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The effects of metabolic syndrome, obesity, and the gut microbiome on load-induced osteoarthritis

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SUMMARY

Objective: Metabolic syndrome is characterized by obesity, hyperglycemia, hypertension, insulin resistance, and dyslipidemia. Metabolic syndrome is associated with osteoarthritis (OA), but it is unclear if the association is attributable to increased mechanical loading on joints caused by obesity or other aspects of metabolic syndrome. Here we examined the effects of altered metabolism, obesity, and the gut microbiome on load-induced OA.

Design: Cartilage damage was induced through cyclic compressive loading in four groups of adult male mice: Toll-like receptor-5 deficient (TLR5KO) mice that develop metabolic syndrome due to alterations in the gut microbiome, TLR5KO mice submitted to chronic antibiotics to prevent metabolic syndrome (TLR5KOΔMicrobiota), C57BL/6J mice fed a high fat diet to cause obesity (HFD), and untreated C57BL/6J mice (WT). Loading was applied for 2 weeks ($n = 10–11$/group) or 6 weeks ($n = 10–11$/group).

Results: After 2 weeks of loading, cartilage damage (OARSI score) was not different among groups. After 6 weeks of loading, HFD mice had increased load-induced cartilage damage, while TLR5KO mice had cartilage damage comparable to WT mice. TLR5KOΔMicrobiota mice had less cartilage damage than other groups. HFD mice had elevated serum inflammatory markers. Each group had a distinct gut microbiome composition.

Conclusions: Severe obesity increased load-induced cartilage damage, while milder changes in adiposity/metabolic syndrome seen in TLR5KO mice did not. Furthermore, the effects of systemic inflammation/obesity on cartilage damage depend on the duration of mechanical loading. Lastly, reduced cartilage damage in the TLR5KOΔMicrobiota mice suggests that the gut microbiome may influence cartilage pathology.

Introduction

Metabolic syndrome is a cluster of conditions including abdominal obesity, hyperglycemia, hypertension, insulin resistance, and dyslipidemia that put an individual at increased risk of developing type 2 diabetes. Recent evidence suggests that abnormalities related to metabolic syndrome may exacerbate osteoarthritis (OA) pathology[1,2]. Understanding the mechanisms relating metabolic diseases and OA has the potential to improve prevention and treatment of joint disease.

Obesity is a well-recognized clinical risk factor for OA. The association between obesity and OA is commonly attributed to increases in joint loading associated with increased body mass. However, rates of OA in non-load bearing joints are greater in patients with obesity[3,4], a finding that suggests that systemic factors contribute to the risk of OA[1,5–7]. Further supporting the idea that systemic factors influences OA, patients with type 2 diabetes and metabolic syndrome have increased risk of OA[5,7–11], although it is unclear if the association with OA is due to increases in body mass or systemic factors.

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Animal models are useful for studying the relationship between OA and metabolic syndrome. Mice fed a high fat diet (HFD) to induce an obese, diabetic state display accelerated progression of OA in both aging and surgical destabilization models of OA, and develop more severe OA after intra-articular fracture[12–14]. Leptin receptor-deficient mice (db/db) display severe obesity and develop more intense degradation of the joint following surgically-induced OA[15,16]. A limitation of these mouse models of obesity (leptin (ob/ob) or leptin-receptor deficient mice (db/db) and mice fed a HFD), is that they display severe obesity and hyperglycemia that is more representative of a diabetic state[17,18] than that of metabolic syndrome (Table I). Furthermore, leptin is involved in mechanisms that affect chondrocyte metabolism and cartilage health[19,20], making it difficult to separate the effects of obesity from those of leptin dependent pathways in the db/db and ob/ob mice. To date the relationship between obesity, metabolic syndrome, and OA has only been studied in animals using post-traumatic, surgically-induced, or aging OA models. Little is known about the effect of *in-vivo* loading models that produce more modest OA pathology.

Obesity and the metabolic syndrome are also associated with changes in the gut microbiome[21]. The gut microbiome is the collection of trillions of micro-organisms that inhabit the gastrointestinal tract and play a key role in host metabolism, immune function, and nutrition[22]. The Toll-like receptor 5 deficient mouse (TLR5KO) spontaneously develops a metabolic syndrome phenotype due to alterations in functions of the gut microbiome. TLR5 is the receptor for bacterial flagellin, and does not have an endogenous ligand. TLR5KO mice exhibit mild hyperglycemia, mild insulin resistance, and a mild obesity[23]. TLR5KO mice do not develop metabolic syndrome when raised germ-free (never exposed to microbes) or when submitted to chronic oral antibiotics that disrupt the gut microbiome. Therefore, the TLR5KO mouse provides a model of metabolic syndrome that can be averted by manipulating the gut microbiome.

Although prior work has indicated that metabolic abnormalities in severe obesity can influence OA development and severity, no studies have examined a mouse model of metabolic syndrome without severe obesity. Additionally, few studies have attempted to understand how manipulation of the gut microbiome may influence OA. In the present study, we tested the hypothesis that metabolic syndrome without severe obesity exacerbates the development of OA. Specifically, we used a non-surgical, load-induced model of OA and a gut-microbiome dependent model of metabolic syndrome to determine: 1) how the metabolic syndrome affects the development of load-induced OA pathology; and 2) how modification of the gut microbiota to prevent the metabolic syndrome phenotype affects the development of OA pathology.

Material and methods

Study design

Animal procedures were approved by the Cornell Institutional Animal Care and Use Committee. C57BL/6J and TLR5KO (congenic strain B6.129S1-Tlr5tm1Flv/J) were acquired (Jackson Laboratory, Bar Harbor, ME) and bred via homozygous mating in a conventional animal facility. Animals were housed in plastic cages filled with ¼-inch corn cob bedding (The Andersons' Lab Bedding, Ohio), provided standard laboratory chow (Teklad LM-485 Mouse/Rat Sterilizable Diet) and water *ad libitum*, and provided a cardboard refuge environmental enrichment hut (Ketchum Manufacturing; Brockville, Ontario). Male mice were divided into four groups): 1) control C57BL/6J mice (WT); 2) TLR5KO mice that develop metabolic syndrome; 3) TLR5KO mice receiving broad-spectrum antibiotics in their drinking water (1.0 g/L ampicillin, 0.5 g/L neomycin) to prevent the development of the metabolic syndrome phenotype (TLR5KOΔMicrobiota)[23]; and 4) C57BL/6J mice fed a HFD (60% energy from fat, Test Diet 58Y1) to induce severe obesity and a diabetic-like state (HFD)[17]. Antibiotic use and HFD began at 4 weeks of age and continued until euthanasia. The antibiotics used are poorly absorbed in the gut and therefore target the gut microbiota without influencing other regions of the body. Animals were housed 3–4 to a cage with others from the same treatment group.

