TNF Produced by Inflammatory Blood Mononuclear Cells Directly Contributes to Cartilage Damage in Arthritis

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Research article

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TNF Produced by Inflammatory Blood Mononuclear Cells Directly Contributes to Cartilage Damage in Arthritis

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Abstract (<350)

Background: Anti-TNF therapies are effective at preventing inflammation and structural damage in rheumatoid arthritis (RA). However, the role of TNF in cartilage destruction in RA is not well understood. Therefore, we studied the effects of TNF on cartilage and compared TNF production by different cell types involved in joint pathology.

Methods: Primary human chondrocytes and cartilage explants were cultured with recombinant TNF. Bovine cartilage was co-cultured with activated human peripheral blood mononuclear cells (PBMCs) or fibroblast-like synoviocytes (FLS). Expression of cytokines and metalloproteinases (MMPs) was assessed by qPCR and MSD, and proteoglycan depletion from cartilage was assessed using histomorphometry and colorimetric detection in tissue culture supernatants. D2E7 was used to block TNF both *in vitro* and *in vivo* in a human TNF transgenic (hTNF-Tg) mouse model of arthritis.

Results: TNF elicited strong pro-inflammatory and catabolic effects on isolated human chondrocytes and cartilage explants leading to upregulation of IL-6 and MMPs, as well as proteoglycan depletion from bovine cartilage explants. In an effort to identify cellular sources of TNF, we challenged chondrocytes, FLS and PBMCs with inflammatory stimuli present in RA joints and found that PBMC that were used to model inflammatory cell infiltration produced significantly higher levels of TNF. Moreover, co-culture with activated PBMCs resulted in proteoglycan depletion from bovine cartilage explants. In sharp contrast with stromal cells, TNF failed to induce high amounts of IL-6 and MMPs in PBMCs, suggesting that different cell populations play distinct roles in the triggering and propagation of joint destruction. TNF blockade protected cartilage from damage both in co-culture systems and in a hTNF-Tg mouse model of arthritis.

Conclusions: Our data demonstrate that TNF directly triggers a catabolic program in human chondrocytes leading to cartilage damage and further suggest that neutralization of TNF produced by immune cells infiltrating the inflamed joints, decreases catabolic activity of chondrocytes and fibroblasts, which, in turn, contributes to the cartilage protective effects of anti-TNF biologics in arthritis.

Keywords

TNF, D2E7, chondrocytes, cartilage, fibroblast-like synoviocytes, rheumatoid arthritis, blood mononuclear cells

Background

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease of unknown aetiology that preferentially targets peripheral joints. RA pathology is highly heterogeneous and involves activation of both adaptive and innate immune responses, dysregulated cytokine expression and hyperactivation of stromal cells that support disease progression (1-3). The use of targeted therapies in clinical studies of RA have elucidated the role of different mechanisms in disease pathogenesis and demonstrated that pathways activated by cytokines, such as tumor necrosis factor (TNF) and interleukin 6 (IL-6), are crucial for RA (1, 3, 4).

Articular cartilage is one of the target tissues in RA. It covers the surfaces of long bones forming the joint and consists of avascular highly organized dense extra-cellular matrix (ECM) populated by chondrocytes. Cartilage ECM is synthetized by chondrocytes and is composed of a collagen network (predominantly type II collagen) and the proteoglycan (PG) aggrecan (5). Several mechanisms contributing to cartilage damage in RA have been proposed. TNF and other pro-inflammatory cytokines found in synovial fluid have been shown to directly damage cartilage by inducing apoptosis of chondrocytes or by increasing production by chondrocytes of proteolytic enzymes, such as metalloproteinases (MMPs) and aggrecanases, even before the development of synovial inflammation (5-9). Cartilage destruction is also associated with synovial inflammation and directly mediated by invasion of hyperplastic inflamed synovial tissue called pannus. Pannus is a highly aggressive tumor-like tissue that mainly consists of activated fibroblast-like synoviocytes (FLS) with infiltrates of immune cells migrating from peripheral blood to inflamed synovium (3, 7, 10). Activated FLS show elevated expression of cytokines, chemokines, adhesion molecules and proteolytic enzymes that propagate an aggressive invasion of the pannus with

destruction of cartilage matrix (5, 11-13). RA FLS have been shown to maintain a unique "transformed" phenotype and retain the ability to invade and damage cartilage both *ex-vivo* when co-cultured with cartilage explants and *in vivo* when injected in mice (11, 14-16).

Clinical studies have demonstrated that anti-TNF therapy inhibits both inflammation and structural damage of bone and cartilage. However, the role of TNF in triggering and propagating cartilage damage in RA is not clear. Results from *in vivo* studies in knockout mice suggest that cartilage destruction is indirectly regulated by TNF via an increase in IL-1β production by hematopoietic cells (17). Other reports suggest that TNF present in synovial fluid directly induces apoptosis of chondrocytes or mediates cartilage damage by inducing production of proteases by chondrocytes (6-9, 18, 19). Recent therapies with synthetic disease-modifying antirheumatic drugs (DMARDs) and biologics have significantly improved clinical outcomes of RA patients and have even induced clinical remission in some cases (20-23). However, even the most efficacious RA treatments that successfully inhibit inflammation and progression of joint destruction are incapable of reversing the structural damage of bone and cartilage and fully restoring joint function (24, 25). In addition, results of clinical and animal studies suggest that synovial inflammation and tissue destruction may be uncoupled and that structural changes in cartilage and bone may occur and progress in the absence of clinical markers of active inflammation (12, 17, 26, 27). Conversely, therapies directed against bone damage helped reduce the radiological damage but had no effect on inflammation (28). Recent elucidation of the relationship between structural damage and functional outcomes have led to dramatic changes in the management of RA. The new strategic approach, treat-to-target, aims to minimize structural damage and achieve the optimal outcome by early aggressive disease

intervention, stringent criteria of remission and tight control of disease activity (3, 29-32).

