Immunometabolism in osteoarthritis

Jong, A.J. de

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.

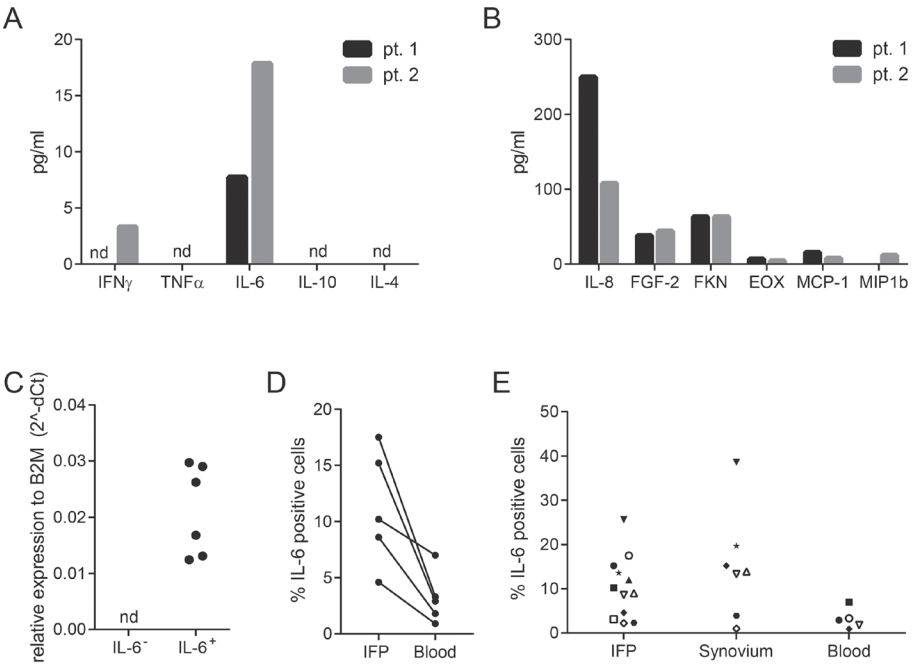


Figure 1. CD4+ T cells from IFP secrete IL-6 *ex vivo*

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.

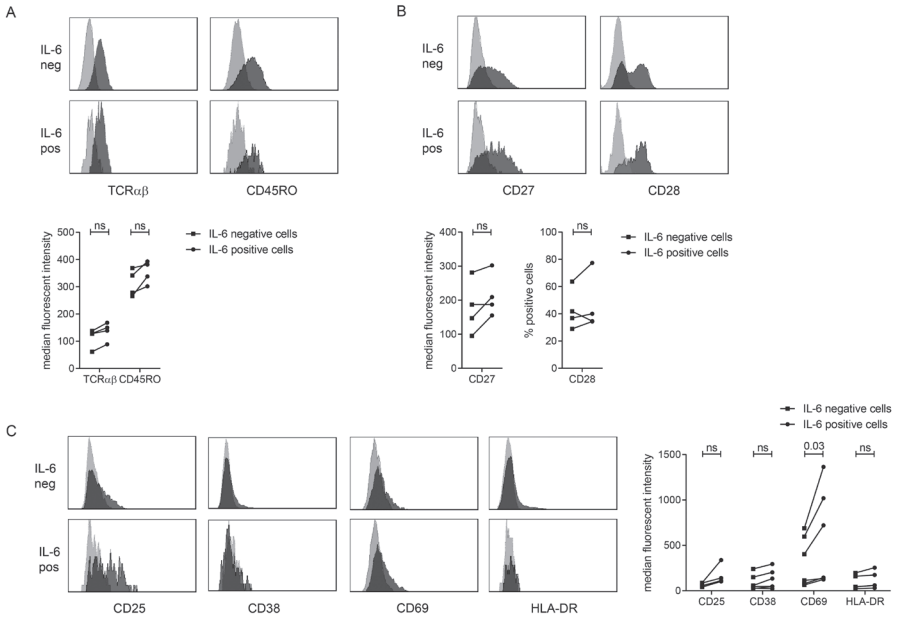


Figure 2. Phenotypic characterization of IL-6+ CD4+ T cells

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.