In-vivo cyclic compression

At 20 weeks of age animals began daily, non-surgical, *in vivo* loading of the left tibia to induce cartilage damage. The procedure has been shown to induce cartilage damage as soon as 2 weeks after loading begins[24]. Compressive cyclic loading was applied to the left tibia with a 4.5N peak load for 1200 cycles at 4 Hz for 5 days per week. The right hindlimb served as a non-loaded contralateral control. The mice were placed under general anesthesia (2% isoflurane, 1L/min) while loading was applied. Animals were exposed to loading for either 2 weeks (n = 10–11/group) or 6 weeks (n = 10–11/group). The sample size was determined through a priori power analysis.

Animals were euthanized after loading was completed (at 22 or 26 weeks of age). Upon euthanasia, right and left limbs and epididymal fat pads were harvested. Blood was collected through cardiac puncture at euthanasia. The knee joints from both limbs were dissected and fixed in 4% paraformaldehyde for 24 h. Fecal pellets were collected on the day prior to euthanasia and stored at −80°C prior to analysis.

Subchondral bone and trabecular microarchitecture

Knee joints from animals loaded for 6 weeks were suspended in 70% ethanol and scanned by microcomputed tomography (μCT35; Scanco Medical AG, Switzerland; 55 kVp, 145 μA, 600 ms integration time, 10 μm voxel size). Images were collected at the subchondral bone plate and proximal epiphysis. An average global threshold for all samples was determined to segment mineralized and non-mineralized tissue[25]. Measures of the subchondral bone plate included thickness and tissue mineral density (TMD). Trabecular bone microarchitecture of the proximal epiphysis was examined in a region extending from the end of the subchondral bone plate to the start of the growth plate. Measurements of trabecular microarchitecture included bone volume fraction (BV/TV), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp).

Table I
Metabolic characteristics of mouse models used to study the effect of obesity, systemic inflammation and/or type 2 diabetes on OA compared to TLR5KO mouse

Metabolic measure (Reported % increase compared to WT control)	C57BL/6J fed high fat diet- 60% calories from fat	Leptin receptor deficient (db/db)	Leptin deficient (ob/ob)	Toll-like receptor 5 deficient (TLR5KO)
Blood glucose	149%[18]	159%[21]	Not reported in OA studies	16%[37]
Body mass	73%[17]; 54%[18]	47%[21]; 210%[22]	252%[22]	23%[37]
Adiposity	251%[17]	229%[22]	219%[22]	173%[37]

Assessment of OA in histology sections

Knee joints were decalcified in EDTA for 2 weeks, dehydrated in increasing ethanol gradients, and embedded in paraffin. Serial coronal sections, 6 μm thick, were taken. Sections spaced at 90 μm intervals were stained with Safranin O/Fast green for histological scoring and assessment of cartilage morphology. The OARSI scoring system was used to assess degenerative changes resulting from loading[26]. Baseline cartilage composition and cellularity in control limbs was assessed by a modified Mankin scoring system[27]. Localized thickness of cartilage was measured on sections used for histological scoring (Osteomeasure, OsteoMetrics, USA).

Metabolic and inflammatory blood serum measurements

Serum was stored at −80°C and sent to the Duke Molecular Physiology Institute Biomarkers Shared Resource for analysis. Serum from 26 week old animals ($n = 6–10$/group) was measured using a custom Proinflammatory Panel (Meso Scale Diagnostics; Rockville, Maryland) measuring IL-1β, IL-6, IL-12p70, KC, IL-10, and TNF-α, a Mouse Metabolic Kit (Meso Scale Diagnostics; Rockville, Maryland) to assess leptin and insulin, and an EndoZyme kit (Hyglos; Bernried, Germany) to measure serum lipopolysaccharide (LPS, a bacterial molecular product). Values for IL-12p70 were below the limits of detection and were excluded (Supplementary Table 1).

Gut microbiota analysis

DNA was isolated from fecal pellets using the Mo Bio PowerSoil DNA Isolation Magnetic kit with the recommended proteinase K step to assist in cell lysis. 16S rRNA libraries were prepared using the Earth Microbiome Project protocol[28] with primers as described previously[29]. Paired-end 150 × 150 reads were imported into QIIME2 (https://qiime2.org)[30] and demultiplexed. The samples were analyzed using DADA2, which removes chimeric sequences, and retains unique *de novo* sequence variants[31]. Taxonomies were assigned using QIIME's machine learning classifier trained on Greengenes sequences.

Statistics

The effect of load and group on OARSI score, micro-CT measures, and cartilage thickness were detected using a 2-factor repeated measures ANOVA with interactions that included individual as a

