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CHAPTER 3

INFLAMMATORY CELLS IN END STAGE KNEE OSTEOARTHRITIS PATIENTS: A COMPARISON BETWEEN THE SYNOVIUM AND THE INFRAPATELLAR FAT PAD (IFP)

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ABSTRACT

Introduction: Synovitis has been associated with radiographic progression of osteoarthritis (OA) and pain, suggesting its involvement in disease pathophysiology. To get a better understanding of inflammatory pathways active in the OA joint, we characterized and compared inflammatory cells in synovium and infrapatellar fat pad (IFP) of knee OA patients.

Methods: Infiltrating **i**mmune cells were characterized by flow cytometry in 76 patients with knee OA from whom synovial tissue (n = 34) and IFP (n = 59) samples were obtained. Pain and Kellgren-Lawrence scores were determined using validated methods.

Results: Macrophages and T cells, followed by mast cells, were the most predominant immune cells in the inflamed synovia and IFP and they were equally abundant in these tissues. Interestingly, both macrophages and T cells secreted mostly pro-inflammatory cytokines even without additional stimulation, indicating their activated state. In line with this, most $CD4^+$ T cells had a memory phenotype and contained a significant population of cells expressing activation markers ($CD25^+$, $CD69^+$). Interestingly, synovial $CD4^+$ T cells had a more activated phenotype than IFP T cells. Preliminary analyses indicated that the number of synovial $CD4^+$ T cells were associated with visual analog scale (VAS) pain (0.55 (0.09–1.02), p = 0.02).

Conclusions: Our data suggest that the immune cell composition of synovium and IFP is similar and includes activated cells that could contribute to inflammation through secretion of pro-inflammatory cytokines. Moreover, preliminary analyses indicate that synovial $CD4^+$ T cells might associate with VAS pain in patients with end-stage OA of the knee.

INTRODUCTION

Knee Osteoarthritis (OA) is a joint disease characterized by radiographic damage and pain. During the last decade, it became clear that synovial inflammation is present in a significant number of OA patients. In several studies, inflammation has been associated with pain[1-3] and radiographic progression[4,5] of the disease, indicating it as an important player in diseases pathogenesis.

The inflamed synovium of OA patients is infiltrated with immune cells, which can secrete various inflammatory mediators (reviewed in[6]) that could mediate the association between inflammation and pain/radiographic progression. Another source of immune cells and inflammatory mediators in the joint is the infrapatellar fat pad (IFP)[7-9]. Since the IFP is situated adjacent to synovium, it is conceivable that a cross-talk exists between these tissues and that IFP could contribute directly or indirectly to disease pathogenesis.

The association between joint inflammation and pain/radiographic progression is primarily established by imaging using (contrast-enhanced) MRI. Signal alterations have been reported both at synovium and IFP level and have been reported to be associated with radiographic damage[10,11] and pain[1-3,12]. However, it is still unclear how these signal alterations relate to histological changes and cellular infiltrate in these two tissues and whether inflammatory changes in synovium and IFP are related. Moreover, the relative contribution of these two tissues to clinical parameters such as radiographic damage and pain in OA remains to be established.

To get a better insight into inflammatory changes in these tissues, we compared the abundance and phenotype of the main immune cells populations in synovium and IFP. Furthermore, we used this knowledge to get insight into the association of different immune cell populations with radiographic damage and pain in a hypotheses generating setting using a well-defined cohort of knee OA patients.

METHODS

Patients and study design

Patients with osteoarthritis (OA) (n = 76), undergoing joint replacement surgery were recruited into the study and synovial and IFP samples were obtained and immediately processed.

Forty two of the patients included in the present study are part of the ongoing geMstoan study (GEneration of Models, Mechanism & Markers for STratification of OsteoArthritis patieNts), an observational study in knee OA patients to find new biomarkers for OA progression. The geMstoan patients were included between 2008 and 2013, had symptomatic radiographic

primary knee OA, following the ACR criteria[13], and attended the orthopaedic department of the LUMC or the orthopaedic department of the Diaconessenhuis in Leiden. In this study, patients with other rheumatic diseases, using immunosuppressive drugs or having knee injections (corticosteroids, etc) in the past 3 months were excluded. Written informed consent is available from all geMstoan patients. From the remaining 34 patients, leftover synovial tissues and IFP samples were obtained during arthroplasty, thus no additional clinical data were available, besides diagnosis, age, gender and BMI. Both studies were approved by the ethical committee of the Leiden University Medical Center (LUMC).

Isolation of synovial cells and the IFP-derived stromal vascular fraction (SVF) fraction.

Synovial tissue was cut and subsequently digested with collagenase type II (Sigma, Germany) for 90 min 37°C under continuous rolling. The digested tissue was filtered through a cell strainer with a pore size of 70µm with and the cell suspension was stained with the appropriate antibodies. Isolation of SVF cells from IFP was performed as previously described [7].

