Male IL-6 gene knock out mice developed more advanced osteoarthritis upon aging

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Summary

Objective: Interleukin-6 (IL-6) is expressed in osteoarthritic joints but its function in osteoarthritis (OA) is unknown. To study this, spontaneous and experimental OA were evaluated in IL-6 deficient (IL-6−/−) mice.

Design: Histology of knees of 18–23-month-old wild type (wt) and IL-6−/− mice was compared for signs of OA. Cartilage proteoglycan (PG) density was measured by image analysis on safranin-O stained whole knee sections. Chondrocyte PG synthesis was measured ex vivo by 35S-sulfate incorporation. Knee bone mineral density (BMD) was measured by dual energy x-ray absorptiometry. In young mice (3 months), OA was induced by intra-articular injection of collagenase.

Results: The incidence of extensive cartilage loss at both lateral and medial sides was markedly higher in old IL-6−/− mice, but not in females, as compared to their wt controls. Compared to age-matched wt mice, reduced ex vivo PG synthesis was found during aging in IL-6−/− males, without affecting their cartilage PG density. IL-6−/− males showed more extensive extracellular matrix deposition in the collateral ligaments and subchondral bone sclerosis, predominantly at the medial side. Total knee BMD decreased more in IL-6−/− (−23%) than in wt (−10%) males during aging. Collagenase-induced OA showed a similar degree of joint pathology in both strains, implying that OA susceptibility was not different at younger age.

Conclusions: Upon aging, IL-6−/− male mice developed more severe spontaneous OA. Reduced PG synthesis and BMD values might be indicative for an impaired repair response in IL-6−/− mice. This suggests a protective role for IL-6 in age-related OA in male mice.

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Key words: Interleukin-6, Knock out mice, Osteoarthritis, Proteoglycan synthesis.

Introduction

Osteoarthritis (OA), the most common of all joint disorders, is a disabling disease whose incidence increases with age. The cause of OA is unknown but several mechanisms could be involved including genetic/age-related alterations in extracellular matrix (EM) components, biomechanical stress or an imbalance in synovial homeostasis. The disease is characterized by breakdown of the cartilage matrix followed by development of fibrillations, fissures and this ultimately can lead to complete loss of articular cartilage. Another characteristic of OA is hypertrophy of the bone. Thickening of the subchondral bone can lead to increased stiffness and reduced shock absorbing capacity of the bone. Another complication related to bone is the formation of osteophytes in OA joints. Whether changes in the bone cause cartilage pathology or vice versa is still a matter of debate.

The production of both anabolic and catabolic cytokines and growth factors by the articular chondrocytes and synovial lining cells could contribute to the coexistence of repair and destructive processes in OA joints (reviewed in Refs.4,5). Increased experimental OA in IL-1β deficient mice suggested recently that catabolic cytokines could also have beneficial effects and could contribute to joint homeostasis in OA. One other cytokine whose expression is increased in affected joints of OA patients is the multifunctional cytokine interleukin-6 (IL-6). IL-6 production has been found in chondrocytes from OA cartilage but its exact function in this disease is not clear. IL-6 could reduce PG synthesis in vitro but this was investigated with normal and not with OA cartilage. IL-6, in contrast, had no effect on PG metabolism itself but depressed IL-1α induced PG breakdown in OA chondrocytes in vitro. The induction by IL-6 of α1-antitrypsin and Timp-1 in human chondrocytes also suggests a protective role against cartilage pathology. Previously, we had found a cartilage protective role for IL-6 during the onset of zymosan-induced arthritis (ZIA) in mice. IL-6 deficient mice had higher articular cartilage PG loss during onset of ZIA. This PG loss could be normalized by intra-articular injection of IL-6.
Furthermore, IL-6 injections in naive mice could stimulate PG synthesis moderately\(^{13}\).

IL-6 also has properties that could have a negative effect in the joint e.g., in the presence of the soluble IL-6 receptor, IL-6 contributes to osteoclast development\(^{14}\), and this links it to bone erosion. Furthermore, IL-6 plays a role in the development of chronic joint inflammation\(^{15,16}\), and might therefore be involved in OA-associated joint inflammation.