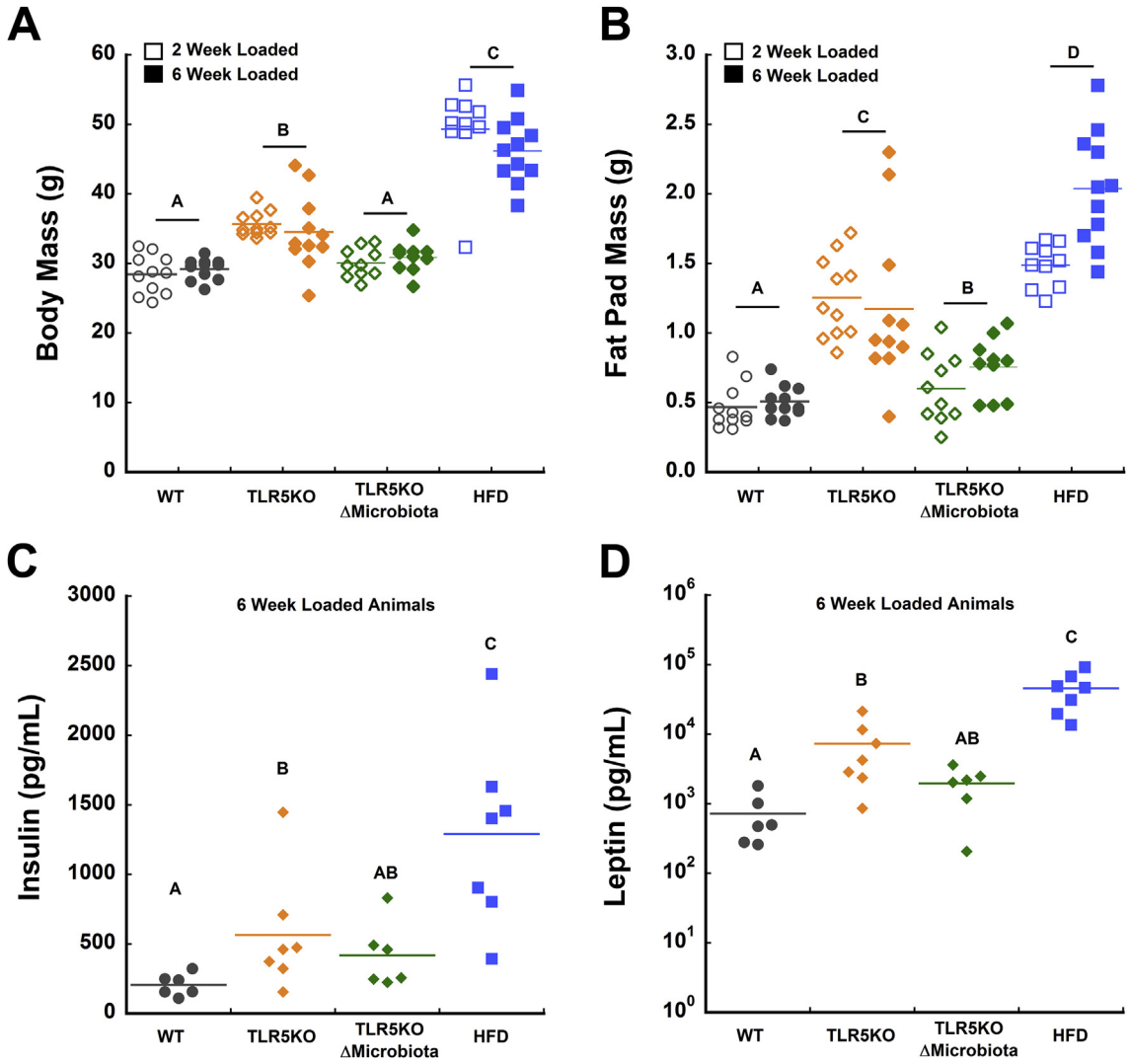

Fig. 1. TLR5KO mice displayed hallmarks of metabolic syndrome including increased (A) body mass, (B) epididymal fat pad mass, (C) serum insulin levels, and (D) serum leptin levels compared to WT mice. HFD mice had increased levels of adiposity, insulin, and leptin. Body mass and epididymal fat pad mass are pooled from 22 week and 26 week old animals. Serum is from 26-week old mice. Solid colored lines on dot plots represent the mean value. Groups sharing the same letter are not significantly different from each other ($p < 0.05$).

random effect (JMP Pro 9.0.0). Group differences between histological scores, micro-CT measures, serum measures, body mass, and fat pad mass were determined using a one-way ANOVA with group as the factor followed by the Holm correction for multiple comparisons with $\alpha = 0.05$. A multivariate analysis was used to create a matrix of Pearson's product-moment correlation coefficients to identify linear relationships between OARSI and Mankin scores and indicators of systemic inflammation and metabolic syndrome (body mass, fat pad mass, serum markers) within the 6 week groups. An analysis of covariance was performed to determine if any correlations were explained by group. A Pearson's product-moment correlation analysis was used to identify relationships between loaded limb OARSI scores and control limb subchondral bone measures.

Results

Body mass, fat pad mass, and metabolic profile

Body mass and serum markers of metabolism exhibited the following patterns: severe obesity in HFD mice, mild obesity in TLR5KO mice, and normal body mass in WT and TLR5KOΔMicrobiota mice. Body mass and fat pad mass were greatest in HFD mice [Fig. 1(A) and (B)]. Body mass and fat pad mass were greater in