Flow cytometric analysis

For surface staining, 100,000 SVF or synovial cells were stained with mixes of the following antibodies (Abs): phycoerythrin (PE)-conjugated CD3; fluorescein isothiocyanate (FITC)-conjugated CD45, CD8 and CD45RA; PacificBlue (PB)-conjugated-CD4; Allophycocyanin (APC)-conjugated CD117 and CD8; and phycoerythrin-Cy-7 (PE-CY7)-conjugated CD14, CD25 (all Abs were from BD Biosciences, The Netherlands). All incubations were performed at 4°C, for 30 min. For intracellular cytokine staining, SVF or synovial cells were plated o/n in medium containing 50 IU/ml IL-2 in the presence or absence of 3 µg/ml brefeldin A. The next day, cells cultured in the absence of brefeldin A were activated with 20ng/ml PMA and 200ng/ml ionomycin for 5 hours and 10 µg/ml brefeldin A was added the last 4 hours of culture. Cells cultured in the presence of brefeldin A were stained directly the next day. Approximately 400,000 SVF or synovial cells were stained using the BD intracellular cytokine fixation/permeabilization solution kit (BD Biosciences, The Netherlands), according to the manufacturer's instructions. The following antibodies were used: AlexaFluor700 (AF700)-conjugated CD3; PB-conjugated CD4; APC-conjugated CD8; FITC-conjugated CD45RA; PE-CY-7-conjugated CD14 and IL-4; PE-conjugated IFNγ, TNFα, IL-10 and IL-6 (all from BD Biosciences, The Netherlands). Cells were fixed with 1% paraformaldehyde and analyzed with a LSR II flow cytometer using Diva 6 software (BD Biosciences). Dead cells were excluded based both on FSC/SSC and staining with the Dead cell discrimination kit (Miltenyi Biotec, Germany), as indicated. Single cells were selected based on FSC/SSC analyses, as described in the gating strategy.

Scoring knee radiographs
In the geMstoan patients X-rays of the affected knees (posterior anterior (PA) fixed flexion) were obtained and scored, blinded for patient characteristics, by an experienced musculoskeletal radiologist (HK) according the Kellgren- Lawrence (KL) scale[14]. The intraclass correlation coefficient (ICC, with 95% confidence interval) was based on a randomly selected sample of 36 radiographs (18 right and 18 left knees) and was 0.99 (0.98-0.99). The knees with KL < 2 were rescored in consensus between HK and an experienced rheumatologic reader (MK).

Clinical data
In geMstoan patients, demographics and disease characteristic were collected via standard questionnaires. Measurement of pain was performed preoperatively using 3 different questionnaires investigating various dimensions of pain for the affected knee. First, general assessment of self-reported pain was assessed by the visual analogue scale (VAS, 0-100) for the affected knee, a one-dimensional measure of pain intensity, in which a score of 100mm represents worst possible pain.. Second, the measure of Intermittent and Constant OsteoArthritis Pain (ICOAP) [15,16] was used. Higher scores indicate worst pain experience. Thirdly, the Knee injury and Osteoarthritis Outcome Score (KOOS subscale pain, 0-100) [17,18] was used. In contrast to VAS and ICOAP, a KOOS score of 0 equals the worst pain experience.

Statistics
Associations between percentages of immune cells and pain scales were determined by linear regression analyses and were adjusted for age, gender and BMI when necessary. Log transformations were used when appropriate and log transformed variables are indicated. Correlations were determined by Spearman's rank test. A correlation < 0.3 was considered as weak, 0.3-0.7 as moderate and > 0.7 as strong. A p-value <0.05 was considered significant. The statistical package for the social sciences (SPSS) version 20.0 was used (SPSS, Chicago, IL- USA) for analyses.

RESULTS

Immune cell populations in synovium and IFP
First, we characterized the immune cell population in synovium and the stromal vascular fraction (SVF) of the infrapatellar fat pad (IFP) in detail. Due to the limited amount of tissue, not all experiments could be performed with each tissue sample. Therefore, variable numbers of samples were included in different experiments.

Flow cytometric analysis of the SVF revealed a heterogeneous forward/side scatter (FSC/SSC). In supplementary Figure 1A we describe the performed gating strategies. To determine the abundance of various cell types we used specific cell-surface markers. Macrophages, mast cells and T cells were readily detectable (Figure 1A, B, F, G), while B cells ($CD19^+$ cells) were virtually absent (less than 1%) in both tissues *(data not shown)*. Within the $CD3^+$ T cell population, the ratio between $CD4^+$ and $CD8^+$ T cells was similar to the one usually found in blood for both tissues (Fig. 1C, H). Further phenotypic characterization of $CD4^+$ and $CD8^+$ T cells in IFP and synovium revealed that these populations consisted mainly of memory cells (Fig. 1D, I). Interestingly, a substantial percentage of both $CD4^+$ and $CD8^+$ T cells had an activated phenotype and expressed CD25 and CD69 (Fig. 1E, J).
To directly compare the abundance of different cell populations in synovium and IFP to detect possible tissue specific effects, we have selected and compared paired synovium-IFP samples (Fig. 2). We found that T cells ($CD3^+$ cells) were more abundant in IFP compared to synovium (Fig 2A) However, this difference disappeared when percentages of $CD3^+$ T cells within the $CD45^+$ cell population were compared, indicating that the differences were probably due to the presence of tissue-resident cells (fibroblasts) in the synovial digest (Fig. 2B). Moreover, no differences were found in percentages of macrophages (Fig. 2C), mast cells (Fig. 2D), memory T cells or T cells bearing the late activation marker CD25 (data not shown) between tissues. Interestingly, percentages of T cells positive for the early activation marker CD69 were significantly higher in synovium-derived $CD4^+$ T cells compared to IFP-derived T cells and a trend was present for synovium-derived $CD8^+$ T cells (Fig. 2E).

