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Effects of hyaluronic acid (HA) viscosupplementation on peripheral Th cells in knee and hip osteoarthritis

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SUMMARY

Objective: Determine Th lymphocytes concentration in patients with knee or hip osteoarthritis (OA). Evaluate their change after HA viscosupplementation.
Methods: Patients with early primary knee or hip OA (ACR Criteria) were recruited in two groups: group A was only observed longitudinally, group B was treated with a course of three weekly intra-articular injections of HA. A healthy control group gender and age matched was enrolled too. All subjects were followed for 3 months. Flow cytometry was performed from blood samples to assess T cells sub-populations (CD3, CD4, CD8, CCR6, CD38, CxCR3, HLA DR) at baseline and at 3-months visit.
Results: 86 patients were recruited with OA: 49 in Group A (35 knee OA, 14 hip OA), 37 in Group B (24 knee OA, 13 hip OA). 23 in Control Group. Activated CD4 T cells ($CD4^+CD38^+DR^+$, $CD4^+CD38^-DR^+$), Th2 ($CD4^+CXCR3^-CCR6^-$),Th1 ($CD4^+CXCR3^+CCR6^-$) were higher at baseline in group A and B than in control group. After the HA course activated T cells were lower in group B than in group A ($P = 0.01$). Th17 ($CD4^+CXCR3^-CCR6^+$) at baseline were higher in groups A and B than in control group and decreased levels in Group B after the HA course were observed ($P = 0.03$).
Conclusion: The presence of activated T cells in patients with OA confirm that OA is a disease with an immunological/inflammatory involvement. Our preliminary results seems to show that HA injections could lower the levels of activated T cells, and so regulate the articular milieu.

Introduction

Osteoarthritis (OA) is a chronic, degenerative joint disease characterized by the progressive destruction of articular cartilage, joint space narrowing, subchondral bone remodeling, joint marginal osteophyte formation and synovitis. OA causes joint pain, stiffness, swelling and reduced range-of-motion having a serious impact on health related quality-of-life, showing several characteristics with rheumatoid arthritis, including joint destruction and synovitis.

OA can be diagnosed by the presence of joint irregularities and deformities on X-radiographic images. The grade of joint degeneration reflects disease severity. Non-invasive biochemical analyses have been developed to evaluate disease progress and severity, and provide a nonradiographical alternative for the early detection of OA. Many factors contribute to an increase risk of OA and include obesity, genetics, aging, and trauma to the joint.

Although the etiology and pathophysiology of OA are both poorly understood, it is believed that secreted inflammatory molecules (such as proinflammatory cytokines and adipokines) are among the critical mediators of the disturbed processes implicated in OA pathophysiology. Humoral and cellular immunity, both innate and adaptive immune response, are known to be involved[1]. In particular, in many studies on OA, it has been demonstrated that an inflammatory synovium/synovitis has linked to increased cartilage damage and pain[2,3]. Synovial tissue of OA patients show infiltrates of immune cells including T-cells, B-cells and macrophages. Immunoglobulins and immune complexes against cartilage components are detected in cartilage, synovium and plasma in OA patients[4,5]. Finally, key role of complement activation in OA synovium has been identified[6].

From data literature, $CD4^+$ T lymphocytes (particularly Th17 subtype) and their cytokines have been reported to play a major

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role to activate rheumatic diseases inflammation as rheumatoid arthritis or OA. In a proinflammatory milieu, chondrocytes become metabolically active and initiate inflammatory processes that degrade articular cartilage and subchondral bone. Chondrocytes secrete several inflammatory cytokines that work synergistically to stimulate synthesis of enzymes that break down cartilage. Normally, synovial fluid contains high levels of hyaluronic acid (HA, a polysaccharide produced by the chondrocytes and synoviocytes) that help to maintain high fluid viscosity and the normal integrity of the joint by attenuating inflammation and preserving the normal cartilaginous matrix. In OA, the synovial fluid viscosity and elasticity are decreased. While HA may help to lubricate and cushion the joint, it can help maintain cartilage matrix and minimize inflammation. In OA, the molecular weight and concentration of HA are reduced, thereby lowering fluid viscosity and elasticity. Protection against articular injury is compromised and OA damage ensues. Intra-articular injections of HA (i.e., viscosupplementation) are approved worldwide for the treatment of pain associated with OA of the knee. In addition to a purely mechanical effect due to the viscosity of the products, intra-articular HA viscosupplementation is thought to provide a range of biological actions including anti-inflammatory effect. *In vitro* data suggest that supplemental HA can suppress IL-1 production, and may increase synovial fluid viscosity[7]. We hypothesize that intra-articular HA can suppress not only the local intra-articular proinflammatory milieu, but also can reduce the overall inflammatory cytokine response. Primary purpose of this study was to evaluate the circulating levels of activated $CD4^+$ and $CD8^+$ lymphocytes in patients with OA, comparing with healthy control. Secondary purpose was to evaluate the changes in lymphocytes after 3 months from an intra-articular HA injection course and the effectiveness in terms of variation of Lequesne Pain-functional index.