In the present study, we addressed the direct role of TNF in RA cartilage damage and compared TNF production by different cell types involved in joint pathology in RA to better understand the mechanism of cartilage protection by TNF blocking therapeutics.

Materials and methods

Cells

Primary human FLS, chondrocytes and human cartilage explants were purchased from Articular Engineering. Human peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation from the whole blood of healthy donors purchased from BioIVT.

Reagents

The following reagents were used as indicated: 10 ng/ml of recombinant human TNF and IL-1β (R&D Systems), 1 ng/ml of lipopolysaccharide (LPS, E. coli 0127:B8; Sigma), 10 μg/ml of recombinant human S100A8 and S1009 proteins (AbbVie), 5 μg/ml of anti-human TNF antibody D2E7 (human IgG1, AbbVie) and control human IgG1 (Sigma).

Cell culture

FLS and chondrocytes were first expanded in T75 flasks in Dulbecco minimum essential medium (DMEM; Invitrogen) or DMEM/F12 (Invitrogen) respectively, supplemented with 10% heat inactivated defined fetal bovine serum (FBS; HyClone), GlutaMAX Supplement, 0.02M HEPES and Pen/Strep (100 units/mL of penicillin and

100 μg/mL of streptomycin; all from Invitrogen), and then were used for experiments between passages 2 and 3. FLS and PBMC were seeded on 12-well plates at 10^5 cells/well and $1.5x10^6$ cells/well, respectively, and cultured in 1.5 ml of DMEM supplemented with 10% FBS, GlutaMAX, 0.02M HEPES and Pen/Strep. Chondrocytes were cultured in 12-well plates at 10^5 cells/well in Advanced DMEM/F12 (Invitrogen) supplemented with 0.02M HEPES, GlutaMAX and Pen/Strep. Following 24 h equilibration, cells were treated with different stimuli and anti-TNF antibody were used in some of the experiments in concentrations indicated above.

In vitro cartilage destruction assay

Bovine cartilage explants (3 mm in diameter) were produced from one metacarpophalangeal joint of a skeletally mature animal (Articular Engineering). Explants were placed in 96-well flat bottom plates (1 explant/well) and human FLS ($5x10^3$ cells/well) or PBMCs ($2x10^5$ cells/well) were seeded over the cartilage pieces in 200 μl of complete Advanced DMEM/F12 or DMEM/F12 media respectively. Cartilage mono- and co-cultures were treated with the same stimuli and Ab as described above for 14 days (4 explants per treatment condition). Media was exchanged every 3 days and saved for future analyses. At the end of culture, cartilage pieces were collected for PG analysis.

Cytokine, MMP and proteoglycan detection

Culture media were collected at various times and levels of cytokines and MMPs were detected using mesoscale discovery (MSD) assays. PG depleted from cartilage into the medium was measured using a sGAG Assay Kit (Kamiya Biomedical Co) following the manufacturer's protocol.

Arthritis model

In vitro effects of D2E7 on cartilage were evaluated in the hTNF-Tg mouse model of arthritis. hTNF-Tg mice (Tg197 strain, C57BL/6 genetic background) spontaneously develop inflammatory, destructive arthritis upon constitutive hTNF overexpression. Both the genotype and phenotype of these mice have been described previously in more detail (33). Briefly, the animals develop joint swelling in the small joints of the front and hind paws between weeks 5 and 6 after birth. This chronic polyarthritis progresses over time and results in severe joint damage. In this study, Tg197 mice heterozygous for human TNF were injected intraperitoneally (i.p.) with 1 mg/kg of anti-TNFα antibody D2E7 (n=12) or placebo (n=6) once a week for 12 weeks. At the end of the study hind paws were collected and processed for histochemistry. Animals were housed and maintained at the facilities of the Hellenic Pasteur Institute, under specific pathogen free (SPF) conditions. All experimental protocols were reviewed and approved by the Institutional Committee for Animal Care of Hellenic Pasteur Institute, and by Abbott's Corporate Animal Welfare Committee.

Tissue processing and staining

***In vitro* studies.** At the end of culture, bovine cartilage explants were washed in phosphate buffered saline (PBS; Invitrogen), fixed in 10% neutral buffered formalin (Alfa Aesar) for 24 hours at room temperature, cut in half, embedded in optimal cut temperature compound (OCT; Tissue-Tek) and kept frozen at -80°C. Frozen cartilage tissues were sectioned (5 μm thick) using a tape transfer technique and were stained with toluidine blue for PG detection. Slides were scanned on the Vectra Imaging system at low power. PG content (as measured by the toluidine blue optical density (OD)) was analysed histomorphometrically as follows: high power (20x) images were selected on each cartilage sample (minimum 2 images per cartilage piece) and

analysed by InForm (Perkin Elmer) image analysis software. The data represent an average of OD of the dye (the amount of absorbing material present at the area of interest) in the image of cartilage pieces that underwent the same treatment (one OCT block).

***In vivo* studies in hTNF-Tg mice.** Hind legs were fixed in 10% neutral buffered formalin, decalcified, embedded in paraffin, and 5 μm ankle joint sections were prepared and stained with hematoxylin (detects nuclei) and alcian blue (PG detection). Total cartilage area and area stained with alcian blue on the proximal and distal ends of talus bones were measured, and PG content was determined as a percent of alcian blue-positive areas within total cartilage area.

Statistical analysis

Statistical analysis was performed using GraphPad Prism analytical software version 8 for Windows. Statistical tests included the nonparametric Mann-Whitney U test, Kruskal-Wallis test with Dunn's post-test for multiple comparisons; repeated measure two-way analysis of variance (ANOVA) with Sidak's post hoc tests for multiple comparisons, two- and one-way ANOVA with Tukey's or Dunn's post hoc tests for multiple comparisons. *P* values less than 0.05 were considered significant.