Investigations on the role of IL-6 in murine OA are limited. Increased IL-6 messenger RNA (mRNA) expression was found in cartilage of C57 black mice with early stages of spontaneous OA\(^{17}\). IL-6 expression, however, did not correlate with histological changes. Male STR/orf mice develop osteoarthritic lesions of the knee joint by 35 weeks of age. In situ hybridization studies showed increased IL-6 mRNA expression in cartilage at the site of the lesions\(^{18}\). It is, however, not clear if this IL-6 expression plays a role in developing cartilage damage in mice. Recently, it was shown that young STR/orf females had a higher expression of IL-6 in their cartilage than males\(^{19}\). Because in the STR/orf strain female mice develop less OA than males, it was of interest to include both male and female mice in our study.

In the present study we have investigated spontaneously developed age-related OA as well as experimentally induced OA in wt and IL-6\(-/\)- mice.

**Materials and methods**

**SPONTANEOUS AND EXPERIMENTAL OSTEOARTHRITIS**

Wild type (wt) C57BL6 (Charles River, Sulzfeld, Germany) and IL-6\(-/\)- mice\(^{20}\), back-crossed eight times with C57BL6, were used in the experiments. Breeding pairs of the IL-6\(-/\)- mice were a kind gift from Dr Manfred Kopf (Basel, Switzerland). During a period of 4 years, groups of healthy mice were followed for the spontaneous development of OA. Knee joints of male and female mice were isolated when they had reached the age of 18 months or older. The mean age in months \(\pm \text{sd}\) for the different groups was 19.8 \(\pm\) 0.6 for wt male (\(n = 36\)), 20.0 \(\pm\) 2.1 for IL-6\(-/\)- male (\(n = 39\)), 19.6 \(\pm\) 2.2 for wt female (\(n = 24\)) and 19.6 \(\pm\) 1.7 for IL-6\(-/\)- female (\(n = 27\)). Isolated knee joints were fixed in formalin and processed for histological evaluation. Male C57BL6 wt and IL-6\(-/\)- mice were used for experimental OA at the age of 3–4 months. Experimental OA was induced by injecting 6 \(\mu\)l of physiological saline containing 1 unit of collagenase (from *Clostridium histolyticum*, type VII, Sigma, St. Louis, MO) in the knee joint of mice. The injection was repeated once at day 2 after the first injection. At day 42 after the start of the experiment, knee joints were isolated, formalin fixed and processed for histological evaluation.

All mice were housed in filter-top cages under standard pathogen free conditions and a standard diet and water were provided *ad libitum*. Experiments were performed according to national and institutional regulations for animal use.

**HISTOLOGICAL EVALUATION OF KNEE JOINTS**

Histological signs of OA were scored in a blindfolded manner on five semi-serial sections of the joint. Cartilage erosion was scored on a scale from 0 to 3 as was published before by Mahr *et al*.\(^{19}\). 0, no cartilage erosion; 1, superficial ruffling; 2, surface erosion and/or fissures; and 3, complete loss of cartilage. Proteoglycan (PG) deposition (red color in safranin-O-stained sections) and bone formation in the ligaments were scored on a scale from 0 to 3: 0, no changes; 1, moderate red staining; 2, extensive red staining; and 3, extensive red staining and bone formation in the ligament. Incidence of mice with erosion and/or PG deposition was expressed as a percentage of the total number of mice in that group. The joint sections were furthermore evaluated for the presence or absence of dislocation of the patella, subchondral bone sclerosis, joint inflammation, osteophyte formation and bone apposition.