Methods

Patients

Patients with hip or knee OA were recruited from the Rheumatology Unit Fornaroli Hospital from January 2012 to October 2013. The inclusion criteria were a diagnosis of bilateral knee or hip OA according to ACR Criteria, with a the Kellgren–Lawrence score of 2–3 and Pain VAS at least 50 mm on a 0–100 mm visual scale. The exclusion criteria were: presence of rheumatoid arthritis or other rheumatic diseases, pregnancy, allergic to hyaluronans, currently experiencing a knee infection or skin infection around the injection site. An age, sex and BMI matched control group was screened for OA and, after exclusion of OA diagnosis, they were recruited too between blood donors of our Hospital; this group didn't meet hip or/and knee OA ACR criteria. Patients with diffuse OA to many joints (e.g., OA of knee associated with hands OA or wright OA or spine OA) were excluded. Weight bearing anteroposterior knee and hip anterior-posterior radiographs were classified according to the Kellgren–Lawrence (KL) radiographic rating scale. All patients, inclused controls, read, understood and signed an informed consent and compiled a pain-functional Lequesne Index. The trial was conducted in accordance with the ethics principles of the Declaration of Helsinki and was approved by the local research ethics committees.

Analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), symptomatic slow acting drugs for OA (SYSADOAs) or disease modifying (DMOADs) if taken before entering the trial were not modified throughout the period of the study. Patients with OA longitudinally evaluated were enrolled in Group A (group A1 with 35 knee OA and group A2 with 14 hip OA) and no intra-articular treatment was performed in these groups. Patients with knee OA or hip OA treated with an HA intra-articular course were recruited in Group B (B1: 24 knee OA and B2: 13 hip OA). 23 subjects were enrolled finally as control-healthy group.

Knee intra-articular injection procedure

The patient sat with the extended knee(s). An anteromedial or lateral approach was performed for these injections. A 20 g needle was used and a syringe of 2 ml of viscosupplement was then delivered. We used for viscosupplementation sodium hyaluronate with a molecular weight of 800–1200 kDa (Sinovial Forte® 1.6% 2 ml IBSA). Two more injections were provided to each patient at weekly intervals for a total of three injections.

Hip eco guided intra-articular injection procedure

Patients were examined supine, with the hip internally rotated by 15–20°. A 10 MHz linear transducer had a sterile device for biopsy attached. Ultrasound scans of the hip joint were taken on an anterior parasagittal axis, lateral to the femoral vessels. The probe was aligned along the long axis of the femur neck to visualize both the acetabulum and the femoral head. Using an anterosuperior approach, a G20 (9 cm) spinal needle was inserted through the biopsy guide into the joint capsule, until the femoral head was reached. Using real time imaging software, the needle was inserted until it was visualized in the articular recess and so HA was injected. A course of three injections with Sinovial Forte® was performed for all patients with hip OA in the group B2.

Flow cytometry

Freshly drawn EDTA blood samples were analyzed by 8-color flow cytometry (FACSCanto II, Becton Dickinson, Milan) with the following conjugated antibody panel: CD45-FITC; CXCR3-PE; CD4-PerCP-Cy5.5; CCR6-PE-Cy7; CD38-Alexa 647; CD8-APC-H7; CD3-V450; HLADR-V500 at the appropriate concentrations (all from Becton Dickinson). After 20-min staining in the dark, 2 ml of ammonium chloride lysing was added for 10 min. After centrifugation at 1500 rpm for 7 min, the pellet was resuspended in 200 μL of cold PBS and immediately analyzed.

