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ORIGINAL ARTICLE

Granzyme-A deficiency attenuates experimental osteoarthritis in mice, but perforin deficiency does not

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ABSTRACT

Objectives: This study aims to assess the development of osteoarthritis (OA) in granzyme A- (gzmA) and B- (gzmB) and perforin- (perf) knockout mice.

Materials and methods: A total of 75 male and female C57BL/6 (eight to nine-week-old) mice were allocated to: gzmA-deficient (gzmA-/-) (11 females, 8 males), gzmB-deficient (gzmB-/-) (9 females, 8 males), perf-deficient (perf-/-) (10 females, 9 males), and control group (10 females, 10 males). Osteoarthritis was induced in the right knee by instability of the meniscus medial ligament. Sham surgery was practiced in the left knee. Knee samples obtained eight weeks after surgery were stained (Safranin-O) and blindly scored in lateral and medial femur and tibia using the Osteoarthritis Research Society International scale (OARSI) (from Grade 0, cartilage intact to 6, deformation), (five stages from 0, no OA to 4, >50% surface involvement); OARSI score (grade x stage); and a semi-quantitative scale from Grade 0 (normal) to 6 (cartilage erosion >80%).

Results: Significantly higher values in all scales in the right knees compared to the left knees in male and female mice were observed ($p<0.05$). Males of all strains showed in the right knee higher values than females on all scales. Deficiency of perforin did not modify OA severity in any sex. The gzmA-/- females presented less degenerative changes than the other groups.

Conclusion: Our study results show that sex plays an important role in the development of experimental OA in mice. Deficiency of gzmA can protect from the development of OA in female mice.

Keywords: Granzyme, osteoarthritis, perforin.

Osteoarthritis (OA) is highly prevalent around the world, being an important cause of disability,[1] and although the involvement of inflammation in the development of other articular pathologies, such as rheumatoid arthritis (RA), has been clearly established, it was some years ago that inflammation was described as playing a key role in OA.[2] Upon mechanical stress and changes in the extracellular matrix produced by an inflammatory stimulus, joint chondrocytes modify their phenotype expressing alarmins and secreting pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), and interleukin (IL)-1.[2] The latter stimulates the production of matrix metalloproteinase (MMP) -1, -3 and -13 and aggrecanases by chondrocytes suppressing the synthesis of proteoglycans and collagen II, and promoting the production of reactive oxygen species.[3,4] The secretion of pro-inflammatory cytokines and chemokines favors the early recruitment of innate immunity cells such as granulocytes and monocytes to the joint, starting a cycle of cartilage degradation and synovial membrane

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involvement that triggers an inflammatory process maintained over time.[5,6] Therefore, inflammation is associated with progression of cartilage loss and signs and symptoms of disease.

Granzymes (Gzms) are a family of serine proteases discovered in the 1980s.[7] To date, gzmA and gzmB are the most abundant and mostly characterized.[8] Granzymes are mainly expressed on CD8 + T lymphocytes and natural killer (NK) cells.[9,10] The main function initially described for Gzms was the induction of cell death on target cells in infected or transformed cells. Perforin (perf) is a pore-forming protein located in the cytotoxic granules of cytotoxic lymphocytes to kill target cells and works together with gzmB,[11] inducing apoptosis in infected or cancerous cells.[12,13] Gzms are involved in the regulation of the inflammatory response, the inactivation of viruses, the modification of the extracellular matrix, and the regulation of activated lymphocytes.[8,13,14] Both pure gzmA and gzmA-expressing cytotoxic cells have been shown to induce the release of pro-inflammatory cytokines.[14,15] Indeed, pro-inflammatory role of gzmA has been demonstrated in several diseases, such as RA and colorectal cancer.[16] Moreover, blocking of gzmA activity reduces inflammation in these conditions and ameliorates disease progression.

Experimental evidences have indicated that Gzms may have an extracellular activity independent of perf, which may be involved in the inflammatory process component underlying aging, atherosclerosis or RA.[12] An increase in the levels of gzmA, gzmB and extracellular gzmK has been observed in several biological fluids of patients suffering from different inflammatory and autoimmune pathologies.[17-19] The detection of elevated levels of gzmB in the serum of patients with chronic inflammatory diseases, such as RA and asthma, suggest that this protease may have extracellular effects related to the inflammatory process.[20] While the extracellular role of gzmB has been described, related to its ability to degrade extracellular matrix proteins in atherosclerosis, skin aging, as well as in wound healing,[18,21] elevated levels of gzmA have been found in patients with RA, both in plasma and synovial fluid,[19] but its exact role is still unclear.

