Research Article

Adipokines induce pro-inflammatory factors in activated CD4$^+$ T cells from osteoarthritis patients†

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Author contributions
All experiments were carried out in the Santiago University Clinical Hospital, NEIRID Laboratory. MS, TP, JC, VA, VL and JP participated in the acquisition, interpretation of the data and drafting the work. MAGG, G-RJJ, AM and RG participated in drafting the work and revising it critically. OG participated in concept and design of the study, in analysis and interpretation of data, drafting and critical revision of the manuscript, and scientific supervision of experiments.
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Abstract

Osteoarthritis (OA) is a chronic systemic musculoskeletal disorder involving inflammation, immunity and metabolic alterations. OA is commonly regarded as non-inflammatory disease; still inflammation is recognized as contributing to the symptoms and progression of OA. New evidence suggests that adipokines are involved in the pathophysiology of OA and might modulate the production of inflammatory mediators including in immune cells. However, the role of immune component in osteoarthritis is still poorly investigated. To gain further insights into the interaction of immune cells in OA and the role of adipokines on these cells, we performed experiments aimed to determine the cytokine profile in activated $CD4^+$ T cells from OA patients. For completeness, we also explored the cross talk between T lymphocytes and chondrocytes in OA by co-culturing human primary chondrocytes with activated $CD4^+$ T cells in two ways: the first by incubating the cells by direct contact (D.C.) or by transwell system. Our results show that the exposure of activated $CD4^+$ T cells to adipokines modulates IL-6, IL-8 and CCL-3 production. In addition, the production of key macromolecules of ECM (aggrecan and collagen-2) and matrix metalloproteinase 13 (MMP-13) in co-cultured chondrocytes with activated $CD4^+$ T cells was altered.

Keywords: Adipokines, Chondrocytes, Inflammation, Immunity, Lymphocytes, Osteoarthritis

Introduction

Osteoarthritis (OA) is a complex disease influenced by multiple risk factors that has been conventionally regarded as a non- inflammatory arthropathy.

However, OA cannot be considered anymore as a simple degenerative disease driven by mechanical events. Actually, a growing body of evidence supports the notion of a leading role of immunometabolic alterations and inflammatory processes, both at local as well as systemic levels in inducing cellular and tissue damage[1-3].

T cell infiltrates are frequently detected in the synovial membrane (SM) of patients with OA[4,5]. Activated T cells can stimulate monocytes to produce cytokines through cell contact–dependent interaction or through soluble mediators[6,7] such as TNF-α and IL-1β. In rheumatoid synovitis, T cells were found to be largely responsible for the production of metalloproteinase[8], proteolytic enzymes with a significant role in cartilage matrix degradation. Moreover, $CD4^+$ T cells were activated during the onset of OA and induced MIP-1γ expression and subsequent osteoclast formation[9]. Thus, recent studies provide evidence that T cells may play an important role in the pathogenesis and progression of OA.

Mechanical overload and systemic inflammation due to the obesity and metabolic syndrome increase the prevalence of OA and contribute to cartilage degradation. Among the inflammatory mediators derived from adipose tissue, adipokines are important factors that contribute to the catabolic mechanism in cartilage degeneration[10-14]. In addition, adipokines have recently emerged as immunometabolic modulators of immune cell responses. Others and we have shown that cartilage, synovium and infrapatellar fat pad from OA patients produce elevated levels of inflammatory mediators in ex vivo cultures when stimulated with adipokines[12,14,15]. These results suggest that chronic low-grade inflammation, supported by adipokines, might influence locally and

systemically the promotion and the progression of OA involving cells of the joint (chondrocytes and synovial fibroblast) but also cells of the immune system.

We therefore hypothesized that the complex interplay between the musculoskeletal system and immune system may be, in part, mediated by adipokines.

Thus, we analysed the effect of adipokines, leptin, adiponectin and lipocalin-2 on activated $CD4^+$ T cells from OA patients. Moreover, we studied the effect of activated $CD4^+$ T cells from OA patients on human primary chondrocytes by using two lymphocytes-chondrocytes co-culture systems.

MATERIALS AND METHODS

Reagents

Fetal bovine serum (FBS) was purchased from Sigma. RPMI 1640 medium, l-glutamine, trypsin–EDTA and antibiotics were purchased from Lonza. Pronase and Colagenase P were obtained from Roche Molecular Biochemicals. Ficoll-Paque™ PREMIUM sterile solution was purchased from GE Healthcare. Dynabeads® Regulatory $CD4^+CD25^+$ T Cell Kit and Dynabeads® Human T-Activator CD3/CD28 were obtained from Invitrogen.