At least 50,000 lymphocytes (defined as $CD45^{+++}$, SSClow cells) were acquired.

The gating strategy included the parallel capture of $CD4^+/CD3^+$ and $CD8^+/CD3^+$ cells in two separate downstream hierarchies. Each parent subset was then further dissected into functional subpopulations, namely $CD4^+$ T cells as Th1 cells ($CD4^+$ $CXCR3^+$ $CCR6^-$), Th2 cells ($CD4^+$ $CXCR3^-$ $CCR6^-$) and Th17 cells ($CD4^+$ $CXCR3^-$ $CCR6^+$), respectively according to Maecker *et al.*, 2012[8]. Both $CD4^+$ and $CD8^+$ cells were divided into quiescent ($CD38^-$ $HLADR^-$) or activated elements ($CD38^+$ and/or $HLADR^+$). Functional subset percentages were calculated over the total lymphocyte population and over the parent $CD4^+$ or $CD8^+$ subsets, respectively, and all values were also recorded as absolute levels per microliter on the basis of total lymphocyte count (Beckman Coulter DXH800, Milan).

Statistical analysis

All the variables collected were normally distributed as stated by Shapiro–Wilk test. Homoscedasticity of variances was assessed with Cochran/Hartley or Levene tests. Continuous variables were presented as mean SD, and compared using two-tailed Student's *t*-test or one-way analysis of variance (ANOVA). Links between continuous variables collected in the groups during the study period were estimated with MANOVA for repeated measures

Table I
Demographic data of whole population

	Knee OA	HIP OA	Controls	*P*
Total patients	35 not treated pts (Group A1) 24 treated pts (Group B1)	14 not treated pts (Group A2) 13 treated pts (Group B2)	23 pts	
Age (±SD)	65.5 ± 1.2 years old	70 ± 4.8 years old	66 ± 0.8 years old	0.2
Sex (female)	30 pts (61.2%)	18 pts (66.6%)	15 pts (65.2%)	0.3
Median BMI (±SD)	25.31 (±4.14)	26.50 (±3.14)	27 (±4.04)	0.1

(Wilk's Lambda). Relationships between variables were examined using nonparametric Spearman correlation coefficients. Statistical analyses were performed with SPSS version 15.0 (SPSS Inc., Chicago, USA) for Windows. *P*-values <0.05 were considered statistically significant.

Results

The study recruited 76 patients with OA randomly: 49 patients were recruited in Group A (group A1 with 21 knee OA and group A2 with 28 hip OA) and only observed longitudinally. 37 patients were recruited in Group B (B1 24 with knee OA and B2 13 with hip OA) and treated weekly with three intra-articular injection of HA. Finally 23 age, sex and BMI matched healthy subjects were enrolled in Control Group. There were no statistically significant differences in age, sex and BMI between patients and controls (Table I demographic table).

Data at baseline

There wasn't any significant difference between group A1 e A2 (OA observed patients) and group B1 and B2 (OA treated patients) in pain-functional Lequesne index ($P = 0.062$), although we observed slightly higher values in the B1 and B2 groups. The mean percentages of cells stained positive for $CD4^+$ (total $CD4^+$ lymphocytes), $CD4^+CXCR3^+CCR6^-$ (Th1 cells), Th2 ($CD4^+CXCR3^-CCR6^-$), $CD4^+CD38^+$, $CD4^+$ HLA-DR^+, $CD4^+$ $CD38^+$ HLA-DR^+ (subtypes of activated $CD4^+$ lymphocytes) were different among the five groups (Table II and Figs. 1 and 2).

Interestingly, while the patients were randomly assigned to Groups A or B, we observed marginally higher values of Th1, Th2 cells, Th 17 and activated T cells in group B1 and B2 towards group A1 and A2 ($P = 0.067$) with a weak relationship with Lequesne index (Spearman Rho 0.34, $P = 0.07$). This bias can be due to small size of the groups.

Data at 3 months

Between baseline and the 3 months, in Group B1 and B2 there was a significant decrease of pain-functional Lequesne from 14.7 (±2.02) to 9 (±1.02) points with a mean variation of 5.7 ± 2.4 points ($P = 0.02$).