Results

TNF induces sustained expression of pro-inflammatory cytokines and MMPs in human chondrocytes and triggers cartilage destruction

Our studies focused on TNF-mediated activation of human chondrocytes and their production of pro-inflammatory cytokines and MMPs that degrade type II collagen and PGs, two major components of cartilage (34, 35). First, we compared inflammatory responses of isolated chondrocytes with that of chondrocytes that TNF directly contributes to cartilage damage in arthritis

remain embedded in cartilage tissue. We cultured cartilage explants or cells isolated and grown as a monolayer in the presence or absence of TNF for several days and evaluated expression of mediators of inflammation and tissue damage. Upon TNF treatment, chondrocytes secreted high levels of IL-6, MMP1 and MMP3 that were sustained and increased over time, reaching maximum levels (ng/ml for IL-6 and μg/ml for MMPs) between 24 and 72 hours after TNF addition to cultures (Fig. 1A and data not shown). The analysis of IL-6, MMP1 and MMP3 mRNA revealed a similar pattern of continuous expression in response to TNF (data not shown). We obtained comparable results with cultured cartilage explants and isolated chondrocytes (Fig. 1A, upper and lower panels), supporting previous findings that isolated cells at early passages (P 2-3) maintain characteristics typical of chondrocytes within the cartilage and thus are suitable for *in vitro* studies of chondrocyte biology (16, 35).

Next, we examined whether increased production of MMPs by TNF-activated chondrocytes translated into cartilage damage. For *in vitro* cartilage destruction studies, we used bovine cartilage as a well-established and readily available experimental model that is commonly used for studies of human cytokines and growth factors (16). Bovine cartilage explants were cultured in the presence or absence of TNF for 14 days. IL-1β, known to be a strong cartilage catabolic factor, served as a positive control. Media with cytokines were replenished every 3 days. At the end of experiments, cartilage pieces were collected, embedded in OCT, sectioned and PG content was detected by toluidine blue staining. TNF treatment resulted in partial depletion of PG from cartilage, as depicted by partial loss of blue colour (Fig. 1B, upper panel), whereas IL-1β-treated tissue was almost completely depleted of PG and did not retain the dye (Fig. 1B, upper panel). Image analysis results from multiple

experiments confirmed that although TNF was less potent than IL-1β, it caused significant depletion of PG from the cartilage as compared to untreated controls (Fig. 1B, lower panel). We also found that IL-1β induced higher levels of IL-6 and MMPs compare to TNF both in isolated chondrocytes and cartilage cultures (Fig.1C). Our results suggest that TNF induces a sustained inflammatory and tissue destructive program in chondrocytes that leads to matrix degradation and destruction of cartilage in vitro.

Co-culture with activated FLS has a marginal effect on proteoglycan depletion from the cartilage

To gain a better understanding of the mechanisms underlying cartilage protection by anti-TNF biologics, we established an experimental system whereby bovine cartilage was co-cultured with activated human cells relevant to RA pathology. RA FLS have been reported to invade articular cartilage *in vitro* and *in vivo* (16, 36) and secrete a wide array of factors that mediate activation and survival of synovial inflammatory cells, enhance synovial neo-angiogenesis, and induce cartilage degradation (11, 37). To evaluate catabolic effects of activated FLS, bovine cartilage explants were co-cultured with human FLS isolated from the joints of RA patients, or from individuals without joint pathology, in the presence or absence of recombinant human TNF, and PG depletion from the explants was measured using toluidine blue. Surprisingly, we found that neither untreated nor TNF-activated RA FLS increased PG depletion, whereas non-diseased FLS treated with TNF marginally increased explant destruction as compared to cartilage monoculture (Figure 2A). We further compared expression of matrix degrading enzymes in activated FLS and found that after stimulation with TNF, RA FLS produced less MMP1 and MMP3 than non-diseased cells (Fig. 2B). This

finding is in-line with the weak catabolic effect of TNF-treated RA FLS on cartilage observed in Figure 2A.

Next, we tested if neutralization of endogenous TNF protects cartilage from damage. To initiate the catabolic program, human FLS/bovine cartilage co-cultures were treated with TNF or IL-1β for three days following the addition of D2E7 monoclonal antibody to block TNF. D2E7 selectively inhibited production of IL-6 and MMPs by FLS in co-culture systems (Fig. 2D and data not shown) and protected cartilage explants from PG depletion in mono- and co-cultures treated with TNF but not with IL-1β (Fig. 2C). Overall, our data demonstrate that co-culture with activated FLS had minimal effect on cartilage destruction and that D2E7 protects cartilage cultures from TNF-dependent damage.

Activated PBMCs produce significantly higher levels of TNF compared to joint stromal cells

Next, we examined whether activated joint stromal cells represent a significant source of endogenous TNF and found that strong pro-inflammatory factors, such as IL-1β and LPS, induced relatively low TNF expression in chondrocytes and FLS (Fig. 3A). Given that D2E7 had very little effect on IL-1β mediated cartilage damage, this suggested that TNF was not downstream of IL-1β catabolic effects (Fig. 2C). Therefore, we investigated additional cellular sources and mediators of TNF in RA joints. In arthritis, activated immune cells migrate to joints from the bloodstream, infiltrate the synovium and propagate inflammation and tissue damage (3, 38). Within inflamed tissues, multiple damage-associated molecular patterns (DAMPs) are released from the dead or activated cells that are capable of recruiting immune cells and maintaining the chronic inflammatory state in the joints (39, 40). Alarmins are a group of endogenous molecules that belong to the DAMP family and are associated TNF directly contributes to cartilage damage in arthritis