Cell culture and treatments

$CD4^+$ T cells were obtained from peripheral blood samples of OA patients or healthy subjects by centrifugation on a Ficoll-Paque density cushion according to standard methods. Next, $CD4^+$ T cells were cultured in 75-cm^2 flasks in RPMI medium supplemented with 10% FBS, 2mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C under 5% CO_2 humidified air. These cells were activated by Dynabeads® Human T-Activator CD3/CD28, according to the manufacturer's instructions.

Primary chondrocytes were harvested from human OA articular cartilage samples obtained from articular joints of patients undergoing total knee replacement surgery as previously described[11,14,16].

$CD4^+$ T activated cells were seeded at a density of 1 x 10^6 cells/well in 6-well plates, stimulated with adipokines and incubated during 3 days at 37ºC in RPMI medium.

For co-culture experiments, $CD4^+$ T activated cells from OA patients were seeded at a density of 1 x 10^6 cells/well in 6-well plates or in transwell in RPMI medium. Human primary chondrocytes were seeded at a density of 3 x 10^5 cells/well in 6-well plates and plated together with activated $CD4^+$ T cells during 3 days.

mRNA isolation and RT-PCR

RNA was isolated from cell culture by Trizol LS, according to the manufacturer's instructions (Gibco BRL Life Technologies, Grand Island, NY).

Human COL2A1, human Aggrecan, human MMP-13, human IL-6, human IL-8, human MIP1-α, human IL-2, human TNF-α and human IFN-γ mRNA levels were determined using SYBR Green–based quantitative PCR. RNA was extracted using a NucleoSpin kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. For relative quantification, we performed an RT reaction with a Thermo Scientific Verso cDNA Synthesis Kit (42°C for 30 min, followed by an incubation at 95°C for 2 min). RT-PCR was performed in a Stratagene MX3005P thermal cycler using a standard protocol (95°C for 10 min followed by 40 cycles for 15 s of denaturation at 95°C and 1 min annealing/extension at 60°C), a SABiosciences Master Mix and specific primers (human COL2A1, PPH02134E; human Aggrecan, PPH06097E; human MMP-13, PPH00121B; human IL-6, PPH00560B; human IL-8, PPH00568A; human MIP-1α, PPH00566E; human IL-2, PPH00172C; human TNF-α, PPH00341F; human IFN-γ, PPH00380C). Results of comparative RT-PCR experiments were analyzed using MxPro software (Stratagene, California, USA).

Statistical analysis

Data are reported as the mean ± SEM of at least 3, 5 or 10 independent experiments, each with at least 2 independent observations. Statistical analysis was performed using analysis of variance

followed by the Student-Newman-Keuls test or Bonferroni multiple comparison test using the Prism computerized package (GraphPad Software). *P* values less than 0.05 were considered significant.

Results

Adiponectin, leptin and lipocalin-2 induce pro-inflammatory factors in activated $CD4^+$ T cells from OA patients

For our studies, we used peripheral blood samples from 10 OA patients and 5 healthy subjects (Table 1). As shown in figure 1, leptin, adiponectin and lipocalin-2 were able to induce the mRNA of pro-inflammatory factors such as IL-6 (Figure 1a, panel A), IL-8 (Figure 1c, panel A) and MIP-1α (Figure 1e, panel A) in OA activated $CD4^+$ T cells but not in activated $CD4^+$ T cells from healthy subject (Figure 1b, d and f, panel A). We also analyzed the effect of these adipokines on IL-2, TNF-α and IFN-γ but we didn't observe any modulation in both OA (Figure 1g, i and m, panel B) and healthy samples (Figure 1h, l and n, panel B).

Co-culture of human primary chondrocytes from OA patients with autologous activated $CD4^+$ T cells

For completeness and in order to gain further insights into the potential interaction of immune cells in OA, we also have explored a cross talk between activated $CD4^+$ T cells and human primary chondrocytes from OA patients.

