Characterization of Synovial Fluid Cytokine Profiles in Chronic Meniscal Tear of the Knee

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ABSTRACT: Concentrations of pro- and anti-inflammatory cytokines in synovial fluid samples collected from patients with chronic meniscal tears were investigated. An acute inflammatory response is generally reported 24–48 h after knee injury, but the largest body of data available in literature concerns anterior cruciate ligament injury and very little information is available about the balance of soluble factors in the synovial fluid of knees with chronic meniscal tears. Sixty-nine patients (46 males and 23 females) with meniscal tear that occurred more than 3 months earlier were enrolled. According to cartilage integrity assessment by arthroscopic examination, patients were assigned to one of the following groups: (i) no chondral damage (n ¼ 18); (ii) chondral damage graded from I to II (n ¼ 15); and (iii) chondral damage graded from III to IV (n ¼ 37). In all groups, levels of IL-10 and inflammatory cytokines IL-6, TNF-a, and IL-8 where greater compared with those reported in the intact population; by contrast, levels of IL-1ra and IL-1b were significantly lower. Interestingly, IL-6 levels were higher in female than male patients. Cytokine levels did not correlate with degree of chondral damage. IL-6 and IL-1ra levels positively correlated with IL-1b, and negatively correlated with TNF-a. Interestingly, levels of IL-1b and TNF-a were inversely correlated. Our data demonstrate increased levels of pro-inflammatory cytokines (IL-6, IL-8, and TNF-a) in the chronic phase of meniscal trauma. This pro-inflammatory state is maintained in the joint from the time of initial injury to several months later and could be a key factor in hampering cartilage regeneration. □ 2016 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 35:340–346, 2017.

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Meniscus tear is one the most frequent knee injuries, with an incidence of approximately 70 cases per 100,000 people.[1,2] The meniscus plays a primary role in shock absorption, lubrication and nutrition of the articular cartilage and is a heterogeneous tissue composed of approximately 70% water, 20% collagen, and few cells. [3–6] Meniscal tears are a major risk factor for the development of post-traumatic arthritis (PTA) and increase, by 50%, the probability of symptomatic osteoarthritis (OA) development within 10–20 years of post-injury repair.[7] Partial excisions and total meniscectomies are associated with articular cartilage loss; subsequently, damage progresses from the articular cartilage to the entire joint structures.[8,9] Consequently, several surgical techniques have been developed to improve meniscal repair, including mechanical abrasion, the application of fibrin clot, and growth factor administration.[10–12] However, these procedures fail to restore meniscal function with a rate from 10% to 27% even in mechanically stable knees with peripheral meniscal tears.[13,14] Several factors influence meniscal healing, including (i) time between injury and surgery, (ii) patient age, (iii) tear morphology, and (iv) location.[13,15–17] It has been proposed that the inflammatory environment within the joint is a primary factor that could strongly influence meniscal repair. To illustrate, elevated levels of inflammatory cytokines have been found in synovial fluid associated with many joint diseases, including knee trauma and OA; however, their pathophysiological role remains inconclusive. Studies which investigated cytokine patterns in anterior cruciate ligament (ACL) injuries have shown the occurrence of pro-inflammatory patterns lasting for several weeks.[18,19] In particular, elevated levels of interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF-α), have been reported with peak levels occurring within 24 h after injury, and with levels remaining significantly higher than normal up to months after the trauma occurred.[18,20] IL-1ß and TNFα are generally acknowledged as primary inflammatory mediators associated with cartilage degeneration, bone changes, and synovial inflammation.[21]

In contrast to the abundance of data obtained in patients with ACL rupture, there are few available regarding cytokine levels in chronic isolated meniscal tear. Inflammatory cytokines appear to have a negative effect on meniscal healing. In vitro, IL-1 suppressed meniscal repair for up to 28 days and both IL-1 and TNF-α activated degradative and pro-inflammatory pathways in the meniscus and joint tissues.[22,23] Consequently, the meniscal matrix begins to degrade and progressively loose its crucial role in load distribution and joint lubrication. IL-1 has also been shown to increase matrix metalloproteinase (MMP)

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activity, and levels of nitric oxide and other catabolic mediators, as well as to decrease the shear strength of meniscal tissue at the repair site in vitro.[24]

The primary goal of this study was to investigate concentration changes of pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) and anti-inflammatory cytokines (IL-1ra and IL-10) in the synovial fluid of human knees with chronic meniscal tears, for comparison with levels observed in previous studies involving chronic tears in the meniscus and anterior cruciate ligament.[18,19,25,26] Our overall intent, however, was to ascertain how specific cytokine concentrations may be correlated with cartilage integrity, and therefore could help in prognostic stratification of meniscal lesions.