**CHONDROCYTE PG SYNTHESIS**

PG synthesis was assessed by \(^{35}\)S-sulfate incorporation in patellar cartilage. Patellae were dissected, with a minimum amount of surrounding synovium, under sterile conditions. The *ex vivo* synthesis assays were performed in RPMI/penicillin 100 U/ml and streptomycin 100 \(\mu\)g/ml/1 mMol/l pyruvate/5% fetal calf serum (Life Technologies, Breda, The Netherlands). The patellae were placed separately in 200 \(\mu\)l medium containing 4 \(\mu\)ci \(^{35}\)S-sulfate and incubated for 3 h at 37 \(^\circ\)C and 5% CO\(_2\). After labeling, the patellae were washed twice with physiological saline and fixed overnight (O/N) in 100% ethanol. Patellae were decalcified in 5% formic acid for 4 h at room temperature. Thereafter, the articular cartilage was stripped from the underlying bone and dissolved O/N in 0.25 ml lumasolve at 60 °C (Lumac, Groningen, The Netherlands). After addition of 1 ml lipoloma (Lumac, Groningen, The Netherlands), the \(^{35}\)S-sulfate content of each patella was measured (counts per minute) by liquid scintillation counting in a Trilux 1450 MicroBeta (Perkin–Elmer Wallac, Turku, Finland).

**CARTILAGE PG BREAKDOWN**

Patellae were isolated and labeled with \(^{35}\)S-sulfate as described for the *ex vivo* PG synthesis. Part of the patellae were fixed after labeling (\(t = 0\)). The rest were, after washing, further incubated for 48 h at 37 °C and 5% CO\(_2\) in medium without \(^{35}\)S-sulfate. Medium was changed after the first 24 h. After 48 h incubation the patellae were washed, fixed and processed as described for the *ex vivo* synthesis.

**ASSESSMENT OF PG DENSITY IN PATELLAR CARTILAGE**

Patellar PG density was quantified by image analysis on histological slides as described previously by Van der Kraan *et al*.\(^{21}\). Images of safranin-O-stained sections were captured using a JVC 3-CCD color video camera (Victor Company of Japan Ltd., Tokyo, Japan) and displayed on a computer monitor. Patellar cartilage was selected and the amount of red staining was measured using the Qwin image analysis system and is presented as a unitless number by this system (Leica Imaging Systems Ltd., Cambridge, UK). For each mouse, three sections were measured.

**MEASUREMENT OF KNEE BONE MINERAL DENSITY**

Bone mineral density (BMD) of murine knee joints was determined by dual energy x-ray absorptiometry (DEXA)\(^{22,23}\). Knee joints were scanned with a Norland bone densitometer XR-46 (Norland Medical Systems, Inc. Fort Atkinson, WI). Calibration of the densitometer was performed on a daily basis with a calibration phantom before measurement and showed less than 0.4% variation among the last 16 calibrations. Scans were performed at...
a speed of 60 mm/s with a resolution of 0.5 × 0.5 mm and included the femoral condyles, patella and tibial plateau. The BMD is expressed as mg/cm².

STATISTICAL ANALYSIS

Statistical analysis between groups was performed with Student’s t test. Distribution of maximal OA scores was analyzed by a chi-square test with a 95% confidence interval. Values of P < 0.05 were considered significant in both tests.

Results

OSTEOARTHRITIS RELATED PATHOLOGY IN OLD MICE

Knee joints were isolated from wt and IL-6⁻/⁻ mice of both sexes at the age of 18 months or older to investigate spontaneous OA (see Table I for number of mice). At the femoral—tibial junction, cartilage surface became affected varying from superficial ruffling to complete erosion of the cartilage layer. PG deposition and, in more advanced cases, bone apposition occurred in the collateral ligaments. Subchondral bone sclerosis was also frequently observed. Inflammation was not observed in the female mice. One out of 36 wt males had an inflamed joint with both an inflammatory infiltrate and an inflammatory exudate while 3 out of 39 IL-6⁻/⁻ males showed signs of previous joint inflammation as demonstrated by a thickening of the synovial lining. Dislocation of the patella was not observed in these old mice.

HIGHER INCIDENCE AND MORE SUBSTANTIATED CARTILAGE DAMAGE FOUND IN OLD IL-6⁻/⁻ MALES

Female mice of both strains had only moderate signs of spontaneous OA with mild cartilage erosion and only in exceptional cases complete cartilage loss was observed [Table I, Fig. 1(A and B)]. Male wt mice also developed spontaneous OA at the same incidence and with comparable degree of OA pathology as the females. However, significantly more IL-6⁻/⁻ male mice showed severe OA with complete cartilage erosion at both medial and lateral sides [Table I, Fig. 1(C and D)]. Further experiments were therefore focused on male mice.