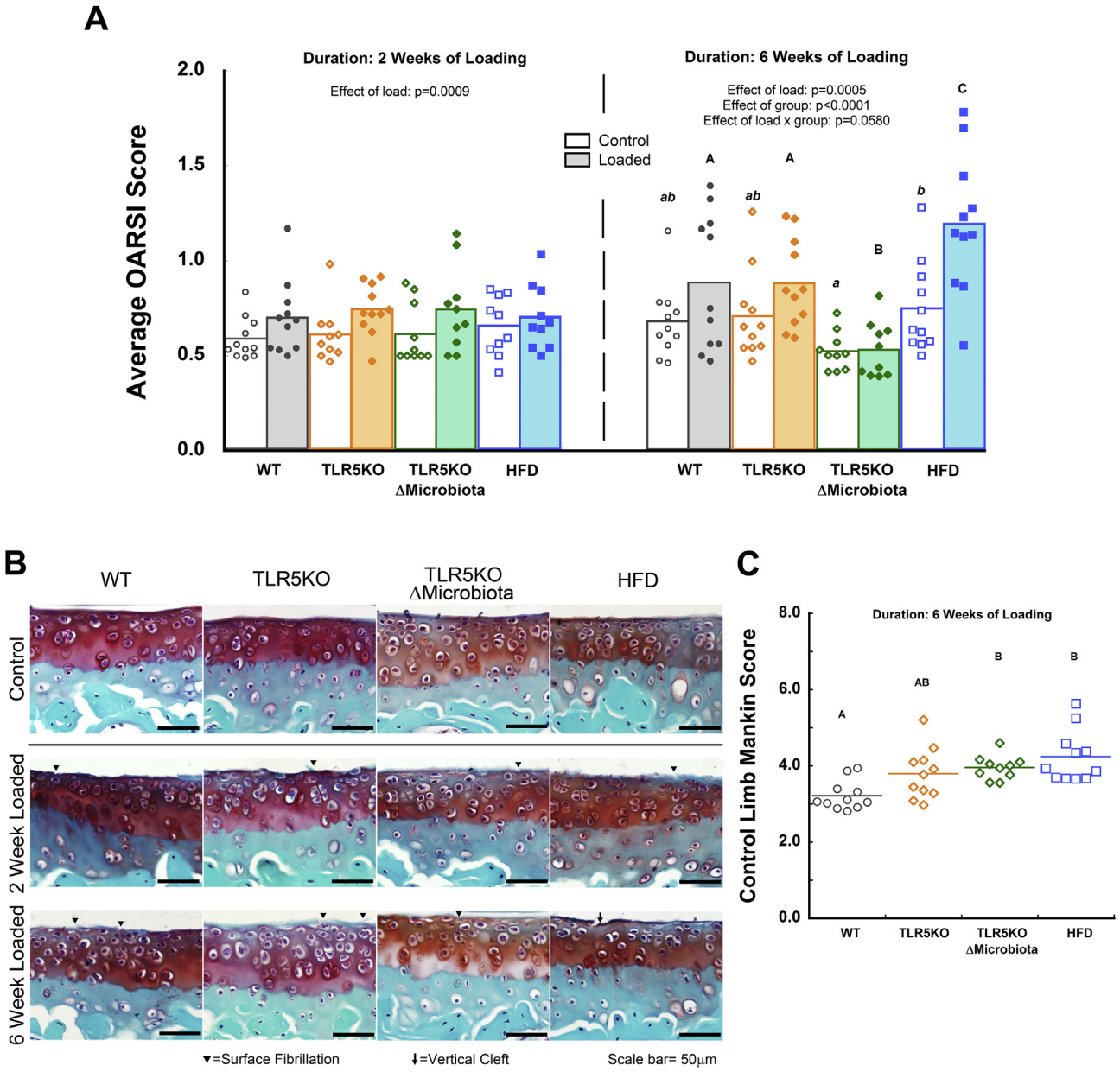

Fig. 2. Twenty-week old male mice were subjected to either 2 or 6 weeks of cyclic mechanical loading to induce OA pathology. (A) After both 2 weeks and 6 weeks of mechanical loading there was an effect of load on OA pathology as measured by OARSI score. No differences the effect of loading were observed among groups at 2 weeks, however, after 6 weeks of loading, HFD mice had elevated loaded limb OARSI scores compared to other groups, and TLR5KOΔMicrobiota mice had lower loaded limb OARSI scores (upper case letters used to denote group differences of loaded limb OARSI scores). Control limb OARSI scores were greater in HFD mice compared to TLR5KOΔMicrobiota mice after 6 weeks of loading (lower case letters used to denote group differences of control limb OARSI scores). (B) Example histology of control and loaded limbs is shown with surface fibrillations and vertical clefts identified. (C) Modified Mankin scores were greater in HFD and TLR5KOΔMicrobiota mice compared to WT. Solid colored bars on plots represent mean. Groups sharing the same letter are not significantly different from each other ($p < 0.05$).

TLR5KO mice than in WT mice or TLR5KOΔMicrobiota mice. HFD mice had the greatest serum levels of insulin [Fig. 1(C)] and leptin [Fig. 1(D)]. TLR5KO mice had greater serum levels of insulin and leptin compared to WT mice. Mean body mass, serum insulin and serum leptin were similar between the TLR5KOΔMicrobiota and WT mice.

Histology and OA cartilage pathology

Mechanical loading caused cartilage damage as measured by OARSI score following either 2 weeks (95% confidence interval of difference between groups: [0.05, 0.18]) or 6 weeks of loading (Fig. 2(A) and (B), [0.10, 0.32]). At 2 weeks of loading, mean OARSI scores were similar among the loaded limbs of the four groups [Fig. 2(A)]. After 6 weeks of loading, HFD mice had greater OARSI scores with more surface fibrillations and vertical clefts in the loaded limbs than other groups [Fig. 2(A) and (B)] and TLR5KOΔMicrobiota mice had lower OARSI scores in loaded limbs compared to the other groups. Control limb OARSI scores were similar among groups at 2 weeks of loading. Control limb OARSI scores at 6 weeks of loading were greater in HFD mice compared to TLR5KOΔMicrobiota mice. Control limb Mankin scores of HFD mice and TLR5KOΔMicrobiota mice were greater than those in WT mice at 6 weeks [Fig. 2(C)], but were similar in animals that received loading for 2 weeks (Supplemental Fig. 1). Cartilage thickness did not differ among groups (Supplemental Fig. 2). No effect of mechanical loading on cartilage thickness was detected.

Subchondral bone plate and cancellous bone morphology

Subchondral bone TMD [Fig. 3(A)] was lower in TLR5KOΔMicrobiota mice than in other groups. Subchondral bone plate thickness in TLR5KOΔMicrobiota mice [Fig. 3(C)] was lower than that in WT and HFD mice. Subchondral bone plate thickness was greater in HFD mice compared to TLR5KO mice [Fig. 3(A)]. Loaded limb OARSI scores were correlated with control limb subchondral bone TMD ($r = 0.69$ [0.40, 0.86], Fig. 3(B)), and control limb subchondral bone thickness ($r = 0.66$, [0.35, 0.84], Supplemental Fig. 3). Epiphyseal bone volume fraction was less in HFD mice than in WT mice [Fig. 3(D)]. Trabecular thickness and trabecular separation were less in TLR5KOΔMicrobiota mice than in other groups (Supplemental Fig. 4). Mechanical loading was not associated with alterations in any other measures of bone [Fig. 3(A),(B),(D)].