METHODS

Subjects

A total of 69 patients were enrolled in this study (46 males and 23 females), aged between 30 and 72 years (mean: 54.4 years ± 10.0) with meniscal tears. Inclusion criteria involved attestation of a chronic meniscal tear occurred more than 3 months earlier (average time since injury 11,2 ± 10,6 months) by a senior orthopaedic surgeon on the basis of (i) present history, (ii) physical examination, (iii) magnetic resonance imaging (MRI) as well as (iv) confirmation by arthroscopic examination. Patients with inflammatory or arthritic diseases, previous history of knee injury or infection, any anterior and/or posterior cruciate or collateral ligament injury, and antecedent intra-articular injection of steroid or hyaluronic acid were excluded. Cartilage integrity was assessed intraoperatively by the operating surgeon and noted in operative reports using the Outerbridge scoring system, where 0 = normal articular cartilage, I = superficial softening, II = superficial fissuring or fibrillation involving <1.25 cm area, III = fibrillation or fissuring involving >1.25 cm area, and IV = full-thickness cartilage wear with exposed subchondral bone.[27] All knees were graded based on the most severe area of chondral damage. Patients were grouped as follows on the base of the Outerbridge classification: group A: patients without chondral damage; group B: patients with meniscal tear associated with chondral damage graded from I to II; group C: patients with OA of grade III or IV.

To investigate the influence of age in cytokines patterns, the sample was resolved into groups as previously reported: (i) <40 years; (ii) 40–59 years; (iii) ≥60 years.[28]

All patients provided written informed consent to the retention of biological material that would have otherwise been discarded. The experimental protocol was approved by the Ethical Committee of the San Gerardo Hospital and conforms to the principles outlined in the WMA Declaration of Helsinki.

Samples

Synovial fluid was aseptically drawn from the knee without lavage at the beginning of the arthroscopic surgery. Synovial samples, collected in tubes containing EDTA, were immediately centrifuged at 3000*g* to remove cellular debris and the supernatant stored at −80°C until assayed.[18,19] The levels of interleukin (IL)-1β, IL-1RA, IL-6, IL-8, IL-10, and tumour necrosis factor (TNF)-α were measured using specific sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (IL-1 β, IL-1RA, IL-10, and TNF-α were from R&D Systems, Minneapolis, MN; IL-6 and IL-8 from eBioscience, San Diego, CA).

Six cytokines were measured by ELISA in the synovial fluid of patients. Detection limit was 18.3, 2.2, 0.8, 5.5, 2, and 1 pg/ml, respectively for IL1-ra, IL-6, IL-8, TNF-α, IL-10, and IL-1β cytokines.

Statistical Analysis

Statistical analysis was performed using MATLAB (Version R2010b; MathWorks). Normality of data distribution was assessed by the Jarque-Bera test. When data were not normally distributed, the influence of a variable of interest on cytokine levels was investigated through the Mann–Whitney test when comparing two groups, or the Kruskal–Wallis non-parametric test for three or more groups. Normal control data were calculated by retrieving mean and standard deviation from literature, and generating in Matlab environment a 100 samples population with the published mean and standard deviation following the equation $x = \text{mean} + \text{standard deviation}^{*}randn(100,1)$, where *randn* is a function that generates random numbers normally distributed. Correlations among various biochemical markers were assessed for significance using the non-parametric Spearman rank correlation coefficient test. Statistical test values of $p < 0.05$ were considered statistically significant.

RESULTS

Cytokines Patterns and Relationship With Chondral Damage

Sixty-nine patients were included in the study, all with verified meniscal tear and with the following clinical diagnosis received on discharge from arthroscopy: Group A (meniscal tear without chondral damage) 18 patients, mean age 50,7 ± 10,9 years, 5 females; Group B (meniscal tear associated with chondral damage graded from I to II) 15 patients, mean age 53,1 ± 9,8 years, 4 females; Group C (meniscal tear associated with chondral damage graded from III to IV) 37 patients, mean age 56,8 ± 9,2 years, 14 females.