Inflammation plays a crucial role in the OA development. Since gzmA and gzmB have been implicated in inflammation and have been identified in other articular pathologies, in the present study, we aimed to assess whether the deficiency of gzmA, gzmB or perf, could influence the development of OA through destabilization of the medial meniscus (DMM) in mice.

MATERIALS AND METHODS

Inbred strains of mice (C57BL/6 (B6)), and deficient strains of mice in gzmA, gzmB or perf on B6 background were bred and maintained at our center. Their genotypes were obtained and periodically analyzed, as previously described.[12] A total of 75 male and female mice (eight to nine-week-old) were used in all experiments. Mice were allocated to: Group A (gzmA-/-: gzmA-deficient mice), Group B (gzmB-/-: gzmB-deficient mice), Group P (perf-/-perf-deficient mice), and Group C (control, wild-type mice). Seventy-five animals were divided into 40 female mice (11 gzmA-/-, 9 gzmB-/-, 10 perf-/- and 10 controls) and 35 male mice (8 gzmA-/-, 8 gzmB-/-, 9 perf-/- and 10 controls).

Surgical procedure

Anonymity of each animal was maintained by means of the allocation of numbers.

Under general anesthesia (inhaled isoflurane), an experimental model of OA was developed by DMM.[22] A longitudinal incision was made in the right knee where the instability of the meniscus medial ligament was carried out. The skin was, then, sutured with 3.0 non-absorbable suture, a subcutaneous dose of 0.05 mg/kg of buprenorphine in 1 mL of sodium chloride was administered. Sham surgery was practiced in the left knee with articular capsule section and, then, sutured. The mice were sacrificed in a carbon dioxide (CO_2) chamber eight weeks after performing the surgical intervention. The operated right knee and left knee were removed with a section at the level of the distal third of the femur and at the level of the proximal third of the tibia and fibula, eliminating the musculature and soft tissues, except for the joint space. The samples were introduced in cassettes. Likewise, the frontal/coronal plane was oriented by placing the knee upwards.

Histopathological analysis

The samples were fixed in 4% formaldehyde and decalcified in ethylenediaminetetraacetic acid (EDTA), processed with the X-PRESS X50 (Sakura Company, Torrance, California, USA 90501) equipment and infiltrated in paraffin with the Leica embedding station. On each slide, three tissue sections were mounted at different levels of 3-μm thick each. The tissue sections were made with the Leica rotary microtome (CM1950). Between each section, 50 μm were roughened to the desired depth.

Once tissue sections were dried in an oven at 37°C overnight, they were deparaffinized, hydrated and stained with the O-Safranin. Once the staining was finished, it was dehydrated and mounted with DPX permanent mounting medium. Microscopic analysis was carried out. The slides were blindly reviewed and scored in lateral and medial femoral condyles and in lateral and medial tibial plateaus in three slides per knee by means of Osteoarthritis Research Society International (OARSI) scale briefly: Grade 0 surface and cartilage intact, Grade 1 surface intact, Grade 2 surface discontinuity, Grade 3 vertical fissures, Grade 4 erosion, Grade 5 denudation, Grade 6 deformation.[23] The OARSI scale establishes five stages: Stage 0 (no OA), Stage 1 (<10% surface OA involvement), Stage 2 (10-25%), Stage 3 (25-50%) and Stage 4 (>50%). OARSI score resulted in multiplication grade per stage.

A semi-quantitative grading scale described by Chambers et al.[24] and modified by Glasson et al.[22] was also used. Briefly: 0 normal cartilage; 0.5: loss of Safranin-O with no structural lesions; 1: roughened articular surface and small fibrillations; 2: fibrillation below the superficial layer and some loss of lamina; 3: fibrillations extending to the calcified cartilage across <20% of the cartilage width; 5: fibrillation and erosions extending from 20 to 80% of the cartilage width; 6: cartilage erosion >80% of the cartilage width. Score 4 was eliminated by Glasson et al.[22]

Statistical analysis

Statistical analysis was performed using the Statview-Statgraphics version 5.0.1 software (SAS Institute Inc., Cary, NC, USA). Descriptive data were expressed in mean ± standard error (SE). Data were analyzed using the non-parametric Mann-Whitney U test to examine differences between male and female mice in each group, while the Kruskal-Wallis and Fisher tests were used to examine differences between the groups. A p value of <0.05 was considered statistically significant.