For analysing the cross talk between T lymphocytes and human primary chondrocytes, we used two types of co-culture: the first by using a cell-cell direct contact and another one by using a transwell

system. In the first system we placed chondrocytes and activated $CD4^+$ T cells in direct contact in 6-well plates. In the traswell system, we seeded chondrocytes in 6-well plates and activated $CD4^+$ T cells in the transwell (Figure 2 A). As shown in the figure 2, mRNA expression of COL2A1 (Figure 2 B) was significantly decreased in chondrocytes seeded in direct contact (D.C.) with activated $CD4^+$ T cells and also in the transwell system when compared with chondrocytes alone. We also observed a decrease of ACAN, but this result is not significant probably due to the high variability among the different patients (Figure 2 C). Moreover, we observed that MMP-13 mRNA expression was significantly increased in D.C. and transwell system when compared with chondrocytes alone Figure 2 D).

Discussion

Several aspects of immune system involvement in OA have been highlighted during the last years. OA is no longer considered a non-inflammatory and/or biomechanical process, but rather an articular chondropathy in which both inflammation and immunometabolic responses play a leading role. Activation of the immune system is likely to be a complex mechanism that might be involved in both initiation and/or perpetuation of inflammatory process of OA. It is reasonably conceivable that a self-perpetuating cycle of joint cartilage degeneration and immune interactions is at play in the pathogenesis of OA. Thus, the extracellular matrix breakdown, due to persistent tissue injury (mechanical, biochemical and/or metabolic etc.) may activate immune response and a subsequent release of pro-inflammatory cytokines that in turn promotes the recruitment of inflammatory cells thus perpetuating the cycle. Although most of the interactions between cartilage and immune cells in OA still remain to be clarified, the involvement of both innate and adaptive immune response in OA is likely to be reasonably accepted. In fact, several studies suggested the role of immune cells in the pathogenesis of OA[5,17-21]. Particularly, $CD4^+$ T cells were found in OA synovium at similar levels as in rheumatoid arthritis (RA) synovium. The Th1 subset of T cells was found to be about 5

times higher than Th2 cells[22] and higher levels of Th1 cytokines, such as IL-2 and IFNγ, have been detected in most of OA patients[23].

Among inflammatory mediators involved in OA, adipokines seems to play an important role in cartilage degeneration. Actually, several studies reported the catabolic effect of adipokines, such as leptin and adiponectin, on chondrocytes[10-14] suggesting a potential role of these adipokines in cartilage degeneration.

In our study we analysed the effect of three adipokines (leptin, adiponectin and lipocalin-2) on activated $CD4^+$ T cells from OA patients compared with the effect on $CD4^+$ T cells from healthy subjects. We observed that these adipokines induced pro-inflammatory factors, such as IL-8, IL-6 and MIP-1α in $CD4^+$ T cells from OA patients but not in $CD4^+$ T cells from healthy subjects. These results suggested that leptin, adiponectin and lipocalin-2 might contribute to increase the immune/inflammatory environment in OA patients.

Our present results might be explained due to the fact that OA patients may have a significant alterations in the blood immune cell composition. This alteration may also reflect differential inflammatory response as well as autoreactivity. To this regard, it is conceivable that this result is due to a specific activation of T cells following their homing at the OA synovial membrane or, alternatively, they might represent activated cells recruited from periphery.

To gain further insights on the role of immune component in OA pathology, we performed a co-culture of human primary chondrocytes from OA patients with the autologous activated $CD4^+$ T cells. It is important to clarify that chondrocytes in normal healthy condition never meet T cells, but in OA this interaction is possible. In the damaged OA cartilage, it has been found that chondrocytes were peeled off from articular cartilage surface and were exposed to the joint cavity and to synovial infiltrates. Moreover, chondrocyte-like cells were present in SF expressing aggrecan and COL2A1[24] suggesting a possible contact in vivo between chondrocytes and T cells.

For this reason, we decide to investigate the response of chondrocytes after activated $CD4^+$ T cells interaction. For our experiments, we used two type of co-culture: the first with the direct contact between T cells and chondrocytes. The second one by separating the two cell types with a transwell system. Notably, in both co-culture systems, we observed that MMP-13 expression in chondrocytes was enhanced whereas structural cartilage specific biomarkers such as COL2A1 and ACAN expressions were decreased.

Our novel data, although preliminary, may contribute to elucidate the role of immune cells in OA, demonstrating that chondrocytes after the interaction with $CD4^+$ T cells showed increased expression of MMP-13 and a decreased expression of COL2A1 and ACAN expression.