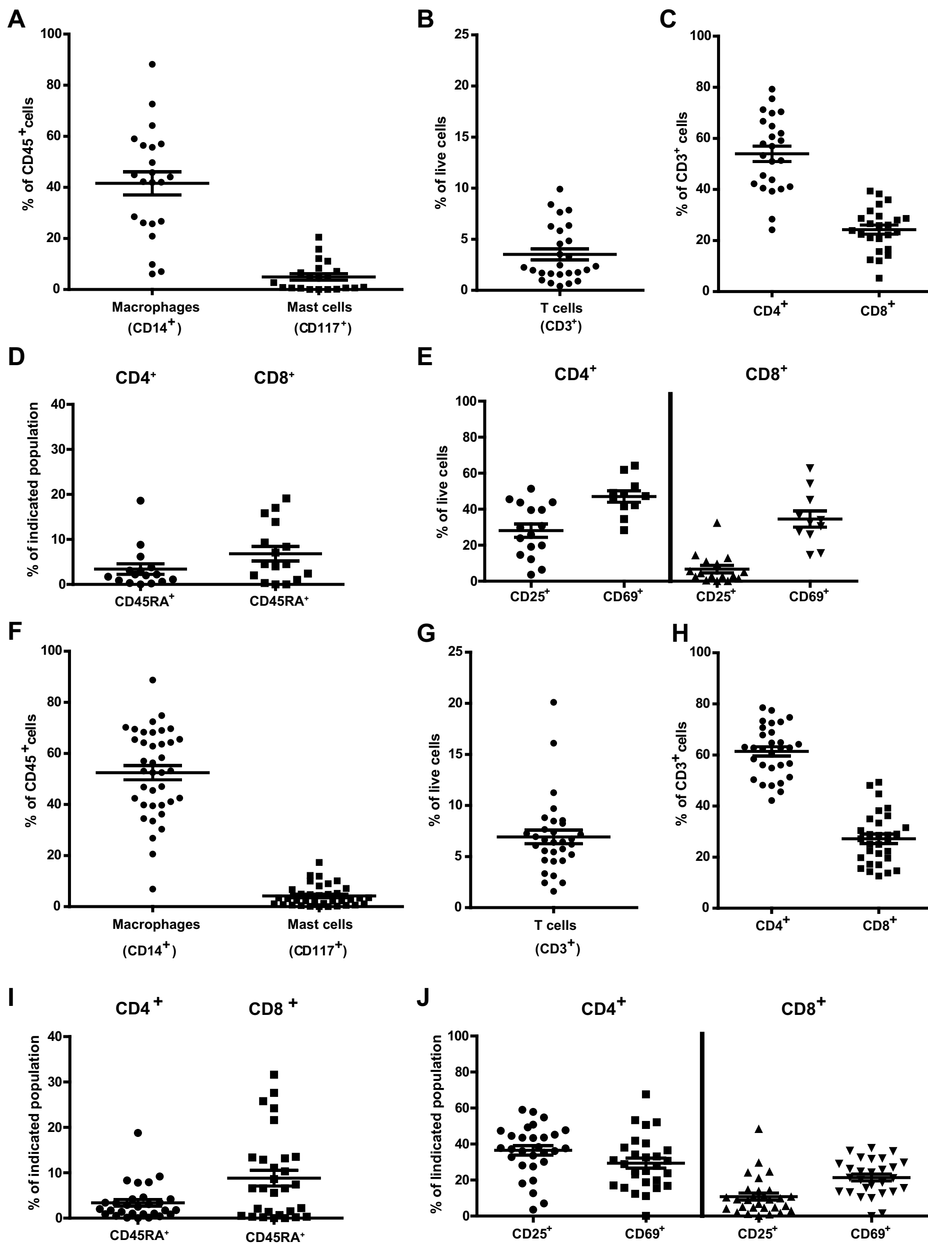

Figure 1. Immune cell characterization 1 of the synovium (A – E) and the stromal vascular fraction of the infrapatellar fat pad (IFP) (F – J) of all end stage OA patients (n = 76) by flow cytometry. Gating strategies were performed as described in suppl. Fig1. Percentages of macrophages (CD45+CD14+), mast cells (CD45+CD117+) (A, F) and T cells (CD3+) (B, G) are depicted. Phenotypic characterization of T cells by flow cytometry; (C, H) Percentages of CD4+ and CD8+ T cells; (D, I) CD45RA expression within the CD4+ and CD8+ T cell
populations; (E, J) CD25 and CD69 expression within the CD4+ and CD8+ T cell populations. Indicated are mean percentage positive cells ± SEM. Each dot represents one patient.