Healthy control didn't' show a significant difference for cells subtype between baseline and the 3 months, indicating the absence of spontaneous variation over time ($P = 0.19$). A non significant tendency for an increase of activated cells count was observed in Group A1 and A2 ($P = 0.067$ and $P = 0.072$ respectively). In Group B1 and B2 there was a significant decrease of $CD4^+$ cells, Th1, Th2, Th 17 and all subtypes of activated T cells ($P = 0.01$) (Fig. 3) and this decrease correlated directly with the pain-functional Lequesne Index (Spearman Rho = 0.56, $P = 0.01$). The ANOVA analysis confirmed the independent influence of activated T cells and Th17 levels on Lequesne value with a significant Eta squared value of 15.6 ± 2.3 and 11.5 ± 1.6 respectively ($P = 0.01$) [Fig. 4(a) and (b)].

Discussion

Our data suggest a biological effect of HA and support a structure-modifying role regulating the systemic pro inflammatory milieu. It's well known that innate and adaptive immune responses are involved in OA pathogenesis[9]. Monocytes/macrophages are among the most abundant cell types present in the cellular infiltrates found in the inflames synovium in OA[4,10,11]. Macrophage-derived cytokines, including IL-1β and TNF-α are the major players in the cartilage breakdown in OA. They up-regulate the expression of MMP family of catabolic enzymes, MMP-1, -3 and -13[12–14] and stimulate the production of ADAMTS-4 and -5 (aggrecanases) in human chondrocytes[15] and in human OA synovial fibroblasts[16]. They also suppress the anabolic mechanism in the cartilage tissue by inhibiting the expression of type II collagen[17,18] and proteoglycans[19,20] in chondrocytes, the two major components of the cartilage extracellular matrix (ECM) in the cartilage.

Mononuclear cell infiltrates in synovial tissues have been reported in OA[21–23] and have been shown to contain primarily $CD3^+$ T cells[24]. The Th1 subset of T cells was found to be about 5 times more than Th2 cells[24] and higher levels of Th1 cytokines, IL-2 and IFNγ, were detected in most of OA patients[25]. T cells infiltrating the synovial membrane express early (CD69), intermediate (CD25 and CD38) and late (CD45RO) activation markers[26].

Saito *et al.* reported a $CD4^+/CD8^+$ ratio in OA ST (synovial tissues) of 5:1 compared to normal ST, where the ratio is 2:1[27]. Steiner *et al.* found that the $CD4^+/CD8^+$ ratio in OA was comparable to RA (Rheumatoid Arthritis)[28]. Several population of T helper (Th) cells can be distinguished in CD4 cells: Th1, Th2, Th17 as well as regulatory cells (Th3 and Tr1) with unique function and unique cytokine patterns[29–32]. In particular, it has been showed that OA synovial tissue is characterized by activated $CD4^+$ T cells and by expression of activation antigens (CD69, CD25, CD38, CD45RO, HLA Class II, CD80, CD83)[29]. In OA synovial tissue the ratio $CD4^+/CD8^+$ is 5:1 and it is higher than in normal ST[29] and it's comparable to RA ST. Also, number of CD4+ T cells is higher in early OA compared to late OA stage[29].

Table II
T cells levels at baseline in the five groups

	Control	Group A1	Group A2	Group B1	Group B2	*P* Value
CD4+	454 ± 123	760 ± 254	877 ± 79	976 ± 267	1227 ± 381	0.01
Th1	164 ± 61	224 ± 71	226 ± 18	273 ± 135	268 ± 118	0.03
Th2	93 ± 19	363 ± 138	412 ± 62	367 ± 148	321 ± 263	0.01
Th17	50 ± 26	88 ± 86	72 ± 8	88 ± 31	85 ± 61	0.01
CD4+DR+	13 ± 4	18 ± 11	20 ± 2.8	33 ± 32	34 ± 8	0.01
CD4+CD38+	85 ± 32	193 ± 63	337 ± 45.2	363 ± 40	662 ± 203	0.03
CD4+CD38 + DR+	6 ± 3	5 ± 2	7.1 ± 0.9	13 ± 10	16 ± 3	0.02