The cartilage erosion occurred in absence of cartilage PG depletion. Image analysis performed on safranin-O stained histological sections showed no significant difference (Student’s t test) between the wt and IL-6⁻/⁻ male mice in the PG density of patellar cartilage [mean ± sd, 68.9 ± 10.2 mg/cm²; n = 13]. IL-6⁻/⁻ males at 20 months of age, in contrast, had a significantly reduced BMD value as compared to age-matched wt mice [mean ± sd, 48.7 ± 5.3 mg/cm²; n = 13] and wt 61.8 ± 6.1 mg/cm²; n = 12, respectively, P < 0.001, Student’s t test]. The reduction in the mean BMD was also greater for IL-6⁻/⁻ males as for wt males (−23% vs −10%) in the period from 3 to 20 months. Histology, however, showed subchondral bone sclerosis in the tibial plateaus, which occurred more frequently at the medial side and had the highest incidence (49%) in old IL-6⁻/⁻ males with spontaneous OA [Table III, Fig. 1(D)]. Although bone sclerosis was found in 11 of the 14 IL-6⁻/⁻ males with complete cartilage loss, it did not co-localize with cartilage erosion in general (compare Tables I and III).

Old IL-6⁻/⁻ female mice (18–19 months), in contrast, did not differ from their wt counterparts in total knee BMD [mean ± sd, IL-6⁻/⁻ 57.3 ± 8.5 mg/cm²; n = 12] and wt 64.0 ± 9.6 mg/cm²; n = 13, not significant, Student’s t test].

Osteophyte formation, another bone-related change during OA, was observed less frequently in both females (wt, 2 of 24; IL-6⁻/⁻, 4 of 27) and males (wt, 1 of 36; IL-6⁻/⁻, 7 of 39) with spontaneous OA.

ENHANCED PG AND BONE APPPOSITION IN COLLATERAL LIGAMENTS OF OLD IL-6⁻/⁻ MALE MICE

Changes in collateral ligaments may jeopardize joint stability and are implicated in the osteoarthritic process. In both sexes of each strain, the apposition of PG and bone in the collateral ligaments occurred mainly at the medial side during spontaneous OA [Table IV, Fig. 1(A and B)].

Table I

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mice (n)</th>
<th>Incidence (%)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Side</th>
<th>Side</th>
</tr>
</thead>
<tbody>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>M</td>
<td>L</td>
</tr>
<tr>
<td>wt female</td>
<td>24</td>
<td>63</td>
<td>9</td>
<td>13</td>
<td>1</td>
<td>1</td>
<td>2(0)</td>
<td>14(1)</td>
</tr>
<tr>
<td>IL-6⁻/⁻</td>
<td>27</td>
<td>70</td>
<td>8</td>
<td>14</td>
<td>2</td>
<td>3a</td>
<td>4(1)</td>
<td>17(3)</td>
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<tr>
<td>wt male</td>
<td>36</td>
<td>66</td>
<td>12</td>
<td>15</td>
<td>5</td>
<td>4</td>
<td>12(4)</td>
<td>18(1)</td>
</tr>
<tr>
<td>IL-6⁻/⁻</td>
<td>39</td>
<td>87</td>
<td>5</td>
<td>15</td>
<td>5</td>
<td>14*</td>
<td>16(11)</td>
<td>26(8)</td>
</tr>
</tbody>
</table>

*Comparison of wt and IL-6⁻/⁻ male mice was analyzed by a chi-square test with a 95% confidence interval. *P < 0.05; ns, not significant.

1Scoring ranged from 0 to 3 as described in Materials and Methods. For each mouse the maximum score, either medial or lateral or equal on both sides, is indicated. This means that the other side could have lower or no signs of cartilage erosion. Incidence is calculated as the percentage of mice with a positive score of 1, 2 or 3.