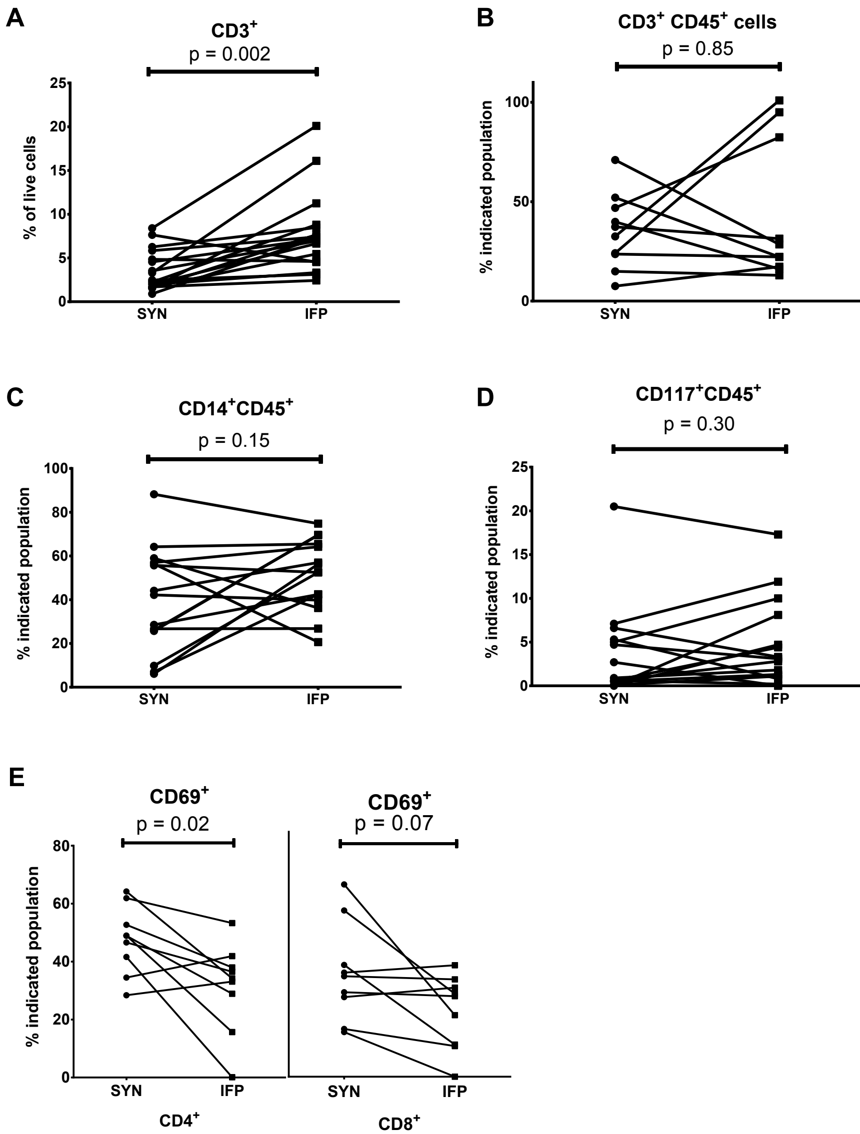

Figure 2. Paired synovium-IFP samples analysis of different cells population for end stage knee OA patients. Paired analysis of T cells (% of CD3+ T cells of live population (A) and % CD3+ T cells of CD45+ cells (B)), macrophages (CD114+CD45+) (C) and mast cells (CD117+CD45+) (D) are depicted. Furthermore, percentages of T cells (CD4+ and CD8+ T cells) positive for the early activation marker CD69 are shown (E).

T cell and macrophage cytokine production

Macrophages and T cells are the most abundant immune cell types present in synovium and in IFP. To assess their possible contribution to the release of inflammatory mediators, we investigated the polarization state of these cells by studying their cytokine secretion.

Cytokines typically associated with different polarization states were investigated. Gating strategies are described in supplementary figure 1B. Intracellular cytokine staining showed that both CD4+ and CD8+ T cell subsets produced mainly IL-6 and IL-4 (Fig. 3A, B) in the absence of extra stimulation. Upon *in vitro* stimulation, these cells were able to produce IFNγ, TNFα, IL-6, IL-4 and very little, if any, IL-10 (Fig. 3C, D). In both tissues, macrophages secreted IL-6 and TNFα, but also some IL-10 *ex vivo* (Fig. 3E, F).