RESULTS

Validation of DMM in OA model

To validate surgical procedure, DMM was performed in the right knees of the mice and sham surgery was carried out in the left knees. Osteoarthritis was clearly induced in the right knees in both males and females while comparing with the left knees (sham surgery) (Figure 1). No significant differences were found while comparing the left knees (sham surgery) between males and females with very low values on the two scales after sham surgery. All these data validated DMM as an adequate surgical method to induce OA in mouse knee.

Role of sex in the development of OA after DMM

After validating DMM as the surgical method, we attempted to assess whether there were differences between the strains according to sex. We compared each group in the four femorotibial quadrants following OARSI (Table I) and Glasson score (Figure 2). While comparing OA severity between males and females, males from the control group showed a greater OA severity than females, both mainly observed in lateral quadrants and medial quadrants of right knees in the OARSI score (Table I). No significant differences were found between males and females in the medial femoral and tibial quadrants where the instability of the meniscus medial ligament was carried. However, significant differences between males and females appeared in the lateral femoral and tibial quadrants according to the OARSI score, and to the Glasson score (Figure 2). However, in medial quadrants where surgical instability of the meniscus medial ligament was carried out inducing OA, no statistically significant differences were observed between male and female mice.

Role of gzmA, gzmB, and perf in the development of OA after DMM

After assessing that OA was appropriately induced, differences in OA severity among different groups were analyzed (Figure 3). Males of all groups had high values on OARSI stage in the right knee without significant differences between the different groups. The gzmA-/- male mice showed less OA

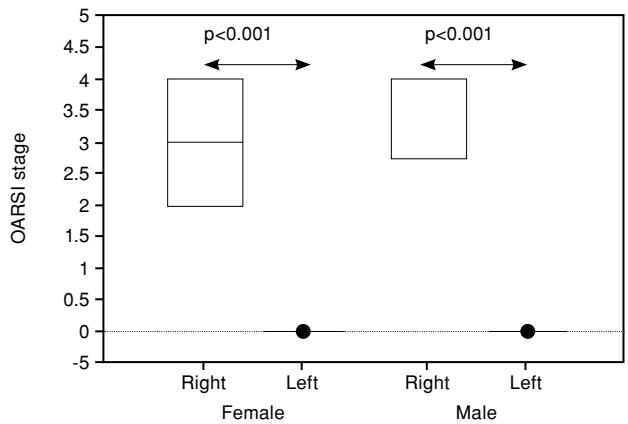

FIGURE 1. Osteoarthritis (OA) severity according OARSI stage was compared in the right (OA model) and left knees in male and female groups.
OARSI: Osteoarthritis Research Society International scale.

TABLE I
OARSI score (grade X stage) in the right knees of control group

	Male	Female
Quadrant	Mean±SE	Mean±SE
Medial femoral condyle	16.4±2.3	12.8±2.8
Lateral femoral condyle	13.7±3.5*	5.0±1.3*
Medial tibial plateau	15.1±3.6	14.5±3.2
Lateral tibial plateau	13.9±3.9	5.5±2.2*

OARSI: Osteoarthritis Research Society International scale; SE: Standard error; * p<0.05.

severity (2.87±0.39) compared to the other groups, but no statistically significant differences were observed compared to the control group (3.33±0.29) (Figure 3).

However, the gzm A-/- female mice showed less OA severity compared to the other groups. Interestingly, female mice with a deficiency of gzmA showed less degenerative changes than control group, being the difference statistically significative according to the OARSI stage classification (p<0.05) (Figure 3, white box plots).

Regarding the gzmB-/- group, although fewer OA changes in both sexes were noticed compared to the control group, no statistically significant differences were observed. Finally, deficiency of perforin did not modify OA severity in any sex (Figure 3).

While comparing the OARSI stage between males and females in the right knee in the different groups only differences between the gzmB-/- male and gzmB-/- female groups were statistically significant (Figure 3).

Finally, OA severity values in the different quadrants (femoral and tibial, medial and lateral) were studied according to the Glasson score (Figure 4). All the quadrants presented significantly worse results in males than in females in Group A, being statistically significant in tibial quadrants, both medial and lateral (Figure 4a). In this line, also in Group B, OA severity was significantly higher in medial and lateral quadrants in males, compared to female group (Figure 4b). No significant differences between male and female mice were observed in the perf-deficient mice (Figure 4c).