1Distribution of the maximal score (either 1, 2 or 3) for cartilage damage over the medial (M) and lateral (L) sides of the knee joint. In case the score is equal for both sides then that joint is counted for medial as well as lateral. The number between brackets indicates the number of joints with maximal damage score 3 at that side.

INCREASED SUBCHONDRAL BONE SCLEROSIS IN OLD IL-6⁻/⁻ MALES

The BMD of murine knee joints was measured by the DEXA technique. Young male mice (3 months) of both strains did not differ in their BMD value (mean ± sd: IL-6⁻/⁻ 63.2 ± 7.7 mg/cm²; n = 12; wt 68.9 ± 10.2 mg/cm²; n = 13). IL-6⁻/⁻ males at 20 months of age, in contrast, had a significantly reduced BMD value as compared to age-matched wt mice [mean ± sd, IL-6⁻/⁻ 48.7 ± 5.3 mg/cm²; n = 13] and wt 61.8 ± 6.1 mg/cm²; n = 12, respectively, P < 0.001, Student’s t test]. The reduction in the mean BMD was also greater for IL-6⁻/⁻ males as for wt males (−23% vs −10%) in the period from 3 to 20 months. Histology, however, showed subchondral bone sclerosis in the tibial plateaus, which occurred more frequently at the medial side and had the highest incidence (49%) in old IL-6⁻/⁻ males with spontaneous OA [Table III, Fig. 1(D)]. Although bone sclerosis was found in 11 of the 14 IL-6⁻/⁻ males with complete cartilage loss, it did not co-localize with cartilage erosion in general (compare Tables I and III).

Old IL-6⁻/⁻ female mice (18–19 months), in contrast, did not differ from their wt counterparts in total knee BMD [mean ± sd, IL-6⁻/⁻ 57.3 ± 8.5 mg/cm²; n = 12] and wt 64.0 ± 9.6 mg/cm²; n = 13, not significant, Student’s t test].

Osteophyte formation, another bone-related change during OA, was observed less frequently in both females (wt, 2 of 24; IL-6⁻/⁻, 4 of 27) and males (wt, 1 of 36; IL-6⁻/⁻, 7 of 39) with spontaneous OA.
significantly higher incidence and more severe PG and bone deposition in the collateral ligaments was found in the IL-6^{-/-}/C255/C255 males as compared to their wt controls [Table IV, Fig. 1(C)]. In 9 of the 10 IL-6^{-/-}/C255/C255 males with maximal apposition in the medial collateral ligament (MCL) (score 3), cartilage erosion was also maximal at the same side.

SIMILAR PATHOLOGY OF EXPERIMENTAL OA IN WT AND IL-6^{-/-} MICE AT YOUNG AGE

Analysis of the spontaneous OA showed a clear difference between old wt and IL-6^{-/-} male mice. To investigate if IL-6^{-/-} mice are even at younger age more prone to develop OA-like changes, we compared young (3 months) wt and IL-6^{-/-} male mice in the collagenase-induced OA model. Ligaments and cartilage were severely affected in both strains [Fig. 2(A and B), Table V]. PG deposition and bone formation in the ligaments were mostly observed at the medial side in both strains. Cartilage damage occurred mostly on the medial side. Subchondral bone sclerosis was scarce and was found only at the medial part in three mice of each group. Patellar dislocation was observed in four wt mice but not in IL-6^{-/-} mice. Bone apposition and osteophyte formation, in contrast, were observed frequently in both groups (9 of 12 in wt and 9 of 11 in IL-6^{-/-} mice).

These results in experimental OA and the results of the spontaneous OA suggest that during aging IL-6^{-/-} male mice start to differ from their wt counterparts in the osteoarthritic response. Intriguingly, chondrocyte PG synthesis (Table II) and BMD also did not differ between young (3–4 months) wt and IL-6^{-/-} mice but started to differ during aging.

Discussion

Expression of the pleiotropic cytokine IL-6 is increased in the joints of OA patients. Chondrocytes, osteoblasts, osteoclasts as well as synoviocytes can respond to IL-6 and the final outcome of IL-6 expression in OA is therefore unclear. Our present study on spontaneous OA in IL-6^{-/-}
and wt mice suggests that especially in males, IL-6 could have a beneficial effect on joint pathology during OA.