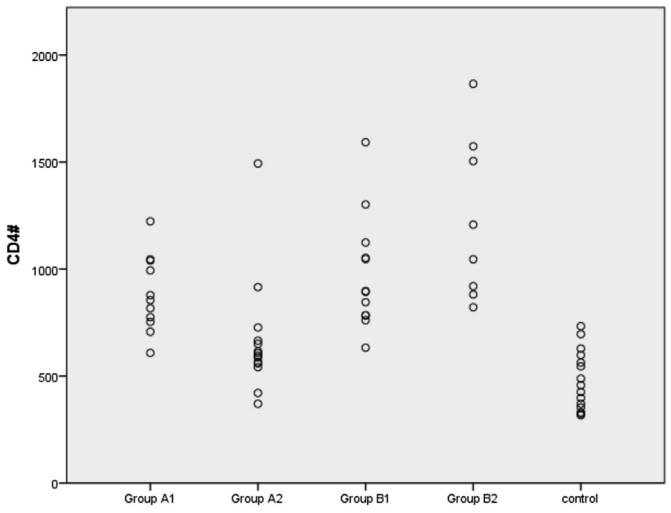

Fig. 1. Global Th CD4$^+$ levels observed in each subject in the five groups at baseline (*$P = 0.01$ between mean values of control group and other OA groups).

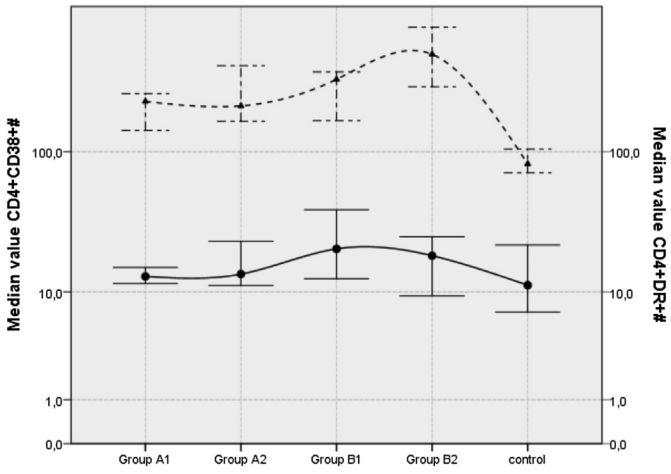

Fig. 2. Activated Th cells CD4$^+$CD38$^+$ (continuous line) and CD4$^+$DR$^+$ (dotted line) levels in the five groups at baseline (*$P = 0.01$ between the CD4$^+$CD38$^+$ levels of control group and OA groups; **$P = 0.04$ between the CD4$^+$DR$^+$ levels of control group and OA groups). Log10 scale used.

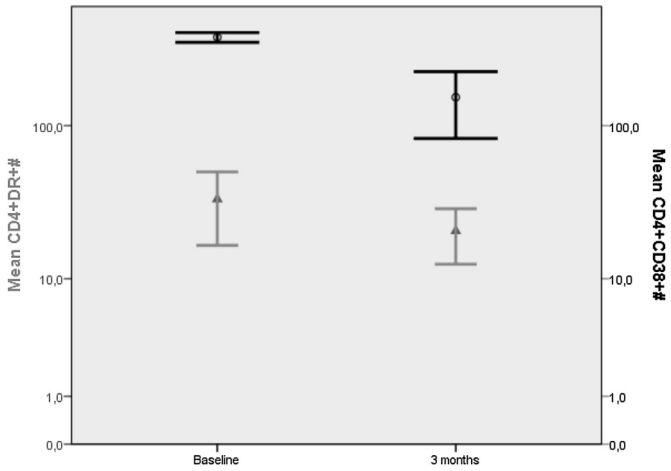

Fig. 3. Variation of Activated Th Cells CD4$^+$CD38$^+$ (black line) and CD4$^+$DR$^+$ (grey line) after the course of HA. Cumulative data of groups B1 and B2. Log 10 scale used (*$P = 0.01$ between the CD4$^+$CD38$^+$ levels; **$P = 0.03$ between the CD4$^+$DR$^+$ levels).