Pronounced degenerative changes in the whole knee and, particularly, on medial side were observed in mice from control groups, mainly in male mice, with denudation and erosion at this location. We observed less degenerative changes on medial side in gzmA-/- female compared to the gzmA-/- male.

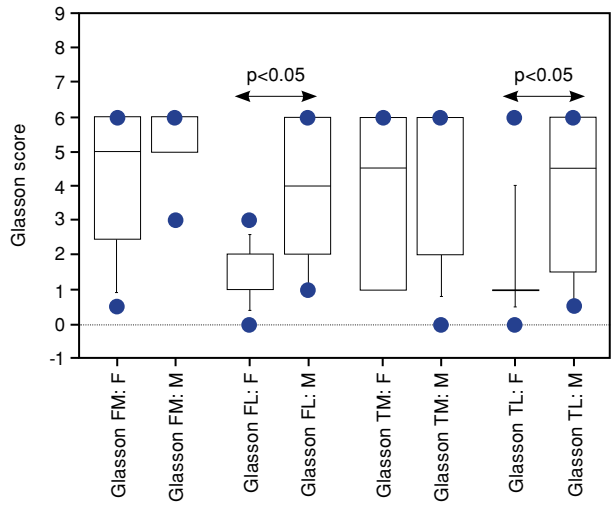

FIGURE 2. Glasson score values in control group in male (M) and female (F) in the different quadrants (femoral medial (FM), femoral lateral (FL), tibial medial (TM) and tibial lateral (TL).

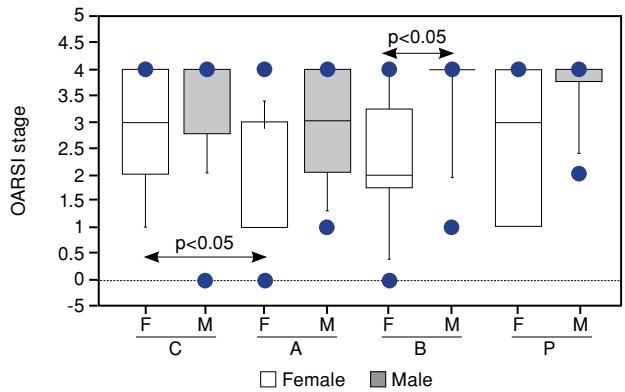

FIGURE 3. OARSI stage in the right knees in wild type (C), gzmA-/-(A), gzmB-/-(group B) and perf-/-(P).
OARSI: Osteoarthritis Research Society International scale.

There were small superficial fibrillations on medial femoral condyle in gzmA-/- female. On the contrary, we observed deformity on medial tibial plateau and denudation on lateral femoral condyle in gzmA-/- male. Regarding gzmB-/- group, denudation in medial side and erosion in lateral femoral condyle were observed in male mice, while female mice showed small superficial fibrillations in medial plateau.

DISCUSSION

In the present study, the role of gzmA, gzmB, and perf in the development of OA in an experimental model of