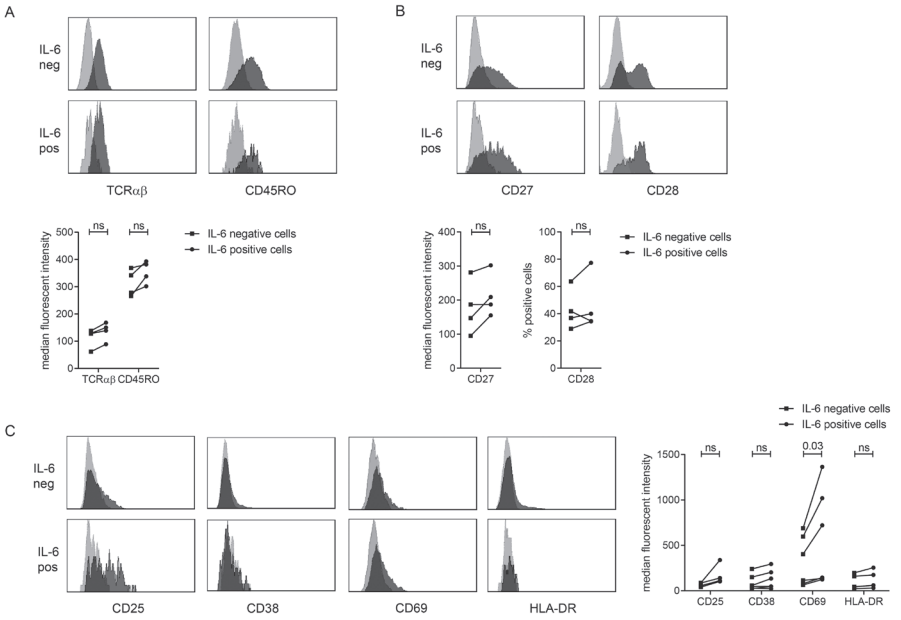


Figure 3. IL-6$^+$ CD4$^+$ T cells cannot be categorized as a conventional T helper subset

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.

