Immunometabolism in osteoarthritis

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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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