranderingen in een vroeg stadium plaatsvinden. Dit wordt ook ondersteund door de vinding dat na 24 uur meer ^{3}H-Thymidine geïncorporeerd is, wat suggereert dat de cellen zich voorbereiden op celdeling.

Om het effect van oliezuur op het metabolisme van de cellen te bepalen hebben we functionele analyses naar het metabolisme uitgevoerd en vonden we dat zowel glycolyse als oxidatieve fosforylering niet beïnvloed werden door het toevoegen van oliezuur. Deze bevindingen suggereren dat oliezuur niet gebruikt wordt als energiebron door de T cel. Uitgebreide analyse van het metabolisme van de T cellen toonde aan dat oliezuur wel een bescheiden verandering opriep in de glycolyse (fosfoenolpyruvaat), tussenproducten van de citroenzuurcyclus (citraat) en de pyrimidine synthese, maar dat oliezuur hier zelf niet voor gebruikt werd. Verder toonde de analyse van het metabolisme aan dat oliezuur niet afgebroken wordt, maar geïncorporeerd wordt in fosfatidylcholinen. Wanneer oliezuur werd toegevoegd in een hogere concentraties werd het ook in triglycerides geïncorporeerd.

Oliezuur toevoeging resulteerde niet in veranderingen in basale levels van de calciumflux, maar de calciumflux was wel verhoogd na T cel receptor stimulatie in aanwezigheid van oliezuur. Ook al was de calciumflux wel beïnvloed door oliezuur, de fosforylatie van ZAP70 was niet beïnvloed.

Toekomstperspectieven

De data beschreven in dit proefschrift laten zien dat obesitas-gerelateerde kenmerken, die normaal gesproken aanwezig zijn in vetweefsel, niet aanwezig zijn in Hoffa's vetlichaam. Echter, dit impliceert niet dat Hoffa's vetlichaam helemaal geen rol heeft in de pathofysiologie van OA. Hoffa's vetlichaam kan nog steeds een belangrijke rol spelen door het uitscheiden van vetzuren of mogelijk andere mechanismen. Om verder uit te zoeken wat de rol van Hoffa's vetlichaam is in de pathofysiologie van OA is het belangrijk om meerdere onderzoekslijnen op te zetten.

Aangezien IL-6$^+$ T cellen een rol kunnen spelen in de pathofysiologie van OA zou verder onderzoek zich moeten richten op de mogelijke samenspraak tussen adipocyten en IL-6$^+$ T cellen in Hoffa's vetlichaam en de daarbij behorende signalen. Daarnaast zou onderzocht moeten worden of vetzuren, die uitgescheiden kunnen worden door adipocyten in Hoffa's vetlichaam in staat zijn om de IL-6 productie van T cellen te verhogen. Deze studies zouden uit kunnen wijzen of deze IL-6$^+$ T cellen een regulatoire rol hebben in Hoffa's vetlichaam.

Verder zou het effect van obesitas op Hoffa's vetlichaam verder onderzocht moeten worden aangezien een recente studie met een populatie van patiënten met een hoog BMI enkele obesitas-gerelateerde kenmerken in Hoffa's vetlichaam heeft aangetoond. Maar de mogelijkheid dat de adipocyten in Hoffa's vetlichaam minder metabool actief zijn dan de adipocyten in subcutaan vetweefsel moet ook onderzocht worden. Daarnaast is het belangrijk om de bron van TNFα te vinden, aangezien deze op een BMI-afhankelijke manier uitgescheiden wordt door het vetweefsel en TNFα betrokken is in de pathofysiologie van OA.

Zoals eerder beschreven kunnen $CD4^+$ T cellen de cellulaire basis zijn voor de associatie tussen synovitis en pijn in de knie van OA patiënten. Vetzuren zouden wellicht het onderliggende mechanisme kunnen zijn dat hier ten grondslag ligt. Daarom is het van belang om de mechanismen waarmee vetzuren T cellen kunnen beïnvloeden te ontrafelen. Uit onze studies blijkt dat oliezuur eigenlijk niet gebruikt wordt als energiebron voor de delende cellen, maar het lijkt wel een bouwsteen voor de dochtercellen te kunnen zijn en de signalering van de T cel te kunnen beïnvloeden. Echter, verder onderzoek is nog nodig. Het blijft namelijk onduidelijk of de verhoogde synthese van pyrimidine door de toevoeging van oliezuur nu belangrijk is voor de progressie door de celcyclus of dat de pyrimidines juist gebruikt worden voor DNA-synthese of lipide synthese. Daarnaast is het onbekend in welke membranen de oliezuur bevattende fosfatidylcholines geïncorporeerd worden. Inbouw van deze fosfatidylcholines in het celmembraan zou de vloeibaarheid kunnen beïnvloeden en daarmee ook de signalering van de cel.