In conclusion, this study demonstrated for the first time that adipokines induce pro-inflammatory factors (IL-6, IL-8 and MIP-1 α) in activated $CD4^+$ T cells from OA patients suggesting new insights into the role of adipokines in OA progression. . Here, we showed a clear interaction between activated $CD4^+$ T cells and human primary chondrocytes from OA patients contributing to a better understanding of immune-mediated mechanisms in OA.

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Conflict of Interest statement

All authors declare that there is no conflict of interest with any financial organization regarding the content discussed in the manuscript.

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Figure legends

Figure 1. Human mRNA expression of IL-6 (a), IL-8 (b), CCL-3 (c) (panel A), IL-2 (g), TNF-α (i) and IFN-γ (m) (panel B) after treatment with Adiponectin (5µg/mL), Leptin (800nM) and Lipocalin-2 (10nM) during 3 days in human activated $CD4^+$ T cells from OA patients. Human mRNA expression of IL-6 (b), IL-8 (d), CCL-3 (f) (panel A), IL-2 (h), TNF-α (l) and IFN-γ (n) (panel B) after treatment with Adiponectin (5µg/mL), Leptin (800nM) and Lipocalin-2 (10nM) during 3 days in human activated $CD4^+$ T cells from healthy subjects. For OA patients: values are the mean ± SEM of at least 10 independent experiments. *p<0.05; **p<0.01; ***p<0.001 vs. Control. For healthy subjects: values are the mean ± SEM of at least 5 independent experiments.

Figure 2. Schematic representation of co-culture systems (A). Human mRNA expression of COL2A1 (B), ACAN (C) MMP-13 (D) in human primary chondrocytes alone or co-cultured with activated $CD4^+$ T cells by direct contact (D.C.) or transwell. Values are the mean± SEM of at least 3 independent experiments. $^{*}p<0.05$, $^{**}p<0.01$.

Table 1. Clinical characteristics of patients enrolled in the study.

Characteristics	**Patients with OA (n=10)**	**Healthy Controls (n=5)**
Age [years, (range)]	70.6 (61-81)	28.8 (25-32)
Sex [n(female/male)]	6/4	4/1
BMI	26.8	20.4
Medication	NSAIDs	-