Depending on the affected joint, human OA occurs either more in females or is equally present in both sexes. In our study, wt male and female mice did not differ significantly in cartilage erosion and ligament PG deposition. A gender difference, in contrast, was seen in the IL-6−/− mice, where males, but not females, developed significantly more severe OA when compared to age-matched wt mice. Androgens have been shown to reduce IL-6 production and androgen receptors have been found in bone and ligaments. Dihydrotestosterone reduced cartilage erosion and ligament PG deposition. A gender difference, in contrast, was seen in the IL-6−/− mice, where males, but not females, developed significantly more severe OA when compared to age-matched wt mice. Androgens have been shown to reduce IL-6 production and androgen receptors have been found in bone and ligaments. Dihydrotestosterone reduced cartilage erosion and ligament PG deposition.

### Table II

<table>
<thead>
<tr>
<th>Mice (n = 6)</th>
<th>Age (months)</th>
<th>Cartilage 35S content t = 0 h (cpm)</th>
<th>t = 48 h (cpm)</th>
<th>% Loss (48–0 h)</th>
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</thead>
<tbody>
<tr>
<td>wt</td>
<td>4</td>
<td>1351 ± 225</td>
<td>369 ± 163</td>
<td>73</td>
</tr>
<tr>
<td>IL-6−/−</td>
<td>4</td>
<td>1361 ± 273</td>
<td>422 ± 62</td>
<td>69</td>
</tr>
<tr>
<td>wt</td>
<td>12</td>
<td>1349 ± 365</td>
<td>261 ± 58</td>
<td>81</td>
</tr>
<tr>
<td>IL-6−/−</td>
<td>12</td>
<td>882 ± 62</td>
<td>228 ± 51</td>
<td>74</td>
</tr>
</tbody>
</table>

*Ex vivo* PG synthesis is shown by the 35S incorporation directly after labeling (t = 0). PG breakdown was determined by comparing incorporation at t = 0 and the amount of 35S that is still present in the cartilage after 48 h incubation in 35S-free medium. n = 6 per group. One of two experiments with similar results is shown. Mean ± sd, **P < 0.005 IL-6−/− mice compared to wt mice of the same age. Student’s t test; ns, not significant.

### Table III

<table>
<thead>
<tr>
<th>Age</th>
<th>Incidence (%)</th>
<th>Medial</th>
<th>Lateral</th>
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<tbody>
<tr>
<td>wt female</td>
<td>24</td>
<td>21</td>
<td>5</td>
</tr>
<tr>
<td>IL-6−/− female</td>
<td>27</td>
<td>26</td>
<td>6</td>
</tr>
<tr>
<td>wt male</td>
<td>36</td>
<td>19</td>
<td>7</td>
</tr>
<tr>
<td>IL-6−/− male</td>
<td>39</td>
<td>49</td>
<td>18</td>
</tr>
</tbody>
</table>

The incidence is calculated as the percentage of mice that have bone sclerosis either medial, lateral or at both sides.

Distribution of bone sclerosis over the medial and lateral sides of the joint in old wt and IL-6−/− mice.

and sex hormones in mice of different ages. Our present study showed that young IL-6−/− male mice were not yet more susceptible to experimental OA suggesting IL-6 involvement only upon aging.

Based on the increase of severe cartilage damage in old IL-6−/− male mice, our present study suggests a modifying or protective role for IL-6 against cartilage pathology in OA. PGs form an important component of the cartilage matrix and, by retaining water, contribute to the shock absorbing capacity of the cartilage. These PGs are continuously synthesized and degraded. In line with previous results, young IL-6−/− male mice did not differ from wt mice in ex vivo cartilage PG synthesis. When these mice got older (12 months), however, PG synthesis decreased in IL-6−/− but not in wt mice. This suggests a positive relation between IL-6 and PG synthesis similar to the positive correlation between IL-6 and cartilage PG synthesis in dogs with experimentally induced OA. Whether this is a direct effect of IL-6 or is indirectly mediated through production of or interaction with other cytokines and growth factors remains to be determined. Preliminary results showed that 24-h incubation of 12-month-old IL-6−/− patellae in IGF supplemented medium restored PG synthesis to wt levels (data not shown). This suggests the presence of lower levels of chondrocyte stimulating factors in older IL-6−/− mice, which should be a subject of further investigation.