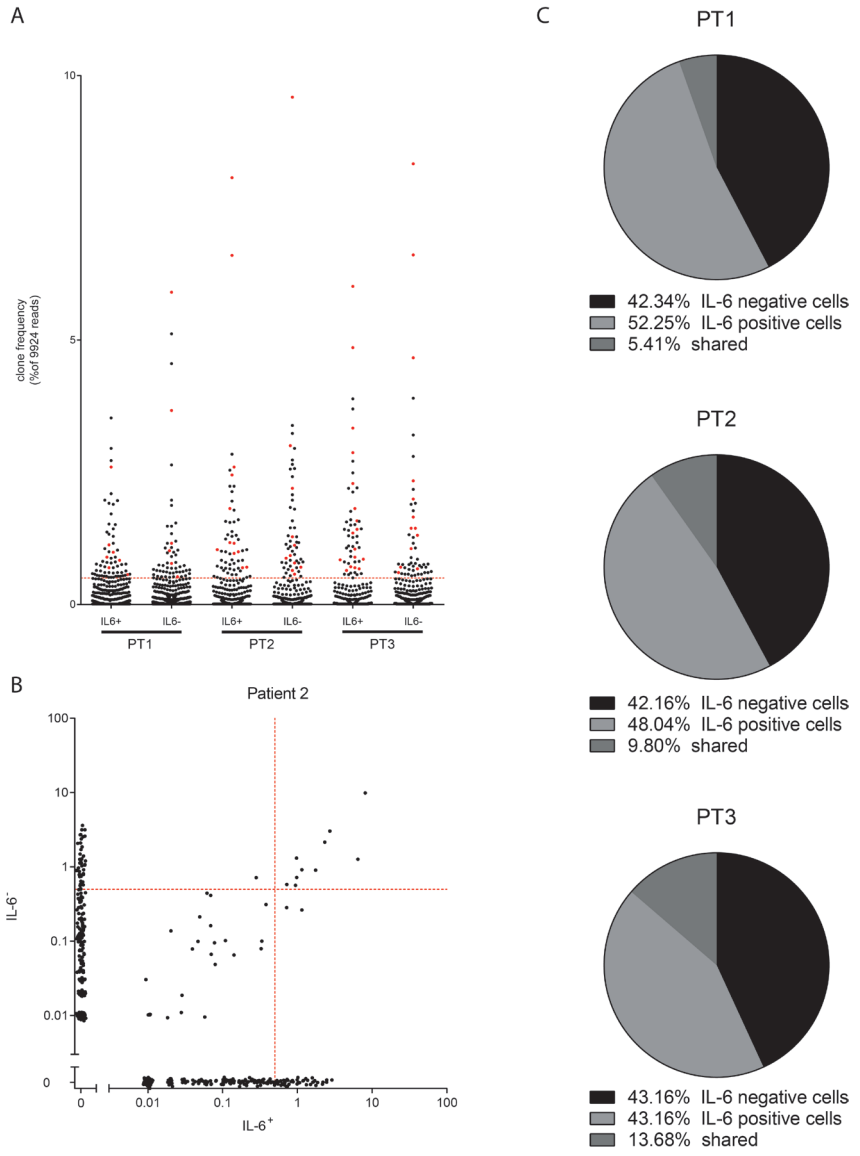


Figure 4. TCRβ repertoire in IL-6+ CD4+ T cells and IL-6- CD4+ T cells

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.