Six cytokines (IL-1β, IL-1ra, IL-6, IL-8, IL-10, and TNF-α) were measured by ELISA in the synovial fluid of patients. All groups of cytokines were assessed for normal distribution through the Jarque–Bera test, and all of them were found to be non-normally distributed. Therefore, all groups were compared with non-parametric statistics. Although levels of IL-1ra and IL-8 only tended to increase with degree of chondral damage, there was no obvious relationship between levels of TNF-α, IL-10 or IL-1β with degree of cartilage degeneration (Table 1). Consequently, specific cytokine values for patient groups were pooled and cytokine data were analyzed and compared independently of the degree of chondral damage.

Comparisons With Cytokines Levels in Healthy Knees and Chronic ACL Associated With Meniscal Injuries

In patients with chronic meniscal tears, synovial concentrations of IL-6, TNF-α, IL-8, and IL-10 were significantly higher compared with their respective normal levels previously reported (Table 2 and Fig. 1).[19,25,26] In

Table 1. Cytokine Levels in the Synovial Fluid of Patient With Meniscal Tears

Type of Lesion	No of Patients		IL-1ra	IL-6	IL-8	TNF-α	IL-10	IL-1β
(A) Meniscal tear	18	Mean	54.93	219.23	65.23	30.43	5.07	4.81
		SD	75.70	359.91	87.58	94.45	3.01	4.03
(B) Meniscal tear associated with chondral damage graded from I to II	15	Mean	103.54	663.69	104.43	8.42	12.03	4.15
		SD	109.85	1774.80	189.69	11.06	19.83	4.43
(C) Meniscal tear associated with chondral damage graded from III to IV	36	Mean	170.83	161.46	456.14	38.96	5.84	2.89
		SD	364.17	273.04	1188.34	144.70	5.13	3.82
p-value			0.6983	0.4155	0.7208	0.1223	0.3671	0.3243

Cytokine concentrations (pg/ml) were measured in synovial fluid of patients with isolated meniscal tears. Chondral damage was scored according to Outerbridge's classification. The influence of chondral damage on cytokine levels was assessed with the Kruskal–Wallis non parametric test. Values are expressed as the mean and standard deviation (SD).

contrast, IL-1ra and IL-1β levels were significantly lower ($p < 0.001$) than those reported in normal knees. We clearly understand that cytokine levels measured in this paper are not straightforward comparable with reported normal levels both because of difference in the assay methods[19,26] and because of differences in the synovial fluid donor (cadaver and normal subjects).[21–23] However, a descriptive statistical approach was used for the interpretation of the data, and it is now possible to get the general picture of cytokine levels at different times after injuries.

The levels of IL-1β and IL-1ra due to chronic meniscal tears reported are significantly ($p < 0.001$) smaller than those reported previously due to chronic ACL- and meniscal tears.[18] On the other hand, we now report significantly ($p < 0.05$) larger synovial concentrations of IL-6, TNF-α, IL-8 in comparison with their levels in associated ACL and meniscal tear.[18] No statistical differences were detected in IL-10 levels between chronic meniscal tears group and chronic ACL and meniscal tear samples (Table 2).

Effects of Age on Cytokine Expression in Chronic Meniscal Tears

Patient groups consisted of: (i) 7 male patients younger than 40 years; (ii) 17 patients aged between 40 and 59 (13 males/4 females); and (iii) 45 patients older than 60 years (27 males/ 19 females). There were no significant age-related differences in the levels of cognate cytokines among patients of different ages with chronic meniscal tear (data not shown).

Effects of Gender on Cytokine Expression in Chronic Meniscal Tears

Cytokine data were further analyzed to investigate whether gender (23 females vs. 46 males) influenced the inflammatory environment of the knee with chronic meniscal tear. Interestingly, statistical analysis shows that levels of IL-6 were significantly greater in females compared with males with meniscal tears ($p = 0.0413$). No other cytokine levels presented any significant gender differences (Table 3).