Met de kennis die voortkomt uit deze onderzoekslijnen zou de rol van Hoffa's vetlichaam in de pathofysiologie van OA verder ontrafeld kunnen worden.

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Chapter 9

List of publications

Curriculum vitae

Dankwoord

List of publications

Anja J. de Jong, M. Kloppenburg, R.E.M. Toes and A. Ioan-Facsinay. Fatty acids, lipid mediators, and T-cell function. Frontiers in Immunology. 2014; 5: 483.

Anja J. de Jong, I.R. Klein-Wieringa, J.C. Kwekkeboom, R.E.M. Toes, M. Kloppenburg, A. Ioan-Facsinay. Inflammatory features of infrapatellar fat pad in rheumatoid arthritis versus osteoarthritis reveal mostly qualitative differences. Annals of the Rheumatic Diseases. Aug 2017

Anja J. de Jong[*], I.R. Klein-Wieringa[*], S.N. Andersen, J.C. Kwekkeboom, L. Herb-van Toorn, B.J.E. de Lange-Brokaar, D. van Delft, J. Garcia, W. Wei, H.J.L. van der Heide, Y.M. Bastiaansen-Jenniskens, G.J.V.M. van Osch, A.M. Zuurmond, V. Stojanovic-Susulic, R.G.H.H. Nelissen, R.E.M. Toes, M. Kloppenburg, A. Ioan-Facsinay. Lack of high BMI-related features in adipocytes and inflammatory cells in the infrapatellar fat pad (IFP). Arthritis Research & Therapy. 2017; 19: 186.
* Both authors contributed equally to the manuscript

Curriculum vitae

Antje Jacoba de Jong (Anja) werd geboren op 15 februari 1989 te Bovenkarspel. Na het doorlopen van de basisschool in Enkhuizen behaalde zij in 2007 haar VWO-diploma aan het RSG Enkhuizen. In datzelfde jaar begon zij aan de Bachelor Biomedische Wetenschappen aan de Vrije Universiteit van Amsterdam met een focus op infectieziekten en immunologie. In het laatste jaar van de Bachelor liep ze stage in de groep van Anje te Velde bij het Tytgat instituut van het AMC. Aansluitend aan haar Bachelor begon zij in 2010 aan de Master Biomedical Sciences aan de Vrije Universiteit waar zij zich verder specialiseerde in infectieziekten en immunologie. In haar Master heeft ze haar eerste stage gelopen in de groep van David Greaves bij de Sir William Dunn School of Pathology van de University of Oxford. Haar laatste stage liep zij bij de groep van Monika Wolkers van de afdeling Hematopoiesis van Sanquin. In 2012 behaalde zij haar Masterdiploma. Van september 2012 tot september 2016 heeft zij haar promotieonderzoek, waarvan de resultaten in dit proefschrift staan beschreven, verricht op de afdeling reumatologie van het LUMC onder leiding van dr. Andreea Ioan-Facsinay, Prof. dr. René Toes en Prof. dr. Margreet Kloppenburg. Op dit moment is Anja naar Londen verhuisd om daar aan nieuwe uitdaging te beginnen.

Dankwoord

Ook al staat er op de voorkant van dit proefschrift maar één naam, zonder de hulp van velen zou het niet tot stand gekomen zijn. Ik wil dus graag van deze gelegenheid gebruik maken om ze te bedanken.

Allereerst gaat mijn dank uit naar mijn promotoren, Prof. dr. René Toes en Prof. dr. Margreet Kloppenburg. Beste René, dank voor je begeleiding en essentiële input tijdens alle besprekingen. Je kennis van de laatste literatuur is ongelofelijk en daar kunnen we allemaal nog wat van leren. Beste Margreet, dank voor je inzichten en klinische blik op fundamenteel onderzoek. Je enthousiasme voor artrose en onderzoek zijn een voorbeeld voor velen.