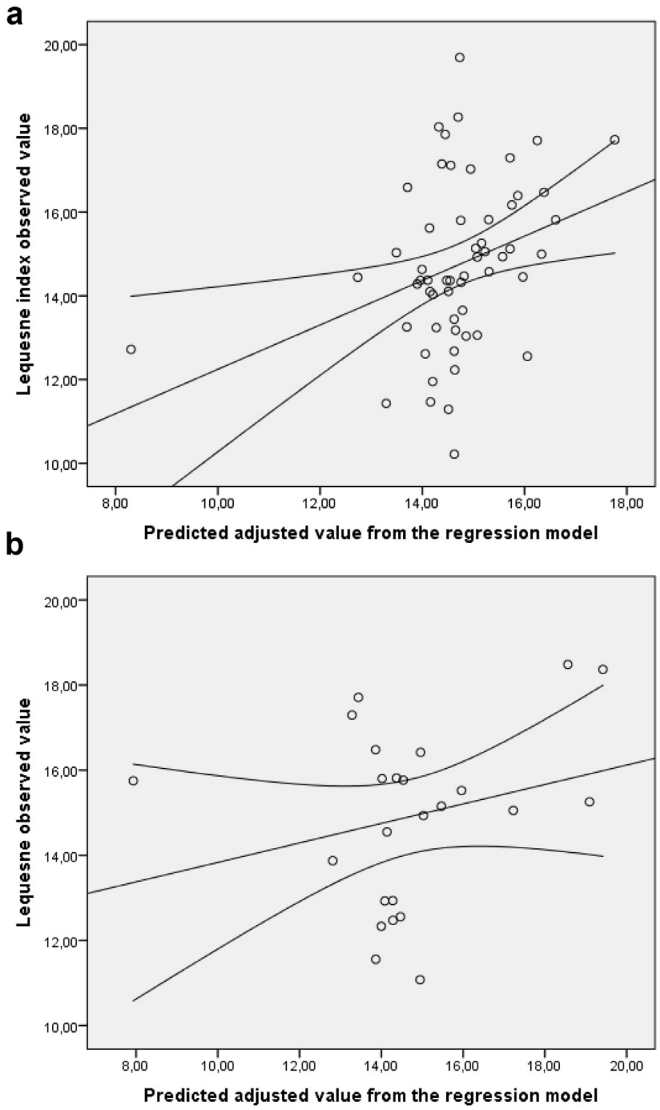

Fig. 4. a and b: Relationship between Lequesne functional pain value and activated Th cells value at baseline and 3-months (linear regression model with observed Lequesne pain value in Y axis and predicted adjusted value in X axis). Eta squared value between activated Th cells and Lequesne index of 15.6 ± 2.3 and 11.5 ± 1.6 at baseline and at 3 months, respectively ($P = 0.02$).

To our knowledge, this study is the first one specifically designed to measure the variations over time of OA T cells phenotype after HA injections.

The current study confirm that Th1, Th2 cells and activated T cells detectable in blood samples from patients with knee or hip OA have increased levels compared with healthy subjects. According to data literature, our study found a significant difference in Th17 cells expression between OA and healthy subjects.

Furthermore, we have demonstrated that HA intra-articular injections may reduce the count of activated T cells, mainly CD38+ and DR + cells, and Th 17 cells.

These data suggest an anti-inflammatory effect of HA. In summary, this exploratory study suggests that HA IA injections may modify the knee or hip joint metabolism in patients with OA resulting in a decrease in proinflammatory T cells concentrations. In particular it can reduce synovial inflammation and restore the rheological properties of synovial fluid. Further large scale prospective placebo-controlled studies, coupling biomarkers and

imaging techniques are needed to confirm these results and to investigate the possible disease modifying effect of HA as suggested in this work.

Declaration of contributions

Alfredomaria Lùrati MD PhD Conception and design Analysis and interpretation of the data.
Antonella Laria MD Collection and assembly of data.
Daniela Mazzocchi MD Drafting of the article.
Re Katia Angela MD Collection and assembly of data.
Mariagrazia Marrazza MD Collection and assembly of data.
Magda Scarpellini MD Final approval of the article.

Conflicts of interest

Authors have no financial or personal relationships with people or organizations that could potentially and inappropriately influence the present study.

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