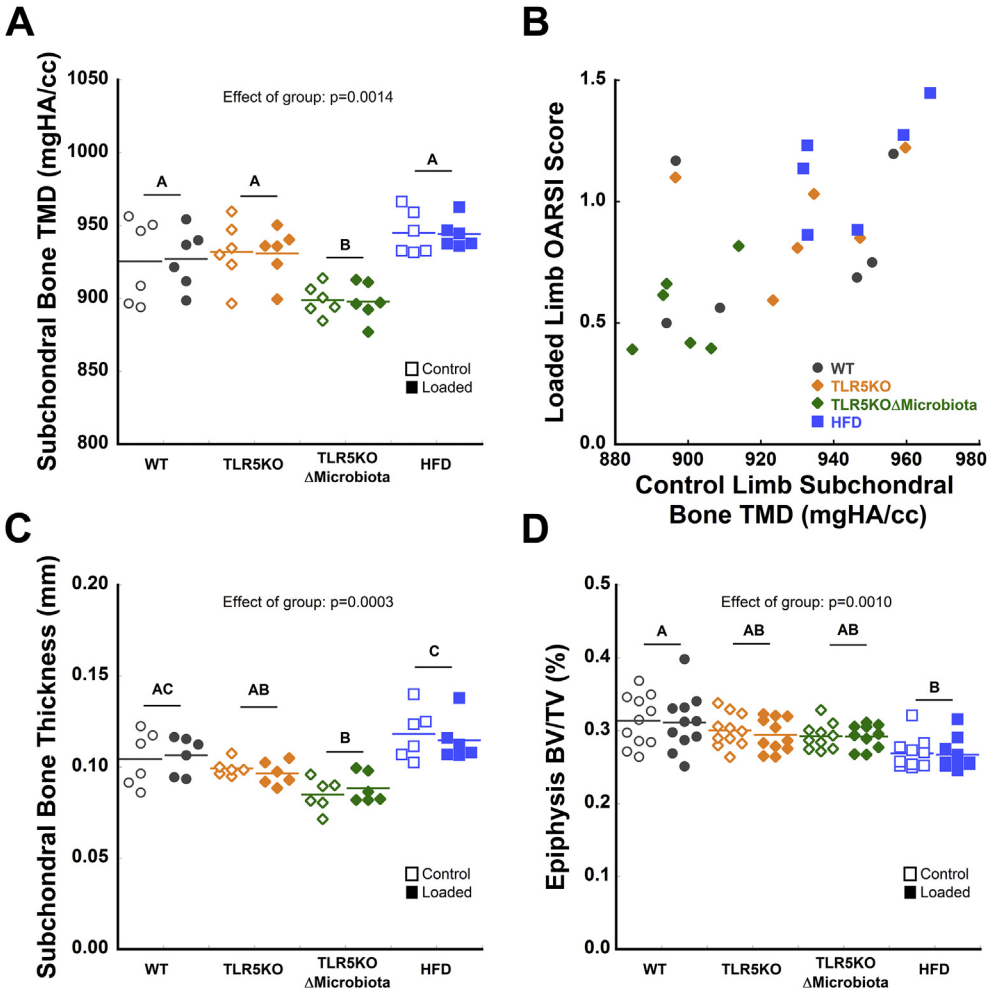

Fig. 3. Measures of subchondral and epiphyseal bone after 6 weeks of loading are shown. (A) TLR5KOΔMicrobiota mice showed lower levels of subchondral tissue mineral density (TMD). (B) Control limb subchondral bone TMD was correlated with loaded limb OARSI scores ($r = 0.69$, 95% confidence interval of correlation coefficient: [0.40, 0.86]). A Pearson's product-moment correlation analysis was used to identify relationships between loaded limb OARSI scores and control limb subchondral bone TMD. (C) TLR5KOΔMicrobiota mice showed lower levels of subchondral bone thickness as compared to other groups. (D) Epiphyseal bone volume fraction was less in HFD mice compared to other groups. Solid colored lines on dot plots represent mean. Groups sharing the same letter are not significantly different from each other ($p < 0.05$).

Serum inflammatory markers from 6 week loaded animals

Serum levels of LPS, KC, and IL-10 were greatest in HFD mice (Fig. 4(A),(C),(D), Table II). Serum levels of TNF-α were greater in HFD mice than in TLR5KOΔMicrobiota mice. Serum levels of IL-6 were lower in TLR5KOΔMicrobiota mice compared to WT mice. Serum IL-1 levels did not differ among groups.

Correlations among histological score and metabolic and inflammatory measures

Loaded limb OARSI scores were correlated with body mass ($r = 0.31$, [0.01, 0.56], Table III, Supplemental Fig. 5), fat pad mass ($r = 0.43$, [0.15, 0.65], Supplemental Fig. 6), KC ($r = 0.39$, [0.00, 0.68]), IL-10 ($r = 0.41$ [0.03, 0.69]), and LPS ($r = 0.54$, [0.24, 0.74]). Of the parameters correlated with loaded limb OARSI scores, LPS was the only parameter that had a significant effect on loaded limb OARSI score when group was included in the regression model. Among the WT, HFD, and TLR5KO groups, LPS explained 44% of the variation in OARSI score across groups ($R^2 = 0.44$, $p = 0.0003$, Fig. 4(B)). Within the TLR5KOΔMicrobiota mice, LPS was not correlated with OARSI score. Leptin and insulin were not correlated with loaded limb OARSI score. Control limb Mankin scores were correlated with body mass ($r = 0.48$, [0.21, 0.68]), fat pad mass ($r = 0.47$, [0.20, 0.68]), LPS ($r = 0.48$, [0.16, 0.71]), IL-6 ($r = -0.41$, [−0.69, −0.03]), insulin ($r = 0.51$, [0.15, 0.75]), leptin ($r = 0.55$, [0.21, 0.77]). Control limb Mankin scores were not correlated with loaded limb OARSI scores.

Gut microbiota analysis

Gut microbiota composition varied dramatically among groups at both the phyla and class level (Fig. 5(A), Supplemental Fig. 7). Gut microbiota composition at the phyla level was dominated by Bacteroidetes and Firmicutes [Fig. 5(B) and (C)]. The relative abundance of Bacteroidetes was greater in WT and TLR5KO mice compared to TLR5KOΔMicrobiota mice. HFD mice had the greatest abundance of Firmicutes. TLR5KOΔMicrobiota mice had the greatest abundance of Proteobacteria [Fig. 5(D)]. Principal coordinate analysis based on Bray–Curtis dissimilarity indicated that each group uniquely clustered together and had a distinct microbial community structure (Fig. 5(E), Supplemental Fig. 8). The diversity of the gut microbiota, as measured by the Shannon Diversity index, was reduced in the TLR5KOΔMicrobiota mice compared to other groups [Fig. 5(F)].

Discussion

In the current study, we examined the role of obesity, a metabolic syndrome-like phenotype, and the composition of the gut microbiome in the development of OA using an *in-vivo* tibial loading model. We demonstrate that alterations in obesity, the gut