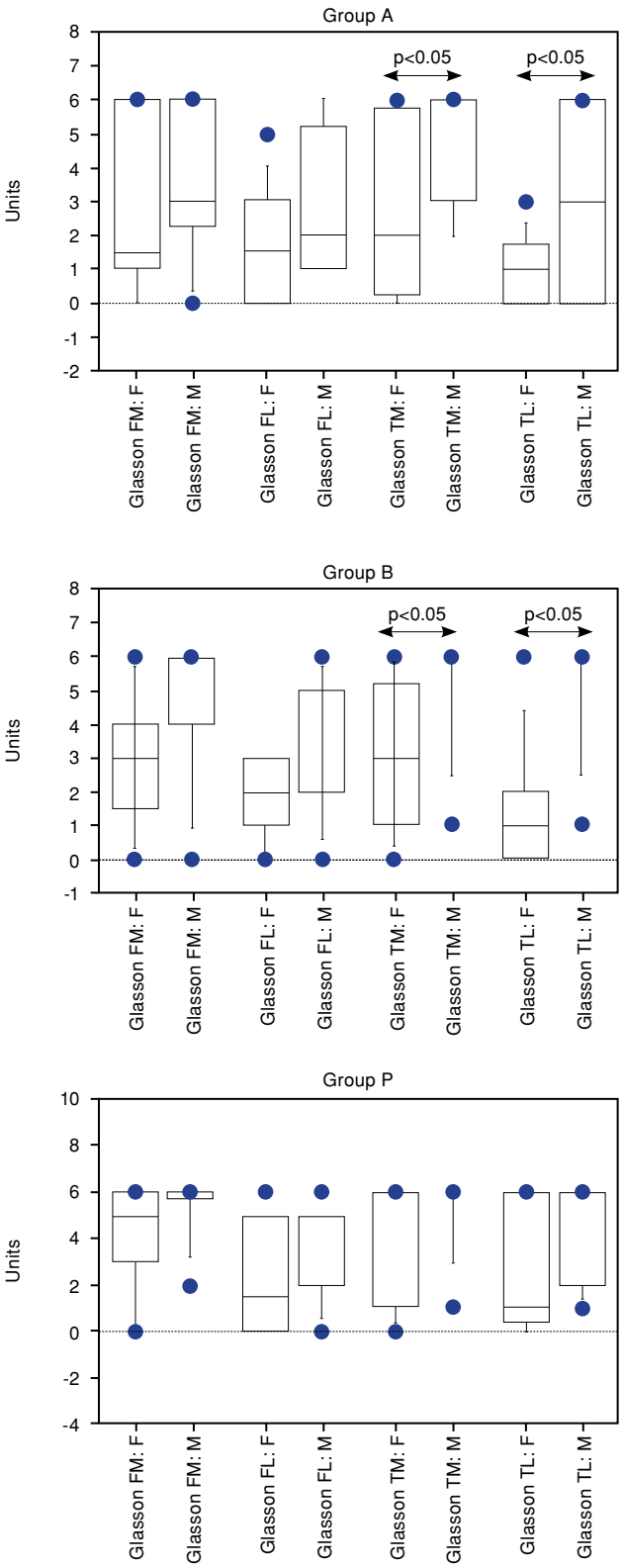

FIGURE 4. Glasson score in gzmA, gzmB, and perf-deficient mice in male and female in the different quadrants.
gzmA: Granzyme A; gzmB: Granzyme B; FM: Medial femoral condyle; FL: Lateral femoral condyle; TM: Medial tibial plateau; TL: Lateral tibial plateau.

DMM was analyzed. To validate surgical procedure, comparison between the right (DMM) and left (sham) knees was performed. Destabilization of the medial meniscus in right knees induced OA development in control groups both in male and female mice after eight weeks, while sham surgery in left knee did not, thereby validating this surgical procedure to induce OA. In this line, differences between males and females on left knees (sham surgery) were minimal in all groups, indicating that DMM model in right knees has sufficient sensitivity to show disease modification in OA.[22]

Differences in OA development depending on sex were also analyzed. Comparing right knees of female and male mice, females developed less severe OA in the control group. Our results are in agreement with those previously reported indicating the chondroprotective role of female sex hormones in OA development.[25] A similar study from another group[26] in a mouse model based on DMM males developed OA more severely than females, suggesting that ovarian hormones decreased severity of OA and testosterone exacerbated OA; ovariectomized females showed more severe OA than intact females and, on the contrary, orchiectomized mice experienced less OA than intact males. In our study, differences between male and female in control groups were observed only in the lateral side (intact meniscus). Based on these findings, we can speculate that the involvement of the lateral side is due to the progression of OA in the most severe cases.

In females, the control group showed the highest OA severity, while the gzmA and gzmB-deficient female mice showed less OA severity than control and also than perf-deficient female mice. We observed less degenerative changes in the OARSI Stage in gzmA-/- and gzmB-/- females, particularly in the gzmA-/- group where differences in OA severity were statistically significant compared to control female group. These results support the important role of extracellular gzmA in the development of degenerative changes of OA induced by DMM. In this line, in an experimental model of collagen-induced arthritis (CIA) in mice, gene deletion of gzmA attenuated arthritis, serum levels of pro-inflammatory cytokines, joint damage, and bone erosion, suggesting that osteoclast activity is reduced in the absence of gzmA;[27] and the severity of polyarthritis in CIA animals was also reduced in more than 50% of mice lacking gzmA.