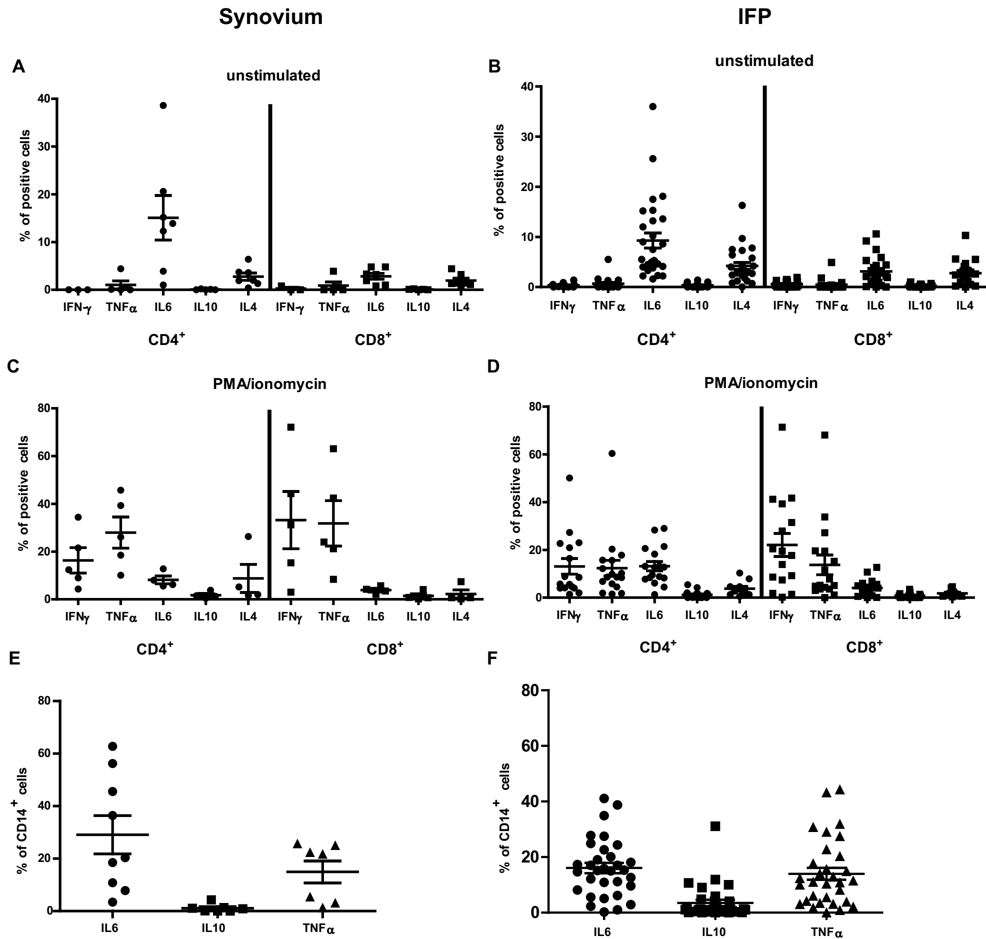

Figure 3. T cell and macrophage cytokine production in synovium (A, C, E) and the stromal vascular fraction of the infrapatellar fat (IFP) (B, D, F). Gating strategies were performed as described in suppl. Fig1. Spontaneous(A, B), or PMA/ionomycin-induced cytokine production (C, D) of CD4+ and CD8+ T cells are depicted. Spontaneous cytokine production by CD14+ macrophages in synovium (E) or IFP (F). Indicated are mean percentage cytokine positive cells ± SEM. Each dot represents one patient.

Association of immune cells with knee pain

To get first insight into the possible contribution of immune cells to clinical characteristics of OA such as radiographic damage and pain, we next investigated whether the synovial- or IFP-derived immune cell populations were associated with Kellgren-Lawrence (KL) scores or self-reported knee pain. For this, we made use of a clinically well-characterized population of patients, participating in the geMstoan study (n=42). No significant differences in immune cell populations were observed between the geMstoan and non-geMstoan samples (data not shown). Furthermore, there were no significant differences in age, gender and BMI between geMstoan patients (n=42) and total group of patients (n=76), indicating that no bias was present in further analysis (data not shown). Patient characteristics are shown in Table 1.

Table 1. geMstoan patient characteristics (N = 42)

Age	63.3 (8.4)
Female, No (%)	22.0 (52.4%)
BMI, median (IQR)	28.9 (25.7 – 31.6)
Fat %	35.98 (11.36)
Fat mass, median (IQR)	29.74 (21.3 - 40.5)
VAS pain (0-100)	65.19 (16.14)
KOOS, subscale pain (0-100)	41.14 (18.49)
ICOAP (0-100), median (IQR)	47.73 (31.82 – 63.07)
KL grade 1, Nr (%)	1 (2.4%)
KL grade 2, Nr (%)	2 (4.8%)
KL grade 3, Nr (%)	17 (40.5%)
KL grade 4, Nr (%)	21 (50.0%)
KL total, median (IQR)	4.00 (3.00-4.00)

Unless specified otherwise, depicted are means (SD); BMI = Body Mass Index; KL = Kellgren and Lawrence; IQR = interquartile range; the visual analogue scale (VAS) for pain; the Knee Injury Osteoarthritis Outcomes Score (KOOS); the Intermittent and Constant Osteoarthritis Pain score (ICOAP).