with RA pathogenesis (40). Thus, we compared TNF production by human PBMCs, FLS and chondrocytes after activation with alarmins S100A8 and S100A9. Alarmins and LPS (positive control) induced significantly higher levels of TNF in PBMCs compared to stromal cells (Fig. 3B). IL-1β induction was observed also, even though levels were in the lower picogram range. Furthermore, in co-culture with cartilage explants, treatment of human PBMCs with alarmins resulted in catabolic activity similar to the effect of TNF treatment, as measured by release of PG into the culture media (Fig. 4A). To obtain additional mechanistic insights into the pathogenic effects of TNF in arthritis, we compared responses of three different cell types to TNF and found that, in contrast to FLS and chondrocytes, PBMCs failed to produce sustained high levels of MMP3 and IL-6 (Fig. 3C). Thus, our findings support a model in which activated immune cells are major producers of inflammatory cytokines, such as TNF and IL-1β, that activate stromal cells in the joints and trigger a cascade of pathologic events culminating in chronic inflammation and tissue destruction in RA.

Neutralization of endogenous TNF attenuates inflammation and protects cartilage from PG depletion *in vitro* and *in vivo*

To confirm that cartilage destruction can be promoted by endogenously produced TNF and can be blocked by an anti-TNF treatment, we added D2E7 or an isotype control antibody to the activated PBMC/cartilage co-cultures and evaluated PG depletion from the cartilage explants. D2E7 protected cartilage from destruction by blocking both exogenously added TNF and endogenous TNF produced by PBMCs activated with S100A proteins (Fig. 4A and B). Next, we evaluated anti-catabolic effects of D2E7 *in vivo* in the hTNF-Tg model of arthritis. In this model, mice continuously express human TNF and develop spontaneous arthritis between weeks 5 and 6 after birth (33). Prophylactic administration of D2E7 attenuated inflammation and cartilage destruction

as depicted by retention of blue dye (asterisk on Fig. 4C, upper panel). Further analysis of alcian blue staining demonstrated a significantly higher content of PG in cartilage of animals treated with D2E7 (Fig. 4C, lower panel).

Collectively, we show that TNF directly triggers a catabolic program in human chondrocytes leading to cartilage damage. Our findings also suggest that neutralization of TNF produced by PBMCs infiltrating inflamed joints decreases catabolic activity of chondrocytes and fibroblasts, which contributes to the cartilage protective effects of anti-TNF biologics in RA.

Discussion

There are several mediators of inflammation that contribute to the pathogenesis and propagation of systemic disease in RA leading to structural damage of joint tissues. Inhibition of major molecular inducers of inflammation, such as TNF, IL-6, IL-1 and chemokines, as well as cellular contributors, such as T cells and B cells, have been shown to be effective in various animal models and in humans (3). As a result, we now have several highly effective drugs in the treatment of RA that inhibit inflammation and reduce structural damage. Anti-TNF biologics are among the effective treatments that have been shown to halt structural damage in articular cartilage and bone. To understand the mechanism of cartilage protection by anti-TNF therapeutics, we studied the effect of TNF on cartilage and chondrocytes, investigated cellular sources of TNF in the joint and explored the outcomes of TNF neutralization on cartilage damage *in vitro* and in a mouse model of arthritis.

It is worth noting that prior to this study, the direct effects of TNF on human chondrocytes and cartilage had not been studied extensively. Most of the published data addressed the role of TNF in animal models of RA or used TNF in combination

with other inflammatory cytokines *in vitro* (9, 16-18). In the present work, we examined responses of isolated chondrocytes and cartilage explants to either exogenously added or endogenously produced TNF. Chondrocytes responded directly to TNF stimulation by secreting IL-6 and the cartilage degrading enzymes, MMP1 and MMP3. Similar results were obtained when isolated human chondrocytes or chondrocytes embedded in the human cartilage matrix of the explants were tested. Interestingly, activation by TNF continued for up to three days, as evidenced by the continuously increasing concentrations of these mediators in the culture supernatants and mRNA levels in chondrocytes. To monitor structural damage, bovine cartilage explants were used as a model, as they are more readily available, have uniform characteristics, an intact surface and are more amendable for studies assessing physical damage. Bovine cartilage explants also have an advantage over human samples typically obtained during autopsy or joint replacement surgery, which have highly variable characteristics. Culturing bovine cartilage explants with TNF for 14 days led to depletion of PGs, a hallmark of cartilage structural damage. In agreement with previous findings, IL-β was more efficient in causing cartilage destruction than TNF (16, 18). The role of IL-β in cartilage and bone destruction in RA is well established, especially in animal models of arthritis (17, 41). Although the IL-1R antagonist anakinra has been approved for RA treatment (42, 43), the overall anti-inflammatory effects of anakinra in RA are moderate and significant improvement is achieved in only about 50% of patients, when compared to anti-TNF treatment. Nevertheless, anakinra demonstrated a strong protective effect in cartilage and bones (43-45).