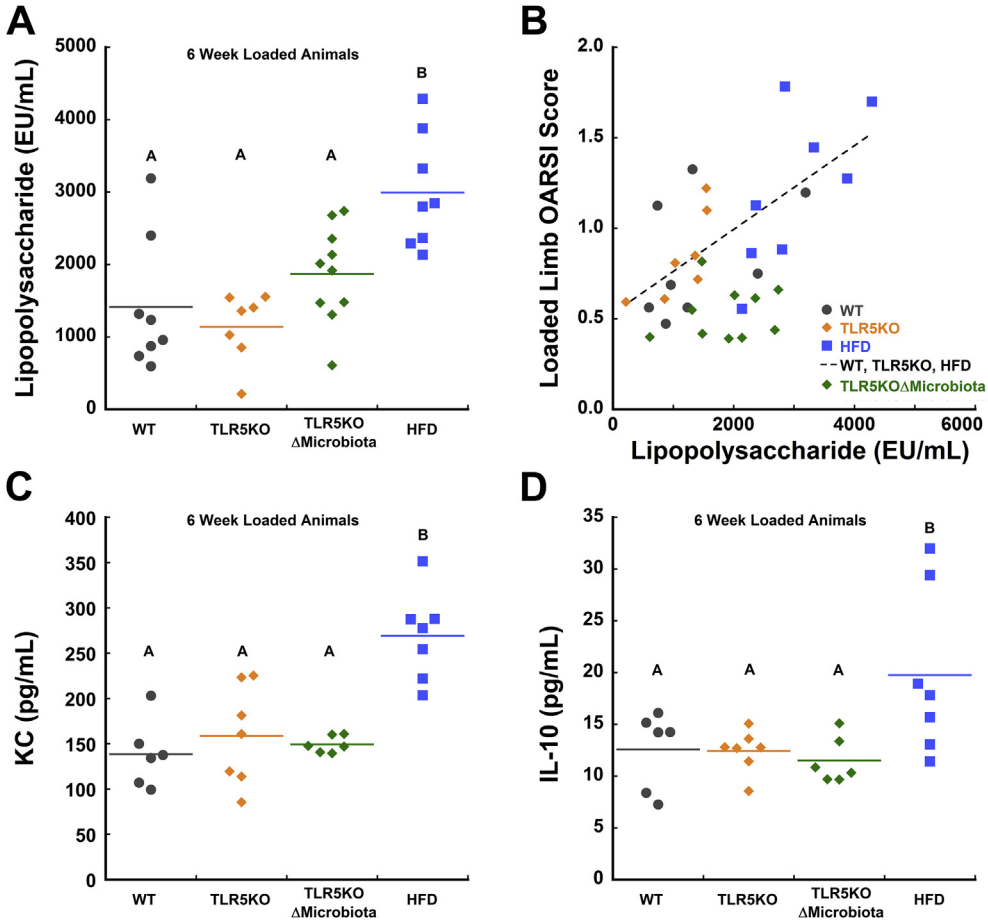

Fig. 4. Serum markers after 6 weeks of loading are shown. Mice fed HFD had elevated (A) serum lipopolysaccharide (LPS). (B) Serum LPS was correlated with loaded limb OARSI scores in untreated animals. Among the WT, HFD, and TLR5KO groups, LPS explained 44% of the variation in OARSI score across groups ($R^2 = 0.44$, $P = 0.0003$). HFD mice also had elevated serum levels of (C) KC and (D) IL-10. Solid colored lines on dot plots represent mean. Groups sharing the same letter are not significantly different from each other ($P < 0.05$).

Table II
Serum markers of cytokines and lipopolysaccharide (LPS)

Serum measure	WT	TLR5KO	TLR5KOΔ Microbiota	HFD
KC (pg/mL)	139 ± 37.0	159 ± 54.8	149 ± 9.26	269 ± 48.8†,‡,§
IL-10 (pg/mL)	12.6 ± 3.75	12.4 ± 2.03	11.5 ± 2.23	19.8 ± 7.94†,‡,§
TNF-α(pg/mL)	7.66 ± 1.31	7.43 ± 1.23	5.94 ± 1.31	9.40 ± 2.32§
IL-6 (pg/mL)	30.2 ± 15.9	21.3 ± 11.5	11.4 ± 2.50*	18.7 ± 10.6
IL-1β (pg/mL)	0.534 ± 0.192	0.350 ± 0.172	0.447 ± 0.198	0.512 ± 0.158
LPS (EU/mL)	1410 ± 909	1140 ± 484	1870 ± 663	2990 ± 779†,‡,§

Values are mean ± SD.
* TLR5KOΔMicrobiota vsWT.
† HFD vs WT.
‡ TLR5KO vs HFD.
§ HFD vs TLR5KOΔMicrobiota.

microbiome, and elevated levels of systemic inflammatory mediators can influence the development of load-induced cartilage damage, although the effects require time to manifest. Additionally, we demonstrate that the metabolic syndrome-like phenotype characteristic of the TLR5KO mouse is not sufficient to increase load-induced cartilage damage.

We attribute a portion of the increased load-induced cartilage damage in HFD mice to increased systemic inflammation. We see elevated levels of serum KC in the HFD mice, consistent with previous reports in HFD mice submitted to post-traumatic OA[13], and in patients with OA that have increased serum IL-8 (the human analog of KC)[32]. The elevated serum levels of IL-10 in HFD mice in the current study may reflect an active anti-inflammatory response to the OA pathology, consistent with findings of elevated levels of IL-10 in the synovial fluid of patients with OA[33]. Serum LPS was elevated in HFD mice, and was the only systemic factor examined in this study that explained differences in OARSI score across groups. Serum LPS has been associated with low-grade inflammation and is thought to play a role in the development of OA in individuals with obesity and metabolic syndrome[34]. Others have shown that mice fed a high fat diet develop an altered gut microbiome and a more permeable intestinal barrier, leading to elevated LPS levels and systemic inflammation[35]. LPS can also initiate local inflammatory responses within the joint that may enhance the effects of mechanical loads[34]. Consistent with our results, serum and synovial fluid LPS levels are associated with signs of OA in patients[36], supporting a possible role of LPS in the pathogenesis of OA cartilage pathology.

Adiposity is thought to influence OA pathology[1,37]. In our study HFD mice had the greatest levels of adiposity and the greatest loaded limb OARSI scores. However, several TLR5KO mice displayed fat pad mass within the range seen in HFD mice, yet did not have increased OARSI scores in loaded limbs like that seen in HFD mice (Supplemental Fig. 6). Additionally, serum factors associated with increased adiposity, such as leptin and insulin, were not associated with loaded limb OARSI score. Hence, our findings suggest that adiposity does not fully explain the increased cartilage damage in the current study.

Our findings suggest that the effects of systemic inflammation, adiposity, and the gut microbiome on load-induced cartilage damage are time dependent. After 2 weeks of loading, loaded limb OARSI scores were similar among groups, but after 6 weeks of loading HFD mice had greater loaded limb OARSI scores. Hence, the additional 4 weeks of loading may be required for systemic inflammation and adiposity to have an effect on OA cartilage pathology. Similarly, TLR5KOΔMicrobiota mice display less cartilage damage after 6 weeks of loading but not after 2 weeks, suggesting that the effect of the gut microbiome is time dependent as well. The effect of time is not surprising as joint degeneration in humans occurs over decades[38]. Our findings may have implications for

Table III
A correlations matrix of histological scores and metabolic and inflammatory measures after 6 weeks of loading is shown