The NK cells from the synovium of patients with OA, presenting an immunoregulatory non-cytotoxic

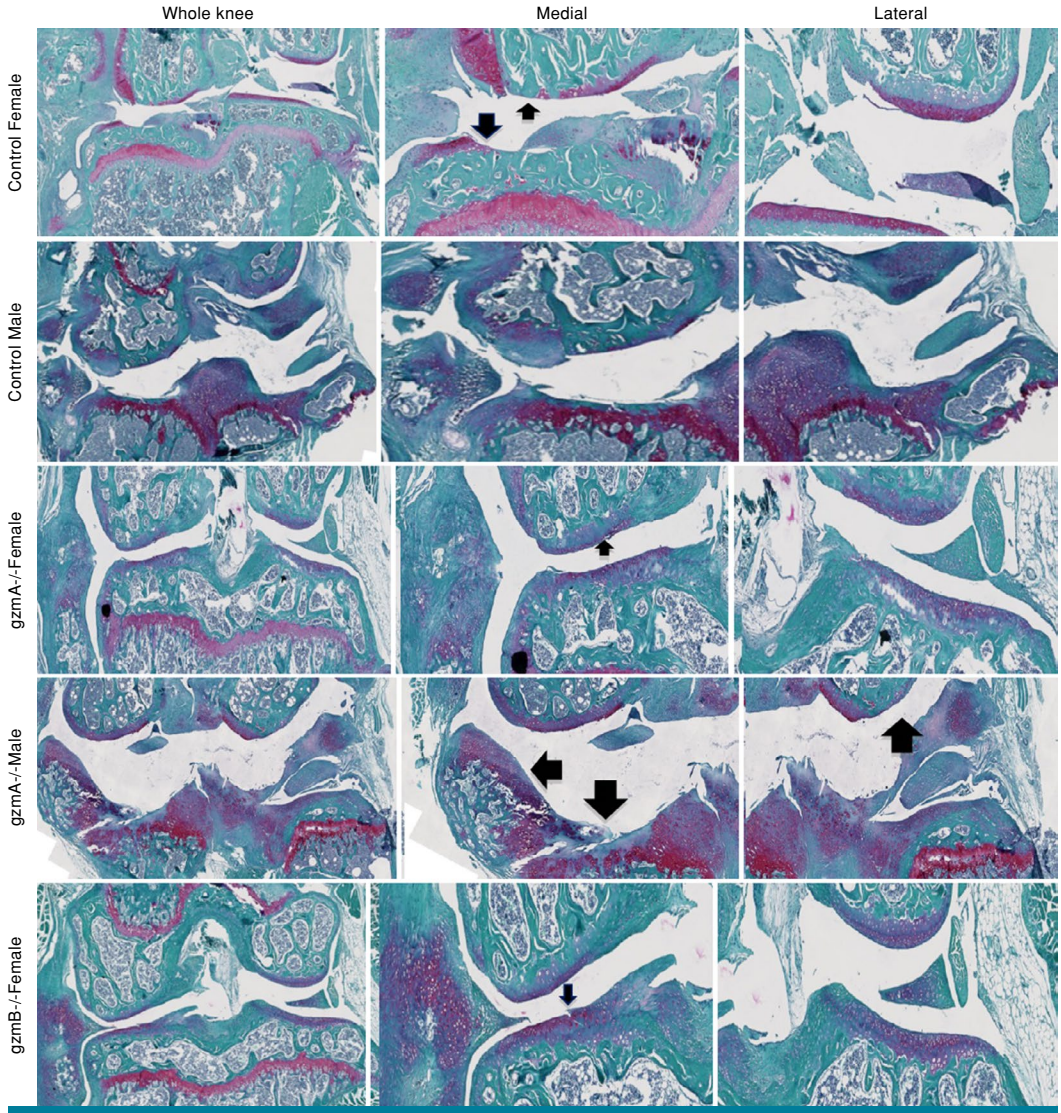

FIGURE 5. Illustrates O-safranin staining carried out in both female and male mice of control, gzmA-/- and gzmB groups. Arrows indicate chondral damage.
gzmA: Granzyme A; gzmB: Granzyme B.