As FLS are in close proximity to the cartilage and are activated and transformed into a tumor-like invasive pannus in RA, FLS from RA patients or healthy controls were co-

cultured with cartilage explants with or without TNF addition. Co-culture with FLS did not show marked PG depletion over monocultures of cartilage irrespective of FLS origin or presence of TNF in the cultures. TNF mediated effects were neutralized by the addition of the anti-human TNF antibody, D2E7. When FLS/bovine explant co-cultures were stimulated with TNF, both diseased and healthy FLS responded by producing IL-6. However, RA FLS secreted less of the tissue degrading enzymes, MMP-1 and MMP-3, as compared to non-diseased healthy control cells. Although these results may appear to be counterintuitive, one should consider the chronically activated state of FLS taken from RA patients, which can cause RA FLS to become unresponsive to further stimulation *in vitro*. Recent studies have suggested that previous exposure to an inflammatory environment can result in long lasting epigenetic memory resulting in altered cellular responses to activation (11). A low response of FLS isolated from the joints of RA patients could also be explained in part by heterogeneity in phenotypes and respective functions of these cells from different layers of the synovium (46, 47). For example, it has been shown in animal models that only fibroblasts from superficial layers exhibited destructive phenotype and function (47). A recent extensive report by Mizoguchi et al assessed synovial fibroblasts from RA and control OA patients using single-cell transcriptomics, flow cytometry, *in vitro* analyses and immunohistochemistry of synovium (48). Three different subpopulations of FLS in RA patients located at distinct anatomical sites and possessing different biological functions were identified. In addition, the authors demonstrated that FLS functional phenotype could not be maintained *in vitro* because of culture conditions and loss of the microenvironment (48). As we did not have information regarding collection area, stage, duration and treatment of disease of the

FLS samples, it is possible that we did not study the relevant FLS subpopulations most important for structural damage.

After demonstrating the catabolic effects of exogenously added TNF on cartilage activation and destruction, we next tested whether endogenously produced TNF elicited the same responses. To determine the cellular sources of TNF, we compared TNF production by PBMCs that were used as a model for immune cells migrating to the inflamed synovium from blood, FLS and chondrocytes activated by the alarmins S100A8 and S100A9, which belong to the DAMP family and are present in inflamed joints (40, 49, 50). The expression of S100A8 and S100A9 is closely correlated with the intensity of inflammation and macrophage activation in mouse arthritis models (51, 52). In addition, mice deficient for S100A9 have reduced joint swelling and proteoglycan depletion relative to wildtype mice, whereas intra-articular injection of S100A8 into wildtype mice induces synovitis, depletes cartilage proteoglycan levels and upregulates synovial levels of mRNAs encoding S100A8, S100A9, IL-1β and MMPs (51, 53). S100A8 and S100A9 treatment resulted in strong TNF production by PBMCs only, whereas TNF production by chondrocytes and synovial fibroblasts was low. Furthermore, S100A8 or S100A9-treated PBMCs induced PG release from bovine cartilage explants into culture supernatants. This effect was blocked by the anti-TNF antibody D2E7, suggesting that the observed catabolic effects were mediated primarily by endogenous TNF produced by activated PBMCs. Consistent with these *in vitro* findings, treatment of the hTNF-Tg mice, Tg197, with D2E7 resulted in protection from TNF-induced depletion of PG from the articular cartilage. Collectively our data provide a mechanistic explanation for the role of TNF in cartilage damage in RA. We demonstrated that activated PBMCs are a major source of TNF, whereas stromal cells are directly activated by TNF and express large

amounts of MMPs and IL-6. MMP production by chondrocytes and synovial fibroblasts triggers PG depletion from cartilage. Our findings are in agreement with the results of an elegant bone marrow transplant study in hTNF-Tg mice that demonstrated the essential role of TNF-dependent activation of the mesenchymal but not the haematopoietic compartment in the development of arthritis (54). Stromal cells produced significantly higher levels of proteases and IL-6 than blood cells, and expression persisted for days. Ineffective termination of inflammatory signalling in RA synovial fibroblasts was demonstrated previously and can be explained by increased chromatin accessibility in the promoters of inflammatory genes (55). We found that, similar to FLS, chondrocytes exhibited sustained responses to TNF. One possible explanation is that, unlike in immune cells or epithelium where homeostatic mechanisms effectively control responses to inflammatory factors, stromal cells that are normally not exposed to the environment mount an exaggerated or continuous response, leading to propagation of joint inflammation and damage in RA.

Conclusions

We demonstrate that TNF directly triggers a catabolic program in human chondrocytes leading to cartilage damage. Our data also show distinct roles for the major cell populations present in the inflamed joints in the triggering and propagation of cartilage damage and suggest that neutralization of TNF produced by PBMCs infiltrating inflamed joints decreases catabolic activity of chondrocytes and fibroblasts, which contributes to the cartilage protective effects of anti-TNF biologics in RA.

Abbreviations

TNF: tumor necrosis factor; RA: rheumatoid arthritis; PG: proteoglycan; FLS: fibroblast-like synoviocytes; PBMC: peripheral blood mononuclear cells; MMP: metalloproteinase; IgG: Immunoglobulin G; FBS: fetal bovine serum; MSD: mesoscale discovery; OD: optical density; hTNF-Tg mice: human TNF transgenic mice; IL: interleukin; LPS: lipopolysaccharide; DAMP: damage-associated molecular patterns; OCT compound: optimal cut temperature compound; ECM: extra-cellular matrix; DMARD: disease-modifying antirheumatic drug

Declarations:

Ethics approval and consent to participate

Experimental protocols were accepted by the Institutional Committee for Animal Care and Use and Experimental Code: S1.53 was given by the Veterinarian of Hellenic Pasteur Institute, fully approved status animal program by Abbott's Corporate Animal Welfare Committee (July 25, 2012). Annual license for this study: 2369/29-03-2012 issued by the Veterinary Department of the Athens Prefecture.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

All authors are employees of AbbVie and authors may own AbbVie stock. The design, study conduct, and financial support for this research were provided by

AbbVie. AbbVie participated in the interpretation of data, review, and approval of the publication.

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Authors' contributions

AY conceived, designed and performed the study and wrote the manuscript. TM carried out IHC and image analysis for the in vitro cartilage destruction assay. ZK conceived and oversaw the project and contributed to the writing of manuscript. All authors reviewed and approved the final manuscript.