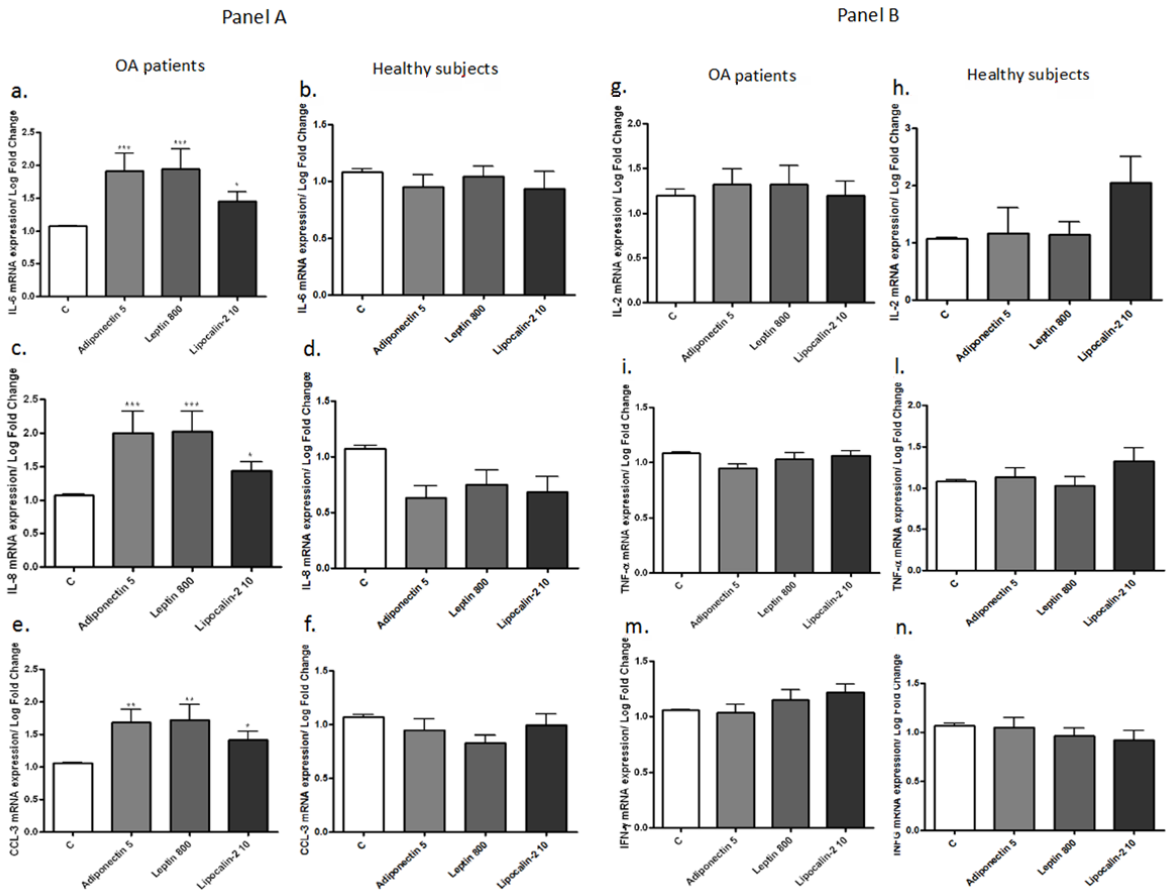

Figure 1

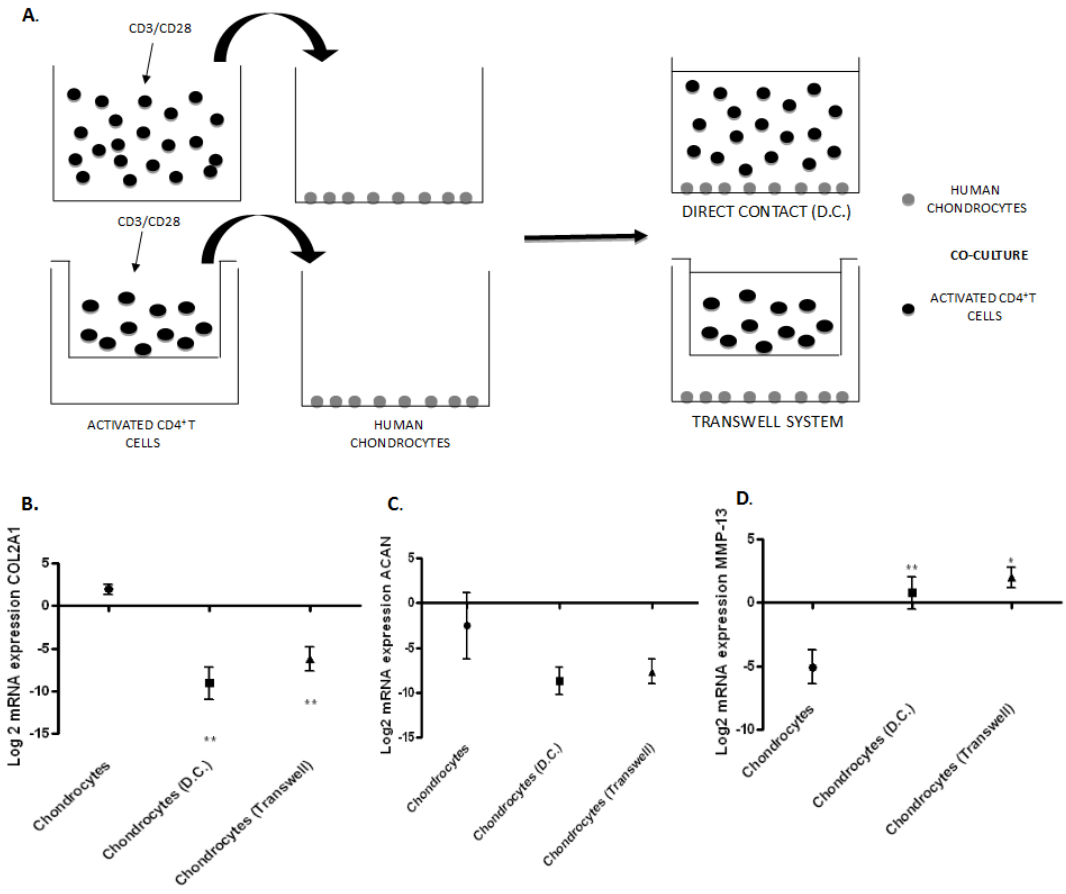

Figure 2