No associations between immune cells and KL scores could be detected (data not shown). Linear regression analyses, adjusted for age and gender, indicated that the percentage of $CD4^+$ T cells in synovium associated with VAS pain (Table 2; Fig. 4),while no such association was found for other immune cells. This association was not present for IFP-derived $CD4^+$ T cells. Because this part of the study was meant as hypotheses-generating study, we did not adjust for multiple testing.

Table 2. Association of immune cells with knee pain.

		Nr. patients	Adjusted B (95% confidence interval)
VAS pain (0-100)			
Synovium	$CD4^+$ T cells	22	0.55 (0.09 – 1.02)*
	$CD8^+$ T cells	22	0.39 (-0.50 – 1.29)
	macrophages	14	-0.26 (-0.60 – 0.08)
	mast cells	13	-0.49 (-1.71 – 0.74)
IFP	$CD4^+$ T cells	24	-0.12 (-0.80 – 0.56)
	$CD8^+$ T cells	24	0.17 (-0.53 – 0.86)
	macrophages	20	0.07 (-0.38 – 0.52)
	mast cells	18	-0.05 (-1.89 – 1.79)
KOOS (0-100)			
Synovium	$CD4^+$ T cells	22	-0.22 (-0.90 – 0.46)
	$CD8^+$ T cells	22	-0.87 (-1.96 – 0.21)
	macrophages	14	0.09 (-0.49 – 0.67)
	mast cells	13	0.29 (-1.67 – 2.25)
IFP	$CD4^+$ T cells	24	0.56 (-0.30 – 1.41)
	$CD8^+$ T cells	24	-0.68 (-1.54 – 0.18)
	macrophages	20	0.14 (-0.49 – 0.76)
	mast cells	18	1.41 (-0.72 – 3.54)
Log ICOAP (0-100)			
Syn	$CD4^+$ T cells	21	-0.002 (-0.04 – 0.03)
	$CD8^+$ T cells	21	0.04 (-0.02- 0.92)
	macrophages	14	-0.014 (-0.03 – 0.004)
	mast cells	13	0.005 (-0.06 – 0.07)
IFP	$CD4^+$ T cells	23	-0.02 (-0.07 – 0.03)
	$CD8^+$ T cells	23	0.03 (-0.02 – 0.08)
	macrophages	19	0.03 (-0.006 – 0.06)
	mast cells	17	-0.006 (-0.07 – 0.06)

*Linear regression analysis of T cells, macrophages and mast cells and the visual analogue scale (VAS) for pain, the Knee Injury Osteoarthritis Outcomes Score (KOOS), and the Intermittent and Constant Osteoarthritis Pain score (ICOAP). Corrections were made for age and gender. *P-value < 0.05*

Interestingly, the percentage of $CD4^+$ T cells in synovium correlated significantly with BMI ($r = 0.45$; $p = 0.03$) in our cohort. Since VAS pain also correlated with BMI ($r = 0.34$; $p = 0.04$), we next investigated whether the association of $CD4^+$ T cells with pain is, in statistical terms, mediated by BMI. These analyses revealed that the association of $CD4^+$ T cells with VAS pain is independent of BMI (Adjusted B (95% CI) = 0.55 (0.02-1.08). None of the other immune cells from either synovial tissue or IFP were significantly correlated with BMI.

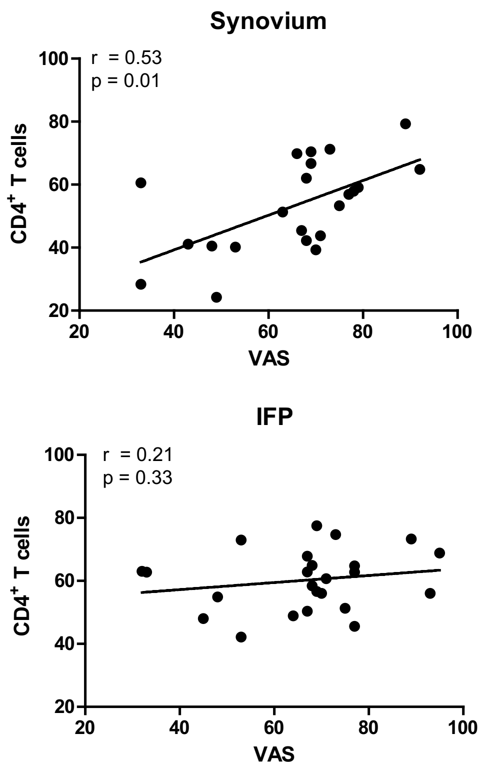

Figure 4. Spearman's rank correlations between synovial (SYN) and Infrapatellar fat pad (IFP)-derived $CD4^+$ T cells and VAS. A P-value ≤ 0.05 was considered significant.