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Figure Legends

Figure 1. TNF upregulates IL-6 and MMP in human chondrocytes and increases PG depletion from bovine cartilage

(A) Levels of IL-6, MMP1 and MMP3 proteins produced by human cartilage explants (upper panel, n=3) or primary chondrocytes (lower panel, n=7) isolated from healthy individuals stimulated with recombinant human TNF (10 ng/ml) for the indicated times were measured by MSD assay. Data are shown as mean ± SEM. Significant differences from untreated control collected at the same time point are depicted: *, $p<0.05$; **, $p<0.01$; ****, $p<0.0001$ (two-way ANOVA with Sidak's post-test for multiple comparisons). **(B)** PG content in bovine cartilage explants (four replicates/condition) cultured with or without 10 ng/ml of recombinant human TNF or IL-1β (positive control) for 14 days was evaluated using toluidine blue staining. The **upper panel** shows representative histological samples where PG in control untreated samples stained dark blue, and loss of blue colour represents PG depletion from the cartilage. The l**ower panel** depicts results of quantitative image analysis of the stained cartilage sections presented as a percent of untreated cartilage (100%, dotted line). Bars show the mean ± SEM of five independent experiments. * = $p<0.05$ as compared to untreated control (one-way ANOVA with Dunn's multiple comparison test).
(C) Effects of IL-1β and TNF on IL-6 and MMPs production by human isolated chondrocytes described in (A) at 48 hours of culture. Data are shown as mean ± SEM, significant differences between two cytokines are shown: * = $p<0.05$ (two-way ANOVA with Sidak's post-test for multiple comparisons).

Figure 2. Effects of co-culture with FLS and TNF blockade on TNF-mediated cartilage destruction

(A) Quantitative analysis of PG content in bovine cartilage explant monocultures (n=5) or co-cultures with FLS isolated from synovium of RA patients (n=4) or individuals without joint disease (n=5). Cultures were exposed to 10 ng/ml of TNF for 14 days or left untreated, explants were processed and stained with toluidine blue as in Fig. 1B. Results are presented as a percent of untreated cartilage (100%, dotted line). **(B)** IL-6 and MMPs production by RA

and non-diseased FLS in supernatants collected from bovine cartilage explant co-cultures as in (A) measured by MSD assay. For both (A) and (B) data are shown as mean ± SEM, significant differences from untreated controls are shown: * = p<0.05; ** = p<0.01; ***, p<0.001; ****, p<0.0001 (repeated measure two-way ANOVA with Sidak's post-test for multiple comparisons). **(C)** Effect of TNF blockade on PG depletion from the cartilage were tested on the same co-cultures of bovine cartilage explants with human FLS as in (A). FLS/cartilage co-cultures were exposed to 10 ng/ml of TNF or IL-1β for 3 days and thereafter, 5 μg/ml of D2E7 or IgG1 (negative control) were added to some of the wells. Explants were processed and analysed as described for panel (A). Results are presented as a percent of untreated cartilage (100%, dotted line). **(D)** Effect of TNF neutralization by D2E7 on IL-6 and MMPs production by FLS in the same co-culture experiments as in (C). For both (C) and (D) data are shown as mean ± SEM, significant differences from IgG1 treated cultures are depicted: * = p<0.05; ** = p<0.01; ****, p<0.0001 (repeated measure two-way ANOVA with Sidak's post-test for multiple comparisons).

Figure 3. Characterization of TNF production and TNF responses in chondrocytes, FLS and PBMCs

All experiments described in this figure were performed with primary human cells isolated from individuals without history of joint diseases. **(A)** TNF production by human FLS (n=4) and chondrocytes (n=3) cultured in the presence or absence of 10 ng/ml of recombinant human IL-1β or LPS for 48 h. **(B)** Comparison of TNF levels produced by human FLS (n=5), chondrocytes (n=9) or PBMCs (n=5) in response to 48 h treatment with 10 μg/ml of alarmins: S100A8 and S1009, 10 ng/ml of IL-1β or LPS (positive control). **(C)** IL-6 and MMP1 production by human FLS (n=6), chondrocytes (n=7) or PBMCs (n=5) in response to stimulation with 10 ng/ml of TNF. All data are shown as mean± SEM, significant difference from untreated control is depicted: * = p<0.05; ** = p<0.01 (Kruskal-Wallis test with Dunn's multiple comparison post-test).

Figure 4. TNF neutralization protects cartilage *in vitro* and *in vivo,* in hTNF-Tg mouse model of arthritis

(A) Bovine cartilage explants were co-cultured with PBMCs isolated from the whole blood of healthy individuals with or without 10 μg/ml of alarmins: S100A8, S1009 or TNF as a positive control. D2E7 or isotype control (hIgG1) were added at 5 μg/ml to some wells. After 7 days of culture, supernatants were collected, and levels of PG depleted from the cartilage into the culture media were measured. Results are presented as a fold over PG levels in untreated samples. Data are shown as mean ± SEM of 3 independent experiments. Dotted line represents untreated control. **(B)** Human PBMCs alone (left panel, n=4) or in co-culture with bovine cartilage (right panel, n=3) were stimulated with 10 μg/ml of alarmins: S100A8 and S1009 or left untreated in the presence of 5 μg/ml of D2E7 or isotype control (hIgG1), supernatants were collected and levels of TNF were measured by MSD assay. Data are shown as mean ± SEM, significant difference between D2E7 and isotype control is shown: * = p<0.05 (two-way ANOVA with Tukey's multiple comparison test). **(C)** Representative alcian blue stained sections from ankle joints of hTNF-Tg mice treated for 12 weeks with 1 mg/kg once a week of anti-TNFα antibody D2E7 (n=12) or placebo (n=6) are shown on the upper panel (magnification 20x). Staining intensity of the articular cartilage (shown by asterisk) correlates with PG content. The lower panel depicts the results of quantitative image analysis of PG content (stained with alcian blue) in the articular cartilage of talus bones (area of analysis is outlined in red). Data for individual animals are shown as a percent of total cartilage area, significant difference between groups is shown: **= p<0.01 (Mann-Whitney U test).