	Loaded OARSI	Control OARSI	Control mankin	Body mass	Fat pad mass	LPS	IL-10	IL-1β	IL-6	KC	TNF-α	Insulin	Leptin
Loaded OARSI		[−0.079, 0.493]	[−0.202, 0.393]	[0.012, 0.560]	[0.153, 0.649]	[0.236, 0.743]	[0.026, 0.688]	[−0.305, 0.478]	[−0.107, 0.611]	[0.003, 0.675]	[−0.241, 0.517]	[−0.166, 0.572]	[−0.057, 0.641]
Control OARSI	0.227		[−0.271, 0.330]	[−0.130, 0.453]	[−0.074, 0.498]	[−0.237, 0.442]	[−0.445, 0.327]	[−0.371, 0.419]	[−0.041, 0.651]	[−0.363, 0.411]	[−0.398, 0.377]	[−0.366, 0.408]	[−0.209, 0.541]
Control mankin	0.105	0.032		[0.209, 0.682]	[0.199, 0.676]	[0.161, 0.705]	[−0.144, 0.587]	[−0.343, 0.445]	[−0.689, −0.028]	[−0.015, 0.666]	[−0.184, 0.559]	[0.149, 0.748]	[0.209, 0.774]
Body mass	**0.312**	0.177	**0.480**		[0.766, 0.926]	[0.052, 0.645]	[0.214.0.776]	[−0.288, 0.492]	[−0.561, 0.181]	[0.601, 0.907]	[0.326, 0.820]	[0.712, 0.936]	[0.844, 0.968]
Fat pad mass	**0.434**	0.232	**0.472**	**0.867**		[0.027, 0.631]	[0.052, 0.701]	[−0.226, 0.541]	[−0.515, 0.243]	[0.252, 0.791]	[−0.112, 0.607]	[0.608, 0.909]	[0.721, 0.939]
LPS	**0.536**	0.116	**0.478**	**0.388**	**0.336**		[−0.081, 0.627]	[−0.292, 0.490]	[−0.520, 0.236]	[0.250, 0.791]	[−0.244, 0.514]	[0.008, 0.678]	[0.256, 0.793]
IL-10	**0.409**	−0.069	0.258	**0.555**	**0.441**	0.316		[0.003, 0.685]	[−0.320, 0.451]	[0.437, 0.858]	[0.390, 0.842]	[0.175, 0.759]	[0.102, 0.726]
IL-1β	0.103	0.029	0.060	0.121	0.173	0.117	**0.397**		[−0.064, 0.648]	[−0.136, 0.603]	[−0.146, 0.597]	[−0.240, 0.531]	[−0.375, 0.415]
IL-6	0.292	0.352	**−0.411**	−0.222	−0.171	−0.167	0.077	0.340		[−0.413, 0.361]	[−0.250, 0.509]	[−0.617, 0.096]	[−0.594, 0.134]
KC	**0.390**	0.029	0.375	**0.802**	**0.570**	**0.581**	**0.705**	0.274	−0.030		[0.334, 0.822]	[0.442, 0.860]	[0.523, 0.885]
TNF-α	0.162	−0.012	0.219	**0.634**	0.332	0.158	**0.675**	0.264	0.152	**0.638**		[0.120, 0.734]	[0.085, 0.717]
Insulin	0.237	0.025	**0.507**	**0.862**	**0.831**	**0.394**	**0.527**	0.172	−0.302	**0.708**	**0.485**		[0.653, 0.922]
Leptin	0.338	0.194	**0.552**	**0.928**	**0.878**	**0.585**	**0.470**	0.024	−0.268	**0.757**	**0.457**	**0.831**	

Bold numbers represent a significant correlation ($p < 0.05$). Bottom left half of table are pairwise correlation value (r). Top left half of table are confidence interval of correlation.

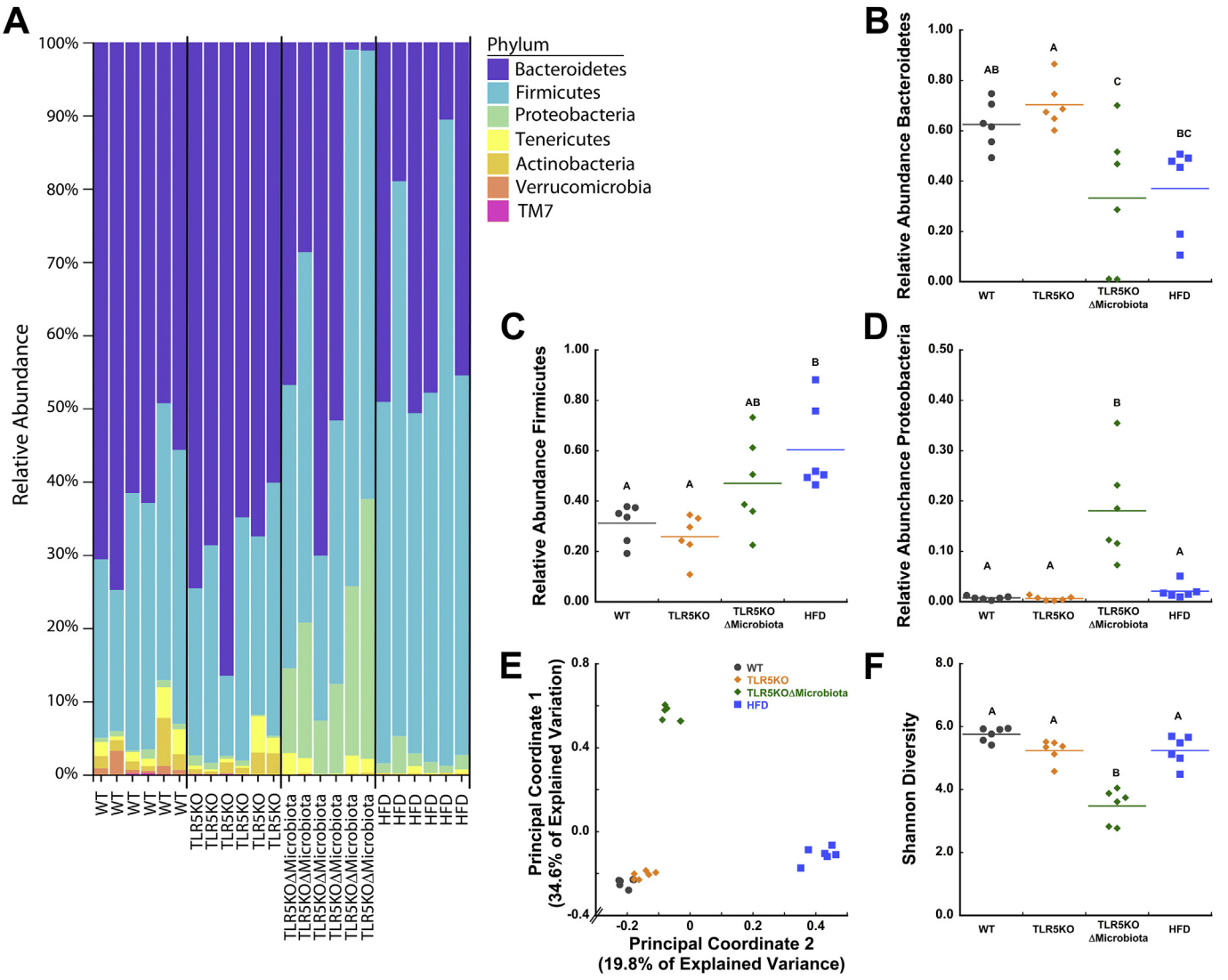

Fig. 5. The taxonomic profile of the gut microbiota from animals after 6 weeks of loading is shown. (A) There are large differences in the relative abundance of organisms at the phyla level. (B) The relative abundance of Bacteroidetes was greatest in TLR5KO and WT mice. (C) The relative abundance of Firmicutes was greatest in HFD mice and (D) the relative abundance of Proteobacteria was greatest in TLR5KOΔMicrobiota mice. (E) Principal coordinate analysis based on the Bray–Curtis dissimilarity shows that each group forms its own distinct clusters from each other. Bacterial diversity was dramatically reduced in TLR5KOΔMicrobiota mice (F). Solid colored lines on dot plots represent mean. * $p < 0.05$.

clinical treatment for patients with obesity or metabolic syndrome. For example, if a patient has a recent load-induced injury subsequent treatments that correct metabolic abnormalities may slow subsequent development and progression of OA.