In an attempt to get more insight into the mechanisms underlying this association, we investigated the relationship between different T cell characteristics and pain. Although the association between different cytokine producing T cells and pain could not be studied due to limited availability of cytokine data in de geMstoan population, we did investigate whether phenotypically distinct $CD4^+$ populations correlated with pain. No correlations could be found between $CD4^+$ T cells expressing $CD45RA^+$, $CD25^+$ or $CD69^+$ with VAS (data not shown).

DISCUSSION

In this study, we investigated in detail the immune cells populations present in synovium and IFP of OA patients. Our data indicate similarities both in composition and phenotype of different immune cells populations in these tissues. Moreover, our exploratory studies investigating the association of different cell types with radiographic damage and pain

suggest that synovial CD4+ T cells might be related to pain perception in patients with end stage knee OA.
Macrophages and T cells are the most predominant immune cell types present in synovial tissue and IFP of osteoarthritis patients, which is in line with earlier observations ([7] and [6]). A direct comparison between synovial cells and IFP has never been performed before. Interestingly, we found that the immune cell composition of these tissues was very similar, with no significant differences in the abundance of different cell populations at the population level. In contrast, we were able to detect differences in T cells and mast cells between paired IFP-subcutaneous adipose tissue samples from OA patients (Klein-Wieringa ARD) in an earlier study. Together, these data could indicate that infiltration of immune cells into tissues might be more affected by the disease process (i.e. knee OA) than by tissue-specific signals. Moreover, these data could indicate, as suggested by other studies [9,19], that a cross talk between synovial tissue and IFP exists, leading to a common inflammatory state. Our analyses further indicate that both tissues are an important source of inflammatory mediators and therefore could both have an important role in the pathophysiology of OA. Indeed, both macrophages and T cells could produce cytokines directly ex vivo, which suggests that they are in an activated state in the tissues. Moreover, they secreted predominantly pro-inflammatory and little or no anti-inflammatory cytokines, which is in agreement with the diseased state and with our earlier finding that haematoxylin and eosin staining of these tissue samples revealed the presence of at least one feature of synovitis (i.e. lining cell layer thickening, stromal activation, presence of cellular infiltrate) in each patient. This was in contrast to synovial samples from OA patient undergoing arthroscopy, in which several samples had no detectable signs of inflammation [20].
In addition to the ex vivo cytokine secretion, CD4+ T cells in both tissues had an activated phenotype, indicated by the expression of CD69, an early activation marker on T cells. Interestingly, percentages of T cells positive for the early activation marker CD69 were significantly lower in IFP-derived CD4+ T cells and a trend was present for IFP-derived CD8+ T cells indicating that T cells in the synovial tissue are more frequently activated than in the IFP. As it is believed that T cells stop secreting cytokines when their cognate antigens is removed and as the expression of CD69 is transient and relatively short-lived, it is tempting to speculate that the activated T cells recognize antigens locally, in the tissues from which they originate. These antigens remain, however, to be determined.
Previous studies have indicated the presence of Th1 cells in synovial tissue. However, IFNγ, the typical Th1 cytokine, proved difficult to detect by immunohistochemistry (reviewed in O&C de Lange), which might indicate that these cells are present in this tissue, but not in an activated state. Our data support indeed this hypothesis, as we could clearly identify IFNγ-secreting Th1 cells upon ex vivo activation, but not in the absence of activation.

Although the number of available samples allows only for a hypotheses-generating study regarding the association of various infiltrating cell types with pain, we believe that the found association of synovial CD4+ T cells with pain is of interest and plausible in view of the literature. Several studies have implicated a potential role for T cells in nociception. T cells have not only been shown to infiltrate the site of nerve injury, elimination of T cells in nude rats attenuated hyperalgesia and allodynia in a model of neuropathic pain [21]. In addition, this effect appeared to be facilitated by T-helper 1 (Th1) cells through the release of IL-2 and IFNγ[21], cytokines that were also secreted by synovial $CD4^+$ T cells in our study. This indicates a possible mechanism by which synovial T cells could be associated with pain in OA. Moreover, in our study, synovial $CD4^+$ T cells secreted also TNFα and IL-6. Interestingly, these cytokines have also been shown to directly affect sensory fiber function [22-25], which further supports the hypothesis that $CD4^+$ T cells could be associated with pain. Therefore, future studies replicating our preliminary findings are certainly of great interest.
Previous studies have shown infiltration of ($CD4^+$) T cells into the OA synovium (summarized in [6]). We confirmed and expanded these findings, by observing BMI-dependent infiltration of $CD4^+$ T cells in synovium ($r = 0.45$; $p = 0.03$), for the first time revealing an associative link between obesity and synovial inflammation. Whether adipose tissue secreted factors or other obesity related changes underlie this association needs to be established.