Uiteraard gaat mijn dank ook uit naar mijn co-promotor, Dr. Andreea Ioan-Facsinay. Beste Andreea, de afgelopen jaren heb ik je leren kennen als een kritisch en stimulerend persoon. Je hebt me de mogelijkheden gegeven om mezelf te leren kennen en er een mooi eindresultaat van te maken.

Het bedanken van het vetgroepje mag natuurlijk niet ontbreken in mijn proefschrift. Als eerste Joanneke, dank je wel voor alles! Mede dankzij jouw pipeteerkunsten en de gezelligheid in het lab is dit proefschrift er nu. Ik vind het dan ook een eer dat je mijn paranimf wil zijn en naast me wilt staan tijdens mijn verdediging! Daarnaast wil ik Inge, Hilde, Stefan en Joost ook heel erg bedanken voor hun inzichten tijdens meetings, maar vooral ook de gezelligheid die we in ons kleine labje hadden!

Het artrose groepje bestaande uit Marion, Badelog, Willemien, Wendy, Féline en Sjoerd wil ik ook graag bedanken voor de maandagochtend besprekingen. Dank jullie wel voor jullie input, visies en natuurlijk de congressen waar we samen naar toe zijn geweest.

Natuurlijk was dit proefschrift niet tot stand gekomen zonder de hulp van de patiënten, de OK-secretaresses en de chirurgen van zowel het Alrijne ziekenhuis als het LUMC. Graag wil ik jullie allen bedanken.

Mede dankzij de gezellige mensen op de reumatologie afdeling zijn de jaren voorbijgevlogen. Op C5 was het altijd een gezellige boel en er was altijd wel

iemand te porren voor een thee of koffie loopje. Ayla, Diahann, Daniel, Hanane, Hendy, Hester, Jacqueline, Jeroen, Jolien, Jurgen, Kim, Leendert, Linda, Marije, Myrthe, Lise, Marieke, Rochelle, Rosalie, Sanne en Tobias dank jullie wel voor de gezellige sfeer. In het bijzonder wil ik Rosanne bedanken voor onze vriendschap, en de steun van de afgelopen jaren. Daarnaast wil ik ook de analisten bedanken: Aleida, Ellen, Gerrie, Linda, Marjolijn, Nivine en Joris, zonder jullie steun, inzichten en kennis op het lab zou het allemaal een stuk lastiger geweest zijn. Marieke, Mandy en Linda, dank jullie wel voor de gezellige tijd die ik heb gehad met jullie begeleiden.

Tijdens een promotieonderzoek kun je natuurlijk niet zonder de momentjes van ontspanning, daarom wil ik graag mijn VU-matties bedanken voor de borrels, etentjes en gezellige momentjes. Natuurlijk wil ik ook mijn VU-mattie van het eerste uur, Amanda, bedanken. Ik vind het een eer dat je na alle practicums samen nu als paranimf naast me wilt staan tijdens mijn verdediging! Joannah, Ivy and Jon, thank you for your friendship and our adventures together, in Oxford, Cyprus, Paris, Amsterdam and Leiden.

Daarnaast wil ik mijn familie bedanken, voor de ontspannen tante Anja dagen, het borrelen in Leiden met de Versteeg mannen en nog vele andere momentjes. Mijn schoonouders, Auke en Els, bedankt dat bij jullie altijd de deur open staat en ik altijd welkom ben.

Mijn ouders, paps en mams, dank jullie wel voor de steun en het luisterend oor de afgelopen jaren. Jullie steun tijdens mijn studie en promotieonderzoek is van onschatbare waarde. Bedankt dat jullie altijd voor mij klaar stonden en staan. Het is een geruststellende gedachten dat ik altijd bij jullie terecht kan.

Tot slot, wil ik jou bedanken lieve Bart, je bent er de afgelopen jaren altijd voor me geweest. Dank voor je onvoorwaardelijke liefde, steun en geduld om mij te helpen met mijn proefschrift. Hoe bijzonder is het dat we nu een dag na elkaar onze verdediging hebben!