HFD and TLR5KO mice both exhibited clear signs of metabolic abnormalities and excess adiposity, but only HFD mice exhibited signs of more severe OA with prolonged loading. Compared to WT, The HFD mice showed severely elevated insulin levels (530% increase) and severe obesity (261% increase in fat pad mass). The TLR5KO mice had mild increases in insulin levels (175% increase) and mild obesity (149% increase in fat pad mass) compared to WT. We attribute the different degrees of cartilage pathology in the HFD and TLR5KO mice to one of three possibilities: First, the severity of metabolic abnormalities in TLR5KO mice may have been insufficient to worsen cartilage pathology. Larger changes in systemic inflammation and/or adiposity may be required to increase load-induced cartilage damage. Second, metabolic syndrome and the related systemic environment in the TLR5KO mice may require more time to become evident in cartilage pathology (just as HFD mice required more than 2 weeks). Last, we must consider the possibility that TLR5 signaling within the joint may contribute to OA; TLR5 is expressed at higher levels in synovial tissue of OA patients compared to healthy individuals[39].

The TLR5KOΔMicrobiota mice display less cartilage damage after 6 weeks of loading compared to other groups and little difference between the loaded and control limbs. The reduced cartilage damage is likely not explained by adiposity or systemic inflammation, two factors that were comparable in TLR5KOΔMicrobiota and WT mice. The antibiotics used here to disrupt the gut microbiota are poorly absorbed at the gut lining, thus making it unlikely that antibiotics have a direct effect on joint tissues. Others have shown that oral antibiotic treatment can lead to reduced OA joint pathology in humans[40] and animals[41]. We consider the most likely explanation for the reduced cartilage damage in the TLR5KOΔMicrobiota mice is alterations in the gut microbiome. We see large differences in the composition of the gut microbiota between TLR5KOΔMicrobiota and WT mice. The gut microbiome may influence distant organs through three different mechanisms: regulation of nutrient/vitamin absorption, interactions with the immune system at the gut lining, and translocation of microbe-

associated molecular patterns (MAMPs) from the gut to the circulation[42]. The TLR5KOΔMicrobiota did not display overt signs of impaired nutrient absorption; TLR5KOΔMicrobiota mice had comparable body mass to WT mice. However, we cannot ignore the possibility that vitamins derived from the gut microbiota may influence cartilage damage mechanisms. Immune regulation at the gut lining may contribute to the reduced OARSI scores, as modification of the constituents of the gut microbiota are known to influence inflammation and/or immune activation at the gut lining and circulating immune cells and cytokines. Additionally, modification of the gut microbiota could have altered the translocation of MAMPs across the gut endothelium and into the systemic circulation. LPS is one example of a MAMP commonly observed in the blood[43]. A larger study with more stringent controls of the microbiome constituents, and that included WT mice treated with antibiotics is required to better understand the specific mechanisms behind the reduced OARSI scores in the TLR5KOΔMicrobiota group. The distinct gut microbial communities may also help to explain the opposite trends in OA cartilage pathology between the TLR5KOΔMicrobiota and HFD mice.

Additionally, it remains possible that the reduced OARSI scores and increased Mankin scores in the TLR5KOΔMicrobiota mice may be secondary to changes in bone tissue and cartilage. Alterations in bone tissue have recently been shown to influence OA cartilage pathology[44,45]. The TLR5KOΔMicrobiota mice had reduced subchondral bone plate TMD and thickness, which may help explain the reduced effect of mechanical loading, since subchondral bone TMD and bone thickness were both correlated with loaded limb OARSI score[46]. We also recently showed that the same modifications to the gut microbiome in the current study were associated with reductions in whole bone strength caused by changes in bone tissue material properties[47]. The relationship between subchondral bone properties and load-induced cartilage damage is complex and warrants further investigation[48]. With regard to cartilage properties, TLR5KOΔMicrobiota mice had increased control limb Mankin scores. It is possible that the alterations to the gut microbiome had a direct effect on cartilage health, although it does not appear that the increased Mankin scores influenced load-induced cartilage damage.

A number of strengths of the current study are worth noting. First, the study is unique in the examination of metabolic syndrome without severe obesity on the development of OA. Previous studies looking at metabolic disease and OA have focused solely on HFD models and leptin/leptin receptor deficient models. Second, the current study is the first to demonstrate an effect of HFD on OA in an *in-vivo* loading animal model without surgery or trauma. Third, the study examines modifications to the gut microbiota that influence load-induced OA. The reduced response to 6 weeks of loading in the TLR5KOΔMicrobiota is interesting and warrants further investigation to understand if the gut microbiome influences OA development[49].

A number of limitations are worth noting. First, the severity of OA cartilage pathology was small compared to more severe OA animal models[13,50]. The use of a greater load magnitude would lead to higher OARSI scores and increased sensitivity to small group differences. However, the milder form of OA cartilage pathology here provides insight into the earlier stages of OA development and/or OA generated by more common, lower magnitude loads. Second, it is not clear in this study if HFD mice had elevated severity or accelerated progression of OA cartilage pathology at 6 weeks. HFD models have been shown to develop both increased severity[13] and accelerated progression of OA[14]. It is possible that if the current study extended beyond 6 weeks, OA cartilage pathology in other groups might become as severe as in HFD mice. Lastly, the metabolic syndrome phenotype of the TLR5KO mice was not completely confirmed as only one direct measure of metabolic syndrome (abdominal adiposity) was assessed. However, the other parameters related to metabolic syndrome (body mass and serum insulin) are consistent with the previous characterization of the TLR5KO mouse[23].

We conclude the following: 1) severe adiposity and systemic inflammation increased load-induced cartilage damage after 6 weeks of loading, while milder adiposity and metabolic abnormalities in TLR5KO mice did not worsen OA pathology; 2) the effect of systemic factors on OA development appeared to be related to the duration of increased mechanical loading; 3) changes in the gut microbiota may contribute to the severity of load-induced OA cartilage pathology and subchondral bone morphology.

Author contributions

Authors' roles: Conceived and designed the experiments: JDG, MCHM, SRG, CJH. Acquisition, analysis, and interpretation of the data: JDG, CJH, SNZ, ML, DTH, TNS, GGG. Wrote and Revised Manuscript: JDG, CJH, MCHM, SRG. Critical revision and final approval of the manuscript: All authors.

Conflict of interest

All authors state that they have no conflicts of interest.

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Supplementary data

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