Figures

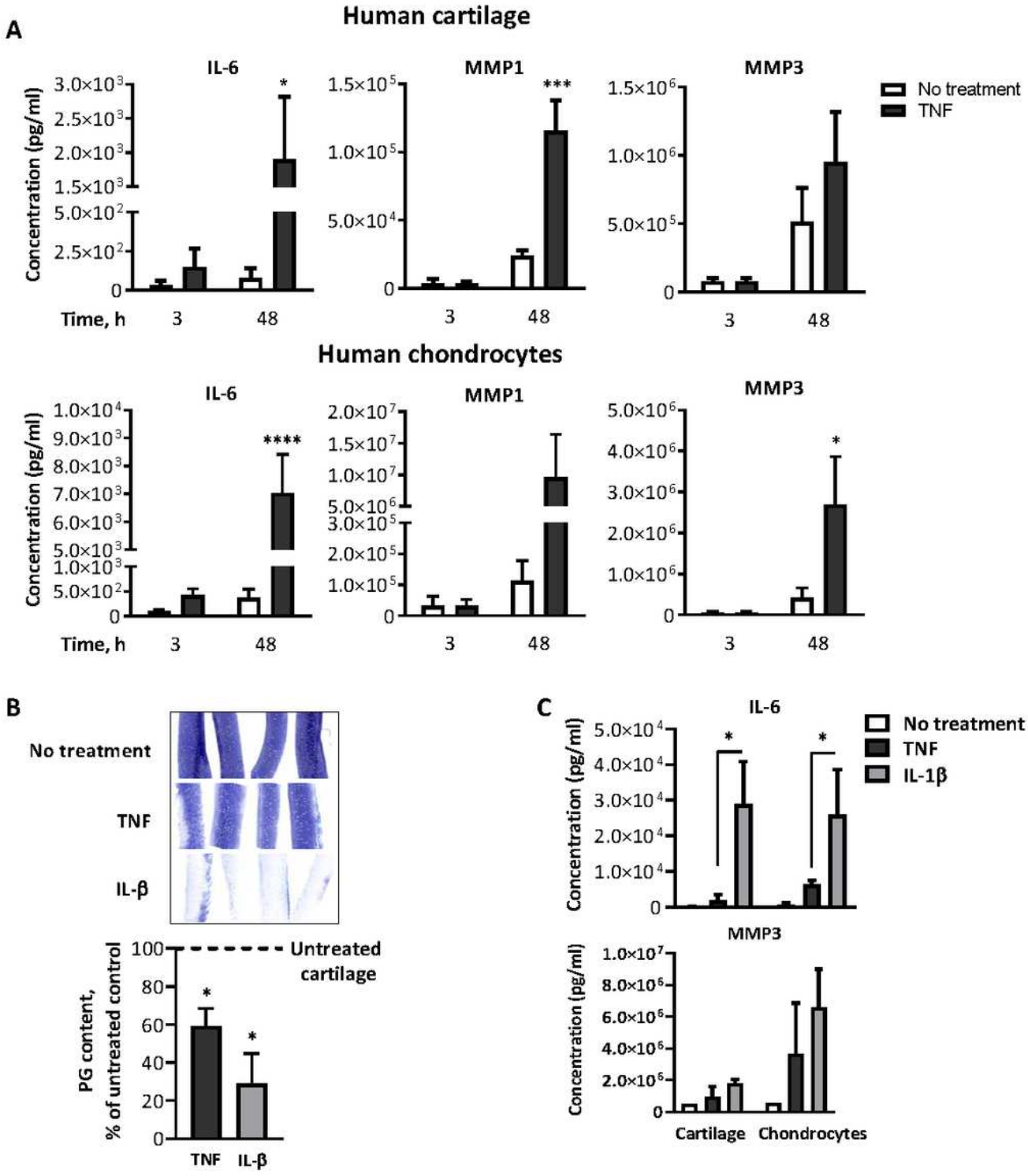

Figure 1

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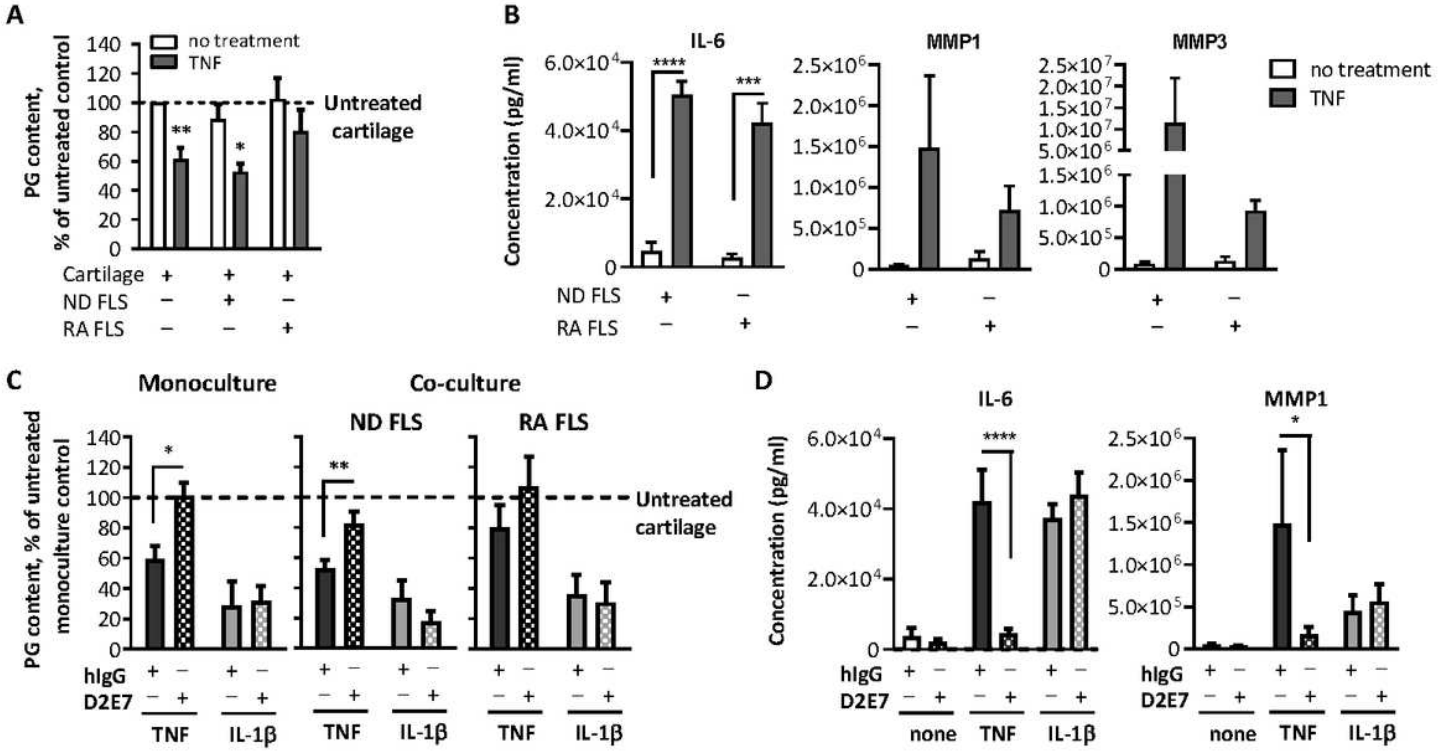