References

1. Ouchi, N., Parker, J.L., Lugus, J.J., and Walsh, K., *Adipokines in inflammation and metabolic disease.* Nat Rev Immunol, 2011. 11(2): p. 85-97.
2. Hotamisligil, G.S., *Inflammation and metabolic disorders.* Nature, 2006. 444(7121): p. 860-7.
3. Yusuf, E., Nelissen, R.G., Ioan-Facsinay, A., Stojanovic-Susulic, V., DeGroot, J., van Osch, G., Middeldorp, S., Huizinga, T.W., and Kloppenburg, M., *Association between weight or body mass index and hand osteoarthritis: a systematic review.* Ann Rheum Dis, 2010. 69(4): p. 761-5.
4. van der Helm-van Mil, A.H., van der Kooij, S.M., Allaart, C.F., Toes, R.E., and Huizinga, T.W., *A high body mass index has a protective effect on the amount of joint destruction in small joints in early rheumatoid arthritis.* Ann Rheum Dis, 2008. 67(6): p. 769-74.
5. Paik, J., Fierce, Y., Treuting, P.M., Brabb, T., and Maggio-Price, L., *High-fat diet-induced obesity exacerbates inflammatory bowel disease in genetically susceptible Mdr1a-/- male mice.* J Nutr, 2013. 143(8): p. 1240-7.
6. Bray, G.A., *Measurement of subcutaneous fat cells from obese patients.* Ann Intern Med, 1970. 73(4): p. 565-9.
7. Sun, K., Kusminski, C.M., and Scherer, P.E., *Adipose tissue remodeling and obesity.* J Clin Invest, 2011. 121(6): p. 2094-101.
8. Salans, L.B., Cushman, S.W., and Weismann, R.E., *Studies of human adipose tissue. Adipose cell size and number in nonobese and obese patients.* J Clin Invest, 1973. 52(4): p. 929-41.
9. Nishimura, S., Manabe, I., Nagasaki, M., Eto, K., Yamashita, H., Ohsugi, M., Otsu, M., Hara, K., Ueki, K., Sugiura, S., Yoshimura, K., Kadowaki, T., and Nagai, R., *CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity.* Nat Med, 2009. 15(8): p. 914-20.
10. Duffaut, C., Galitzky, J., Lafontan, M., and Bouloumie, A., *Unexpected trafficking of immune cells within the adipose tissue during the onset of obesity.* Biochem Biophys Res Commun, 2009. 384(4): p. 482-5.
11. Winer, S., Chan, Y., Paltser, G., Truong, D., Tsui, H., Bahrami, J., Dorfman, R., Wang, Y., Zielenski, J., Mastronardi, F., Maezawa, Y., Drucker, D.J., Engleman, E., Winer, D., and Dosch, H.M., *Normalization of obesity-associated insulin resistance through immunotherapy.* Nat Med, 2009. 15(8): p. 921-9.
12. Feuerer, M., Herrero, L., Cipolletta, D., Naaz, A., Wong, J., Nayer, A., Lee, J., Goldfine, A.B., Benoist, C., Shoelson, S., and Mathis, D., *Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters.* Nat Med, 2009. 15(8): p. 930-9.
13. Kintscher, U., Hartge, M., Hess, K., Foryst-Ludwig, A., Clemenz, M., Wabitsch, M., Fischer-Posovszky, P., Barth, T.F., Dragun, D., Skurk, T., Hauner, H., Bluher, M., Unger, T., Wolf, A.M., Knippschild, U., Hombach, V., and Marx, N., *T-lymphocyte infiltration in visceral adipose tissue: a primary event in adipose tissue inflammation and the development of obesity-mediated insulin resistance.* Arterioscler Thromb Vasc Biol, 2008. 28(7): p. 1304-10.
14. Yang, H., Youm, Y.H., Vandanmagsar, B., Ravussin, A., Gimble, J.M., Greenway, F., Stephens, J.M., Mynatt, R.L., and Dixit, V.D., *Obesity increases the production of proinflammatory mediators from adipose tissue T cells and compromises TCR repertoire diversity: implications for systemic inflammation and insulin resistance.* J Immunol, 2010. 185(3): p. 1836-45.
15. Fabbrini, E., Cella, M., McCartney, S.A., Fuchs, A., Abumrad, N.A., Pietka, T.A., Chen, Z., Finck, B.N., Han, D.H., Magkos, F., Conte, C., Bradley, D., Fraterrigo, G., Eagon, J.C., Patterson, B.W., Colonna, M., and Klein, S., *Association between specific adipose tissue CD4+ T-cell populations and insulin resistance in obese individuals.* Gastroenterology, 2013. 145(2): p. 366-74 e1-3.
16. McLaughlin, T., Liu, L.F., Lamendola, C., Shen, L., Morton, J., Rivas, H., Winer, D., Tolentino, L., Choi, O., Zhang, H., Hui Yen Chng, M., and Engleman, E., *T-cell profile in adipose tissue is associated with insulin resistance and systemic inflammation in humans.* Arterioscler Thromb Vasc Biol, 2014. 34(12): p. 2637-43.
17. Klein-Wieringa, I.R., de Lange-Brokaar, B.J., Yusuf, E., Andersen, S.N., Kwekkeboom, J.C., Kroon, H.M., van Osch, G.J., Zuurmond, A.M., Stojanovic-Susulic, V., Nelissen, R.G., Toes, R.E., Kloppenburg, M., and Ioan-Facsinay, A., *Inflammatory Cells in Patients with Endstage Knee Osteoarthritis: A Comparison between the Synovium and the Infrapatellar Fat Pad.* J Rheumatol, 2016. 43(4): p. 771-8.
18. Klein-Wieringa, I.R., Kloppenburg, M., Bastiaansen-Jenniskens, Y.M., Yusuf, E., Kwekkeboom, J.C., El-Bannoudi, H., Nelissen, R.G., Zuurmond, A., Stojanovic-Susulic, V., Van Osch, G.J., Toes, R.E., and Ioan-Facsinay, A., *The infrapatellar fat pad of patients with osteoarthritis has an inflammatory phenotype.* Ann Rheum Dis, 2011. 70(5): p. 851-7.