Table 2. Cytokine Concentrations in the Synovial Fluid of Normal and Injured Knees

	Chronic Meniscal Tear						
	Cytokine Concentrations (pg/ml)	CV(%)	<LLOQ or 0 (*n*)	Normal Concentrations (pg/ml) From Literature	*p*-Value	Cytokine Concentrations (pg/ml) in Chronic ACL and Meniscal Tears[18]	*p*-Value
IL-1ra	137.9 ± 295.5	204.87	24	2520.0 ± 1469.0[23]	<0.001	1230.499 ± 963.538	0.001
IL-6	289.6 ± 894.9	298.14	20	66.4 ± 102.3[21]	0.0145	83.23 ± 51.868	0.0220
				0.8 ± 0.4[23]	0.0014		
IL-8	283.1 ± 891.6	284.56	3	21.0 ± 5.1[23]	0.0036	68.115 ± 56.768	0.0155
TNF-α	29.6 ± 114.5	364.46	49	<5.0[21]	0.0181	6.69 ± 2.02	0.0439
IL-10	7.1 ± 10.3	141.37	9	3.3 ± 6.3[22]	0.0056	5.457 ± 2.815	0.2149
IL-1β	3.6 ± 4.0	114.67	35	10.0 ± 4.3[23]	<0.001	6.69 ± 2.02	0.001

Cytokine levels from knees with chronic meniscal tear were compared to normal levels reported in literature and to cytokine levels previously measured in knees with anterior cruciate ligament (ACL) and chronic meniscal tears. CV, coefficient of variation; LLOQ, levels below the lower limit of quantification indicated as the value of the lowest point on the calibration curve divided by 2. Control samples in reference[21] have been obtained from six healthy volunteers (five men and one woman), those in reference[23] from 10 volunteers (four men and six women) and in reference[22] from 16 post mortem donors within 24 h after death. For statistical comparison purposes, we randomly generated 100 samples of normal population using the published mean and standard deviation (as described in the Statistic section). Cytokine concentrations in chronic ACL and meniscal tears[18] have been obtained from 6 male patients, and statistical comparison has been performed with raw data. Values are expressed as the mean ± standard deviation. A $p < 0.05$ was considered significant.

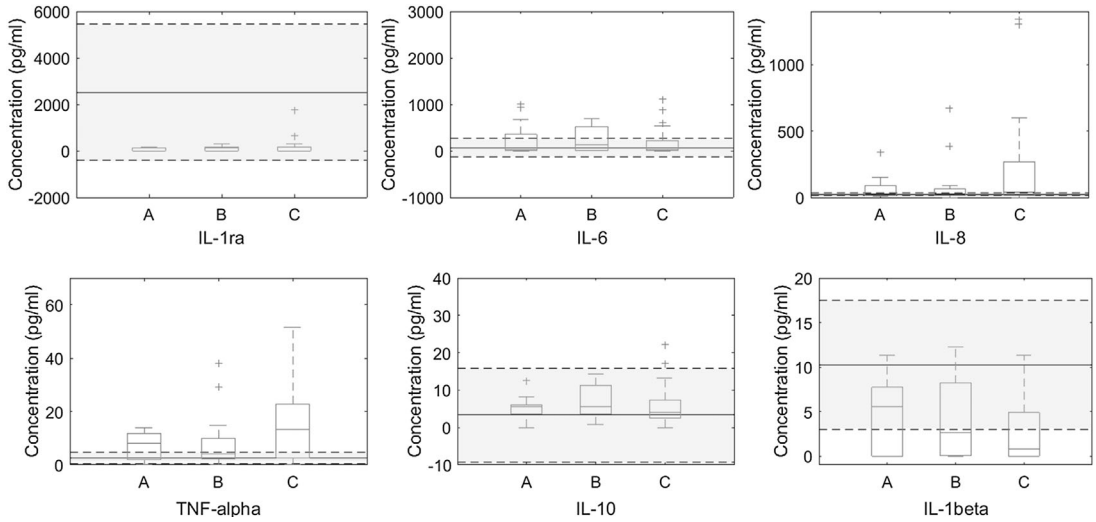

Figure 1. Cytokine levels in synovial fluid of patients with different grades of chondral damage. The mean concentration (gray dots), and standard deviation (bars) of measured cytokines were plotted for the following groups: (A) meniscal tear without chondral damage; (B) meniscal tear associated with chondral damage graded from I to II; and (C) meniscal tear associated with chondral damage graded from III to IV. Chondral damage has been graded following the Outerbridge's classification as described in Methods. Gray shaded area represents normal concentration ranges where the horizontal full line represents the mean cytokine concentration in the control group derived from literature, and the dashed lines are the ± 2 SD interval reported for the control group.