Histological measurement of cartilage PGs revealed no difference in net PG density of old IL-6−/− and wt patellar cartilage. This is most likely to be caused by decreased PG turnover in IL-6−/− mice. Although a reduced PG synthesis in old IL-6−/− mice does not affect the patella, it might be indicative for an impaired capability of the cartilage to respond to damage. In this way, it could make the cartilage more prone to develop OA lesions. This is illustrated by increased inhibition of PG synthesis when patellae of 12–13-month-old mice were incubated in vitro for 24 h with 1 ng/ml IL-1β (wt, 42% inhibition; IL-6−/−, 57% inhibition; n = 6; P < 0.05 for the absolute counts per minute).

A previous study of our group had shown that patellar and tibial cartilage responded in a similar way to experimental osteoarthritic stimuli and the patella was therefore used in the present study to measure the PG content in our old mice. The fact that the patella was not damaged is most likely caused by the fact that it is a non-weight bearing part of the joint which is in contrast to the weight-bearing femur and tibia. Future studies in younger mice will have to investigate PG density in femur and tibia before onset of cartilage erosion.

The collateral and cruciate ligaments contribute to stability of the joint. We had previously found a positive relation between ligament damage and cartilage loss at the medial side in wt mice with collagenase-induced OA. A similar positive relation between calcification and ossification of the MCL and the development of OA lesions has been found in STR/ort mice. In our present study we found more intense PG deposition and bone formation in the MCL of old IL-6−/− male mice. Deposition of this so-called fibrocartilage and subsequent ligament stiffening could contribute to cartilage damage and bone sclerosis by altering the load distribution in the joint. Increased stiffness of the MCLs is also found in OA patients. Future studies should address the relation between IL-6 expression, OA development and age-matched healthy people. Heterotopic osteogenesis of human ligaments has been most studied in the spine where ligament fibroblasts can differentiate into chondrocytes in response to bone morphogenetic protein-2 (BMP-2) signaling. IL-6 can inhibit BMP-2 mRNA expression and it is therefore possible that increased expression of BMP-2 or related factors in human ligaments may contribute to OA development.
growth factors contributes to the ligament damage in the IL-6−/− males. Alternatively to being a cause, fibrocartilage deposition in and stiffening of ligaments might also be a consequence of other remodeling processes that occurred earlier in the joint. Increased collagen remodeling was measured in the anterior cruciate ligament (ACL) of STR/ort mice before radiological signs of OA were detected and the ACL was also weaker than in control mice36. Similarly, a reduced strength and stiffness of the ACL was found in people over 50 years of age when compared to young adults37. Weaker ligaments could contribute to greater joint instability and in this way might increase the risk of development and progression of knee OA38. Ligament lesions in either cruciate or collateral ligaments are frequently found in patients with advanced OA39. During progression of OA and as a response to increased joint laxity, the ligaments might subsequently become stiffer by development of fibrocartilage.

In vitro cultures of pig MCL fibroblasts showed that collagen production is positively correlated with IL-6 production40. This suggests that absence of IL-6 in our study might reduce the MCL collagen content, which might lead to a weaker ligament or impaired response to damage. Experimental ligament damage was induced by collagenase injection. The equal OA development that was observed in wt and IL-6−/− mice with collagenase-induced OA could indicate that during aging under normal conditions, IL-6 plays an important role in maintaining ligament function.