19. Picelli, S., Faridani, O.R., Bjorklund, A.K., Winberg, G., Sagasser, S., and Sandberg, R., *Full-length RNA-seq from single cells using Smart-seq2.* Nat Protoc, 2014. 9(1): p. 171-81.

20. Klarenbeek, P.L., Tak, P.P., van Schaik, B.D., Zwinderman, A.H., Jakobs, M.E., Zhang, Z., van Kampen, A.H., van Lier, R.A., Baas, F., and de Vries, N., *Human T-cell memory consists mainly of unexpanded clones.* Immunol Lett, 2010. 133(1): p. 42-8.

21. Turner, D.L., Bickham, K.L., Thome, J.J., Kim, C.Y., D'Ovidio, F., Wherry, E.J., and Farber, D.L., *Lung niches for the generation and maintenance of tissue-resident memory T cells.* Mucosal Immunol, 2014. 7(3): p. 501-10.

22. Teijaro, J.R., Turner, D., Pham, Q., Wherry, E.J., Lefrancois, L., and Farber, D.L., *Cutting edge: Tissue-retentive lung memory CD4 T cells mediate optimal protection to respiratory virus infection.* J Immunol, 2011. 187(11): p. 5510-4.

23. Mackay, L.K., Minnich, M., Kragten, N.A., Liao, Y., Nota, B., Seillet, C., Zaid, A., Man, K., Preston, S., Freestone, D., Braun, A., Wynne-Jones, E., Behr, F.M., Stark, R., Pellicci, D.G., Godfrey, D.I., Belz, G.T., Pellegrini, M., Gebhardt, T., Busslinger, M., Shi, W., Carbone, F.R., van Lier, R.A., Kallies, A., and van Gisbergen, K.P., *Hobit and Blimp1 instruct a universal transcriptional program of tissue residency in lymphocytes.* Science, 2016. 352(6284): p. 459-63.

24. Becattini, S., Latorre, D., Mele, F., Foglierini, M., De Gregorio, C., Cassotta, A., Fernandez, B., Kelderman, S., Schumacher, T.N., Corti, D., Lanzavecchia, A., and Sallusto, F., *T cell immunity. Functional heterogeneity of human memory CD4(+) T cell clones primed by pathogens or vaccines.* Science, 2015. 347(6220): p. 400-6.

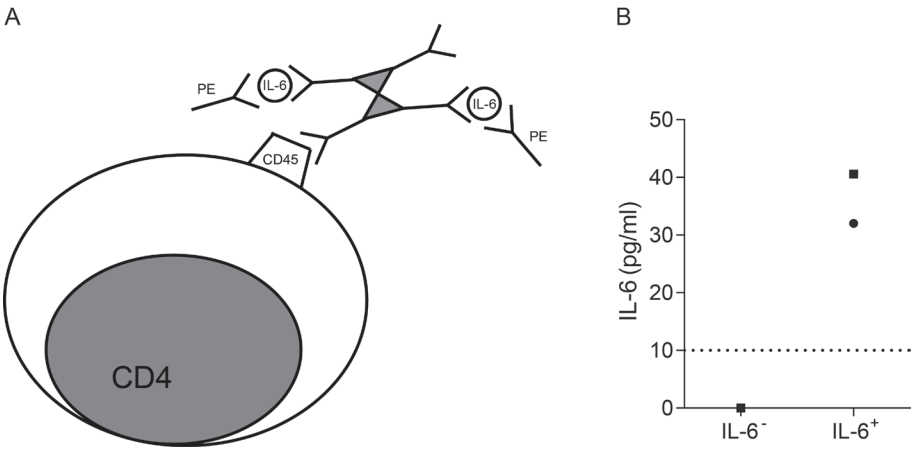
25. Duffaut, C., Zakaroff-Girard, A., Bourlier, V., Decaunes, P., Maumus, M., Chiotasso, P., Sengenes, C., Lafontan, M., Galitzky, J., and Bouloumie, A., *Interplay between human adipocytes and T lymphocytes in obesity: CCL20 as an adipochemokine and T lymphocytes as lipogenic modulators.* Arterioscler Thromb Vasc Biol, 2009. 29(10): p. 1608-14.