Correlations Between Cytokines in Chronic Meniscal Tears

Correlations among cytokine levels in synovial fluid were investigated independently from chondral damage and other previously mentioned parameters (i.e., gender and age). Our results show that the concentrations of IL-1ra were negatively correlated with those of TNF-α (Fig. 2, panel A), but positively correlated with those of IL-1β (panel B). Interestingly, the concentrations of IL-1β were positively correlated with those of IL-6 (panel D), and negatively correlated with those of TNF-α (panel E).

DISCUSSION

The metabolic activity of meniscal cells is strongly influenced by various factors, including (i) mechanical compression and stretch,[29–31] (ii) synovial fluid properties, and particularly (iii) synovial messengers, including specific growth factors and cytokines.[32,33] Meniscal damage itself is a well-known risk factor for knee osteoarthritis (OA), and may be a trigger for synovial activation. Moreover, evidence suggests that age and gender are factors which may interact with the nature of injury pattern, leading to OA.[19] Knee injury leads to the release of several cytokines into synovial fluid, and an acute inflammatory response is initiated, typically lasting 24–48 h, which may occasionally persist several weeks.[9,20] To illustrate, anterior cruciate ligament injury results in prolonged elevation of specific pro-inflammatory cytokine levels in synovial fluid.[18,19,25] However, to the best of our knowledge, there is no information on synovial fluid cytokine levels after the acute phase of isolated meniscal tear. We now present the first information on levels of pro-inflammatory and anti-inflammatory cytokines in synovial fluid collected at least 3 months after an acute (verified) isolated meniscal tear.

Table 3. Effects of Gender on Cytokine Expression in the Synovial Fluid of Knees With Chronic Meniscal Tears

	Female $N = 23$	Male $N = 46$	p-Value
IL-1ra	116.91 ± 174.11	148.00 ± 341.43	0.7594
IL-6	611.34 ± 1480.72	128.74 ± 208.60	0.0413
IL-8	70.95 ± 121.04	401.06 ± 1092.24	0.5433
TNF-α	27.80 ± 80.35	31.26 ± 129.90	0.9361
IL-10	5.41 ± 4.11	7.84 ± 12.23	0.6192
IL1-β	4.12 ± 4.45	3.38 ± 3.83	0.4746

Cytokine concentrations (pg/ml) were measured in synovial fluids of male and female patients with chronic meniscal tears (23 female patients, mean age 57.82 ± 7.53 years; 46 male patients, mean age 52.71 ± 10.72 years). Values were expressed as the mean $\pm$ standard deviation. A $p < 0.05$ was considered significant.

Our results show that synovial fluid concentrations of the pro-inflammatory cytokines IL-6, TNF-α, and IL-8, as well as the modulator cytokine IL-10, remained significantly greater in knees with chronic meniscal tears for several months after the injury compared with reported normal population values, suggesting a very prolonged inflammatory environment. IL-6 and TNF-α are generally considered key players in the initiation and progression of osteoarthritis processes. Coincidentally, the low levels of the anti-inflammatory cytokine IL-1ra imply a suppressed anti-inflammatory response.

The considerable variation in synovial cytokine levels that we currently present support the hypothesis that local inflammatory responses in traumatic knee injuries depend on a variety of factors: (i) time from injury, (ii) the injury pattern, (iii) patient age, and (iv) gender.[25,34,35]