function, showed different phenotype compared to the NK cells from the peripheral blood.[28] Synovial fluid CD56+brightCD16- NK cells with low cytotoxic capacity proportionally expressed higher levels of gzmA than CD56+brightCD16- NK cells of the peripheral blood.[28] Moreover, the presence of CD56+brightCD16- NK cells expressing gzmA was correlated with the increased synovial fluid levels of inflammatory cytokines such as TNF-α and IL-6.[28] In this line, gzmA has been also described as a key pro-inflammatory mediator during chikungunya virus (CHIKV) arthritis.[29] The results obtained in the present study suggest that gzmA may play an important role in the early onset of OA development acting as a pro-inflammatory protease promoting the development of the disease.[30] As granzymes are mainly expressed on CD8 + T lymphocytes and NK cells[9,10] and their main function initially described was the induction of cell death on target cells in infected or transformed cells, complete inhibition of granzymes may deteriorate the defending response against infection or tumors. A downregulation in pro-inflammatory situations would be more beneficial.

Perforin has also been implicated in the development of CIA, but not in the same way as gzmA.[31] In a CIA model, perf-/- mice showed delayed onset and reduced incidence, but some individual

perf-/- mice also developed severe disease.[30] Although other perf-independent cytotoxic death pathways may be involved in CIA development, our results suggest that perf-dependent cytotoxicity is involved in the initiation of tissue damage in arthritis. Perforin is a pore-forming protein that facilitates the intracellular cytotoxic action of granzymes. In the present study, female perf-/- mice developed more severe OA in the operated knees than gzmA-/- and gzmB-/- female mice, indicating that perf does not protect from OA development. These findings suggest that the contribution of gzmA and gzmB to the development of joint pathology seems to be independent of perforin and probably due to their extracellular activity. These results are consistent with previous studies,[27] and confirm the physiological relevance of the studies in which extracellular gzmA is detected in both serum and synovial fluid from humans with other joint diseases such as RA.[19] Moreover, synovial fluid CD56+brightCD16- NK cells expressing high levels of gzmA detected in OA patients in a previous study,[28] showed proportionally low levels of perf and, consequently, exhibited a low cytotoxic activity, suggesting that gzmA plays a role at the extracellular level rather than in a perf-dependent intracellular manner.

Elevated levels of gzmB found in different biological fluids have been related to the progression of different inflammatory diseases.[17,18] In the present study, we observed less degenerative changes in the right knee on gzmB-/- female mice compared to control mice. However, unlike gzmA-/- female group, differences compared to control group were not statistically significant. Nevertheless, a role of gzmB in OA development cannot be ruled out. Results obtained in perf-/- group suggested a perf-independent action of gzms in the DMM model of OA, thus, gzmB could contribute to OA development due to its ability to degrade extracellular matrix proteins previously described.[18,21]

Nonetheless, there are some limitations to this study. This is an experimental study in mice, and the results may be different in humans. Females developed less severe OA in the control group, and our results are in agreement with those previously reported indicating the chondroprotective role of female sex hormones in OA development; however, hormones were unable to be analyzed in the present study. Although mouse strains deficient in gzmA, gzmB, or perf were obtained and checked, gzms levels in the peripheral blood or intraarticular were not studied. The possible long-term negative effects of the complete elimination of gzmA, gzmB, or perf production may be investigated in further studies.

In conclusion, our study results show that sex plays an important role in the development of OA in mice. Gzms, mainly gzmA, also play an important in the development of OA in a DMM model in female mice. This reinforces the thought of the participation of these gzms in joint inflammatory pathologies, not only RA but also OA. Therefore, therapeutic inhibition of extracellular gzmA may reduce joint inflammation in the early stage of OA and may have a beneficial effect in OA development.

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Ethics Committee Approval: All experimental procedures were approved by the Animal Care and Research Committee of the University of Zaragoza (Spain) (PI27/17) comply with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines and EU Directive 2010/63/EU for animal experiments.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: Conceptualization, methodology, software, data curation, formal analysis, writing-original draft preparation, writing-reviewing and editing: J.C.; Visualization, methodology, data curation, formal analysis, investigation, writing-reviewing and editing: L.S.; Visualization, data curation, investigation, writing-reviewing and editing: M.A.; Conceptualization, resources, supervision, formal analysis, writing-reviewing and editing: J.P.; Visualization, formal analysis, writing-reviewing and editing: J.A.; Conceptualization, supervision, formal analysis, writing-original draft preparation, writing-reviewing and editing: L.M.L.; Conceptualization, methodology, investigation, supervision, formal analysis, writing-original draft preparation, writing-reviewing and editing: F.G.A.

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