26. Deng, T., Lyon, C.J., Minze, L.J., Lin, J., Zou, J., Liu, J.Z., Ren, Y., Yin, Z., Hamilton, D.J., Reardon, P.R., Sherman, V., Wang, H.Y., Phillips, K.J., Webb, P., Wong, S.T., Wang, R.F., and Hsueh, W.A., *Class II major histocompatibility complex plays an essential role in obesity-induced adipose inflammation.* Cell Metab, 2013. 17(3): p. 411-22.

27. van Hall, G., Steensberg, A., Sacchetti, M., Fischer, C., Keller, C., Schjerling, P., Hiscock, N., Moller, K., Saltin, B., Febbraio, M.A., and Pedersen, B.K., *Interleukin-6 stimulates lipolysis and fat oxidation in humans.* J Clin Endocrinol Metab, 2003. 88(7): p. 3005-10.

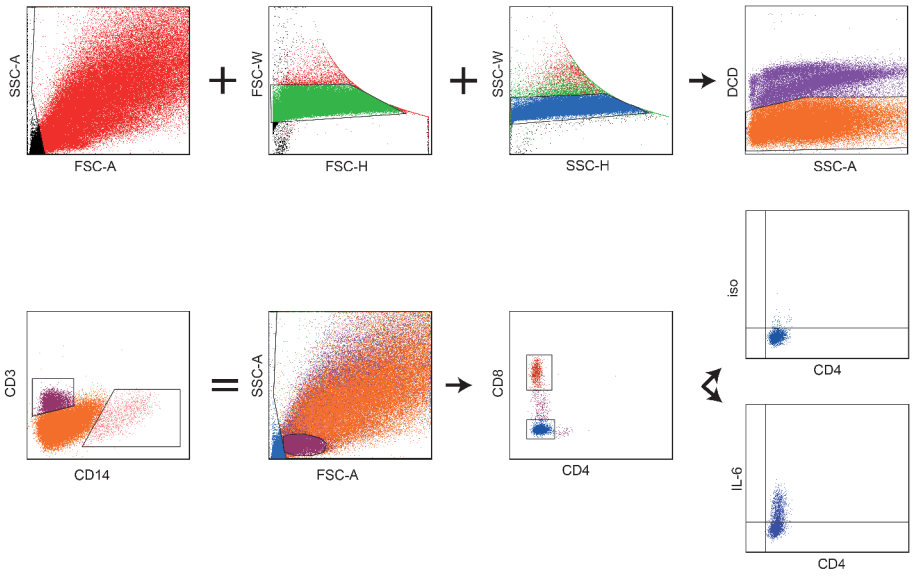
28. Trujillo, M.E., Sullivan, S., Harten, I., Schneider, S.H., Greenberg, A.S., and Fried, S.K., *Interleukin-6 regulates human adipose tissue lipid metabolism and leptin production in vitro.* J Clin Endocrinol Metab, 2004. 89(11): p. 5577-82.

Supporting information



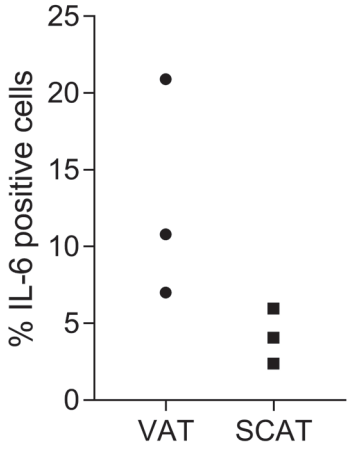
Supporting Figure 1. Capture complex

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).