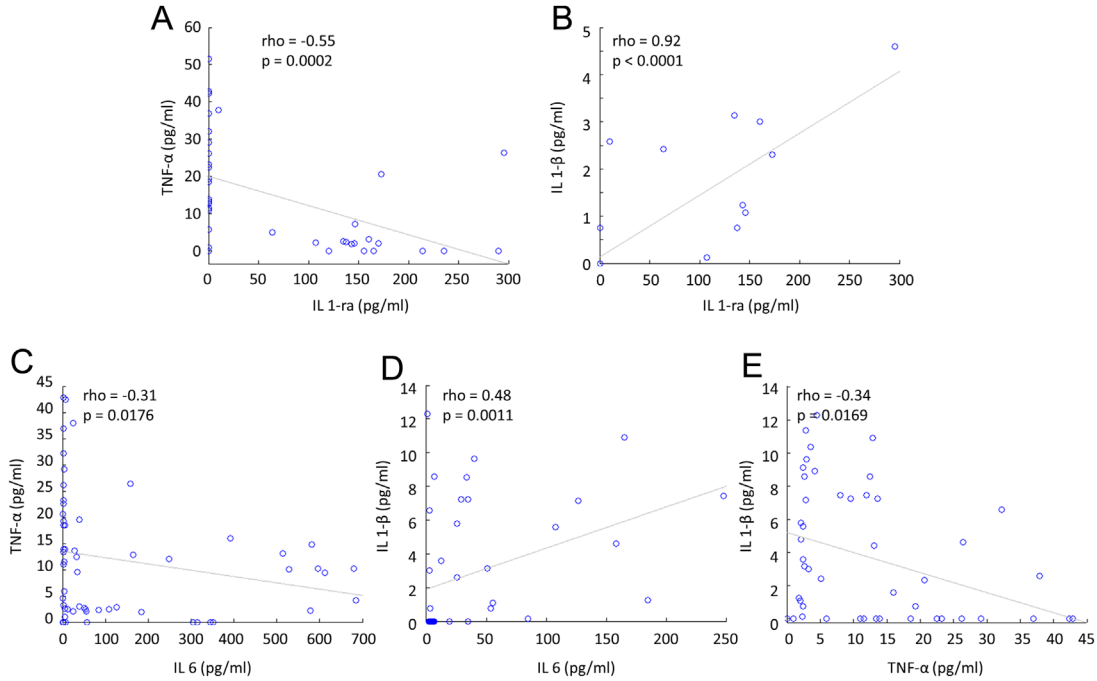

Figure 2. Correlations between cytokines in the synovial fluid of knees with chronic meniscal tears. Panels illustrate correlations that were statistically significant. Correlations among cytokines were investigated using the non-parametric Spearman rank correlation coefficient test. Calculated regression lines are shown.

Among inflammatory mediators, TNF-α has been implicated in the chronic inflammatory process associated with an isolated ACL tear or ACL tear associated with meniscal tear.[18,25] The role of this cytokine in the chronic stage of knee inflammation is also supported by our current results which demonstrate that the concentrations of TNF-α, IL-6 and IL-8 are greater in chronic meniscal tears without ACL injury compared with those associated with coincident ACL and meniscal injury.

With respect to IL-1β and IL-1ra concentrations in our samples (meniscal tear only) were smaller than those in reported in literature for patients with chronic combined ACL and meniscal tears. Thus, our findings support the hypothesis that differential injuries of the articular environment induce divergent cytokine responses. Cytokines belonging to the IL-1 family appear to play a primary role in the period immediately following joint trauma; in fact, increased IL-1 expression has been documented very shortly after mechanical joint injury and is correlated with severity of cartilage damage.[36,37]

By comparison, greater concentrations of IL1-β, and TNF-α, MMP-13 and other chemokines like CCL3 and CCL3L1 have been reported in meniscal tissue of patients with combined ACL and meniscal tears compared with isolated meniscal tear.[34]

From the clinical perspective of arthritis, there is a close relationship between meniscal tear and (i) premature onset of osteoarthritis,[38] and (ii) increased levels of cytokines, metalloproteases and cartilage degradation products, all of which characterize the joint environment after a trauma.[25,39] In the present study we did not find any correlation between the concentrations of inflammatory cytokines and the grade of chondral damage assessed at arthroscopy. This finding suggests that following knee injury the inflammatory response could lead to structural cartilage damage, but that the direct contribution of cartilage to cytokine levels in the synovial fluid is relatively limited. In particular, ACL tear is associated with the up-regulation of pro-inflammatory mediators involved in cartilage loss.[40]

Patient age may play a role in the expression of soluble factors after joint trauma. The meniscus in young individuals is more prone to inflammatory changes after an isolated meniscal tear.[34] In the present study, patients who were (i) under 40 years, (ii) those aged between 40 and 59, and (iii) those older than 59 were compared with respect to cytokine levels in the synovial fluid. Our results fail to demonstrate age-related differences among cytokine responses measured. This could be due to the very small size of the group of young patients (only 7 under the age of 40) enrolled in this study. Moreover, the measurement of cytokine mRNA levels directly in the meniscal tissue[34] is a more sensitive method to study the inflammatory process specifically in the meniscus, whereas measurement of cytokine levels in synovial fluid provides a more general picture of the joint environment.