Table IV

<table>
<thead>
<tr>
<th>Strain</th>
<th>Incidence (%)</th>
<th>No. of mice per score</th>
<th>Incidence (%)</th>
<th>No. of mice per score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>wt female</td>
<td>24</td>
<td>4</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>IL-6−/− female</td>
<td>27</td>
<td>14</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>wt male</td>
<td>36</td>
<td>32</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>IL-6−/− male</td>
<td>39</td>
<td>19</td>
<td>4</td>
<td>6</td>
</tr>
</tbody>
</table>

* Scoring ranged from 0 to 3 as described in Materials and Methods. For each mouse the maximum score, either medial or lateral or equal on both sides, is indicated. This means that the other side could have lower or no signs of EM deposition. Incidence is calculated as the percentage of mice with a positive score of 1, 2 or 3.

** Distribution of the maximal score (either 1, 2 or 3) for ligament PG deposition/bone formation over the medial (M) and lateral (L) sides of the knee joint. In case the score is equal for both sides then that joint is counted for medial (M) as well as lateral (L). The number between brackets indicates the number of joints with maximal damage score 3 at that side.

Subchondral bone sclerosis can occur in human OA and was also in our study the most prominent bone-related change. Subchondral bone sclerosis could lead to a reduced shock absorbing capacity that finally leads to cartilage erosion. In vitro IL-6 expression could divide human OA osteoblasts in normal and high IL-6 producers41. This study, however, could not relate IL-6 expression to pathological findings. In osteophytes, IL-6 mRNA has been detected in active osteoblasts42. In trabecular OA bone, decreased IL-6 mRNA expression was associated with enhanced mRNA expression for the bone formation marker osteocalcin43. The increased bone sclerosis in the old IL-6−/− males could be in line with this last result.

Although the resolution of the bone densitometer did not allow us to evaluate the subchondral bone in detail due to the small size of the murine joint, DEXA measurements revealed a reduced BMD in knees of old, but not young, IL-6−/− male mice when compared to wt mice. Old IL-6−/− females, in contrast, did not differ from old wt females in their knee BMD values. Our data, therefore, suggest a BMD preserving role for IL-6 in male mice. In a previous study, Poli et al.44 had found that 4–5-month-old IL-6−/− females had a similar bone mineralization as their wt controls. Our data in the young males are in agreement with their results in females and furthermore show that the effect of IL-6 deficiency on BMD is only seen later in life. The reduced BMD might seem conflicting with the increased sclerosis in the IL-6−/− males but studies on human OA suggest that it is not. Although bone volumes can increase in OA, the subchondral bone of these patients is less mineralized than in control individuals.

![Fig. 2. Collagenase-induced OA in wt and IL-6−/− male mice. Severe OA at day 42 after the first collagenase injection in both wt (A) and IL-6−/− (B) male mice. Complete cartilage erosion is seen at the femur. Osteophytes are formed at the tibia and femur. PG deposition is seen in the collateral ligament. F = femur, L = ligament, T = tibia, O = osteophyte, M = meniscus. Safranin-O staining. Original magnification: 50×.](image-url)
normal bone (reviewed in Hunter and Spector\textsuperscript{45}). Different reports showed a reduced BMD in OA subchondral bone\textsuperscript{35,46,47}. Longitudinal studies relating BMD and OA development showed that high BMD at non-joint sites is associated with increased risk of OA. Once people have OA, in contrast, a low BMD and high bone turnover appear associated with enhanced progression of the disease (reviewed in Hunter and Spector\textsuperscript{46}). This could explain the coexistence of reduced BMD, bone sclerosis and enhanced OA development in old IL-6\textsuperscript{−/−} males.

In our old wt and IL-6\textsuperscript{−/−} males bone sclerosis, ligament ossification and complete cartilage erosion coincided in the most severe cases of spontaneous OA but only at the medial side. Bone sclerosis and ligament ossification were almost completely restricted to the medial side in all groups. Cartilage erosion, in contrast, occurred at both sides and had the highest incidence among all pathological changes. Together with the reduced PG synthesis that started already at younger age, this suggests onset of OA in the cartilage. Histological and functional studies with mice at a younger age might identify the location of the first pathological change. Independent of the disease etiology, our present data clearly showed more severe cartilage erosion and ligament ossification in IL-6\textsuperscript{−/−} male mice.

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### References

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