A limitation of our study was the preferential inclusion of patients who underwent arthroplasty, as the only possible source of IFP and of sufficient synovial tissue for exhaustive analyses. Therefore, all patients included in the present study had synovial inflammation and experienced pain in the presence of advanced radiographic knee OA, which could bias our data. Although no patients free of pain were included, the variation in all pain scores (ranges of pain measures; VAS 32-95, KOOS subscale pain 2.78-86.11 and ICOAP 0-72.73) were sufficient to enable us to study associations between immune cells and pain. Other limitations include the lack of healthy control subjects, due to technical difficulties, and the low number of samples available for cytokine analysis which precluded the study of the association between cytokines and pain measures.
In summary, our study indicates that the inflammatory cell composition and phenotype is similar between synovium and IFP of OA patients and that part of the infiltrating cells have an activated phenotype and secrete pro-inflammatory cytokines. Moreover, while our data awaits replication, this study indicates that synovial $CD4^+$ T cells could contribute to the perception of knee pain in patients with end-stage knee OA offering a cellular bases for the association between synovial inflammation and knee pain in OA patients.

Conflict of interests: the authors have no conflicts of interest

Author contributions:

Study conception and design: MK, AIF, GJEO, AMZ, VSS, RGHHN, REMT; Acquisition of data: BJELB, IRKW, EY, SNA, JCK, HMK; Analysis and interpretation of data: BJELB, IRKW, AIF, SNA, JCK, MK, REMT. All of the authors were involved in drafting the article or revising it critically for important intellectual content, and all of the authors approved the final version to be submitted for publication. Dr. AIF had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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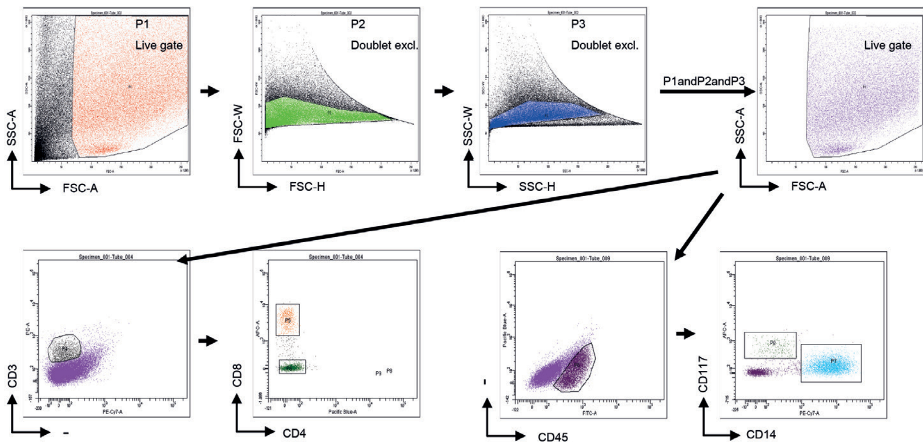
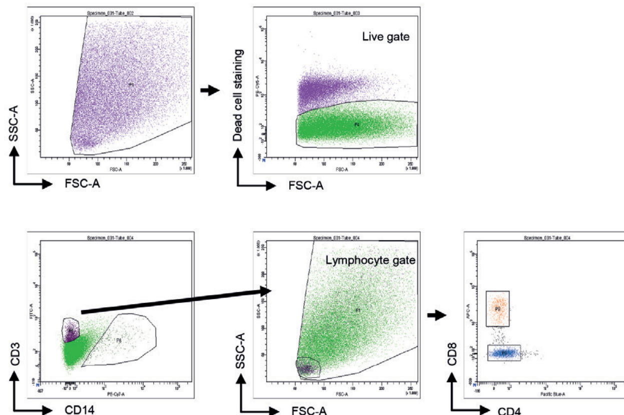
SUPPORTING INFORMATION





Supplementary Figure 1. (A) Gating strategy for Fig 1 and 2. A live gate based on FSC-A/SSC-A was first set on stromal vascular fraction cells (SVF), followed by two gates to exclude double cells. (B) The intersection of this gate was used for gating the lymphocytes positive for CD3 and the hematopoietic cells positive for CD45. Lymphocytes positive for CD3 were selected and, within this population, the CD4$^+$/CD8$^+$ cells were further analyzed as depicted in Fig.1. Hematopoietic cells were selected and within this population the CD117$^+$ and the CD14$^+$ populations were analyzed.

(B) Gating strategy used for Fig 2. A live gate based on cell 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 as described in “Materials and Methods”. CD14$^+$ cells in this live gate were further analyzed as depicted in Fig. 2. The lymphocyte population was gated based on FCS-A/SSC-A. Lymphocytes positive for CD3 and negative for CD14 were selected, the CD4$^+$/CD8$^+$ cells were further analyzed as depicted in Fig. 2.