Gender may also have a role in knee pathology. It is well documented that OA is a more prevalent condition among women than among men[41] and that musculoskeletal-pain is a cause of disability especially

in women with low levels of estrogen, for example, during menopause.[42] Indeed, our current data show greater concentrations of IL-6 in female synovial fluid compared with those in males; by contrast, there were no significant gender differences in the concentrations of all the other cytokines measured. We did not, however, collect menopausal data from our female patients; nonetheless, the mean age of our female sample group (57.8 ± 7.5; mean $\pm$ SEM, $n = 23$) is comparable with the estimated mean age for menopause of 50 years among Italian women.[43]

Consequently, it is now not surprizing that low levels of estrogens such as 17β-estradiol lead to upregulation of IL-6 production[44] and that high levels of IL-6 are coincident in OA. IL-6 levels are increased in the synovial fluid and serum of patients with rheumatoid arthritis.[45] IL-6 is a pro-inflammatory cytokine with a key role in MMP production synthesized by synoviocytes, meniscal, and articular chondrocytes[46,47] and it is widely recognized as a potent stimulator of osteoclast-driven bone absorption in the context of chronic inflammation and estrogen deficiency.[45] Other cytokines like IL-1, TNF-α, and INFγ are also able to upregulate IL-6 synthesis.[46,48] These finding collectively imply a primary role of IL-6 in driving articular cartilage degeneration. We speculate that for preventing the development of OA in meniscal tears, it could be important to neutralize IL-6 as soon as possible after the trauma. In fact, suppressing IL-6-induced production of chemokines and adhesion molecules could reduce the progression of the inflammatory pattern into the injured joint.

Interesting correlations among cytokine responses have been already described in consequence to ACL injury, either isolated or combined with meniscal tear.[18,19,36] In this study, we show that large concentrations of pro-inflammatory IL-1β are directly correlated with IL-1ra and IL-6. IL-1ra inhibits the catabolic activity of IL-1β and protects the joint environment; however, IL-1ra concentrations are smaller than reported in normal knee (Table 2). IL-1β and IL-6 are involved in the stimulation of expression of MMP and other pro-inflammatory cytokines (e.g., IL-1α, IL-1β, IL-6) in normal and osteoarthritic meniscus cells.[46] It is interesting to note that after the acute phase of inflammation, IL-1β levels follow a pattern toward normalization, whereas IL-6 and TNF-α levels remains elevated for several months after meniscal (this study) and ACL tears.[49,50] The inverse correlation between IL-1β and TNF-α levels that we have found in this study is of difficult interpretation and deserves further attention in future studies. The existence of correlations between different cytokines suggests the presence of a complex network, which will require more specific and targeted investigations.

We regret that the nature of our patient population did not allow for a longitudinal consideration of cytokine responses, since no repeated sampling was made within individuals. Longitudinal studies, incorporating repeated sampling, beginning closely to onset of injury and projecting to complete healing or onset of OA are strongly needed to investigate the modification of cytokine responses in isolated meniscal tears in order to formulate potential clinical interventions to mitigate the onset and severity of consequent OA.

In conclusion, our findings suggest that elevated cytokine levels indicate an inflammatory and catabolic response in patients with meniscal tear, either in an isolated meniscal tear, or in presence of high chondral damage. Females presented higher levels of IL-6 than males, consistent with a greater female predisposition to progression of OA. The complex and cytokine-mediated inflammatory mechanisms revealed in this study provide compelling rationale for further research in support of better understanding of the high failure rate of meniscal repair and development of novel therapies for knee OA.

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AUTHORS' CONTRIBUTIONS

M.B., M.T., D.G., V.L., R.J.O., A.T. designed the study. M.B., M. T., A.C., M.P., D.G., P.S., S.F. performed the experiments. M.G., A.P., M.T., A.C. E.B., A.T. analyzed the results. M.B., M.T., M.P., D.G. drafted the manuscript. Manuscript final revision by P.S., R.J.O., V.L., A.T., M.G.. All authors approved the final version of manuscript for submission.

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