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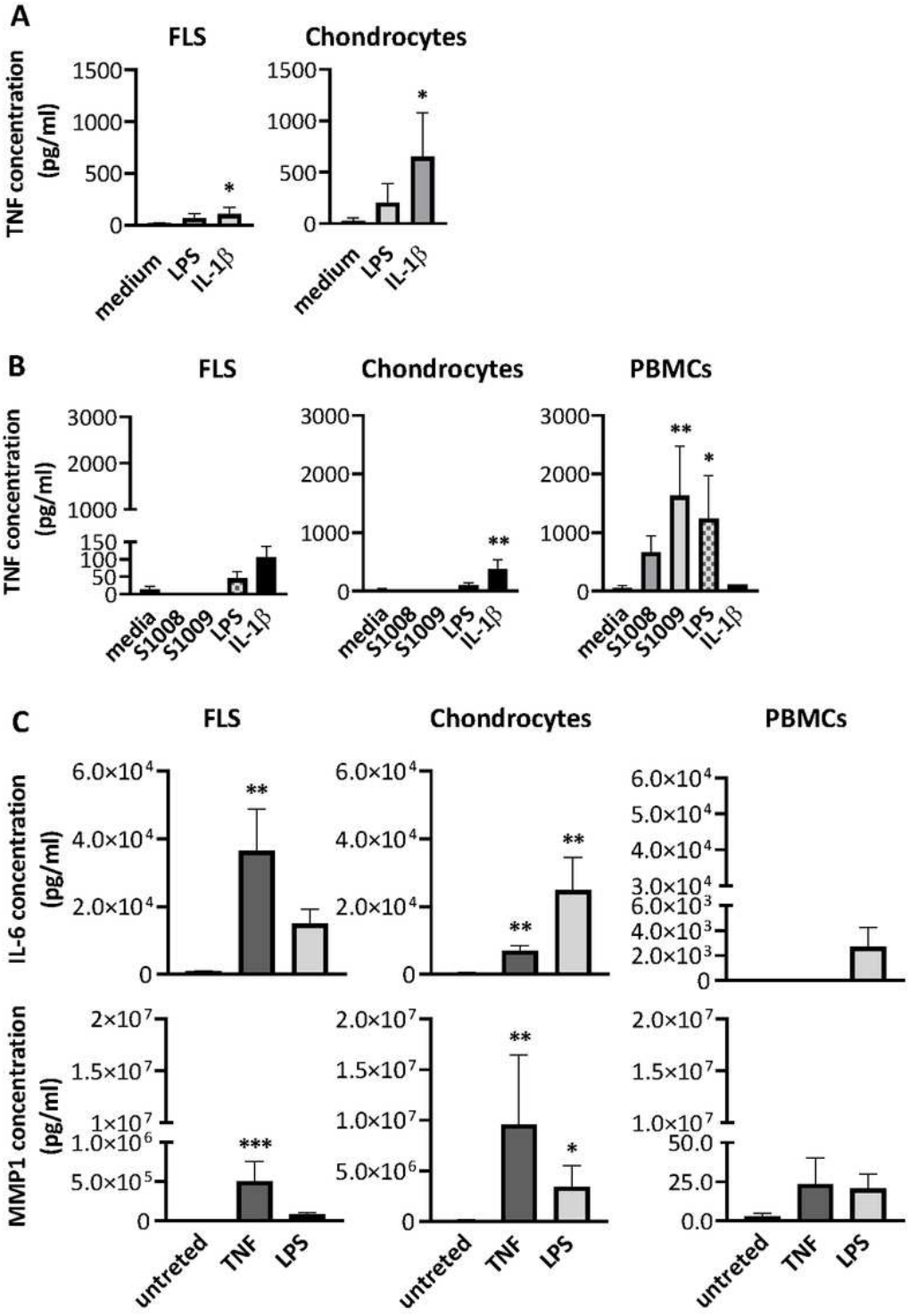

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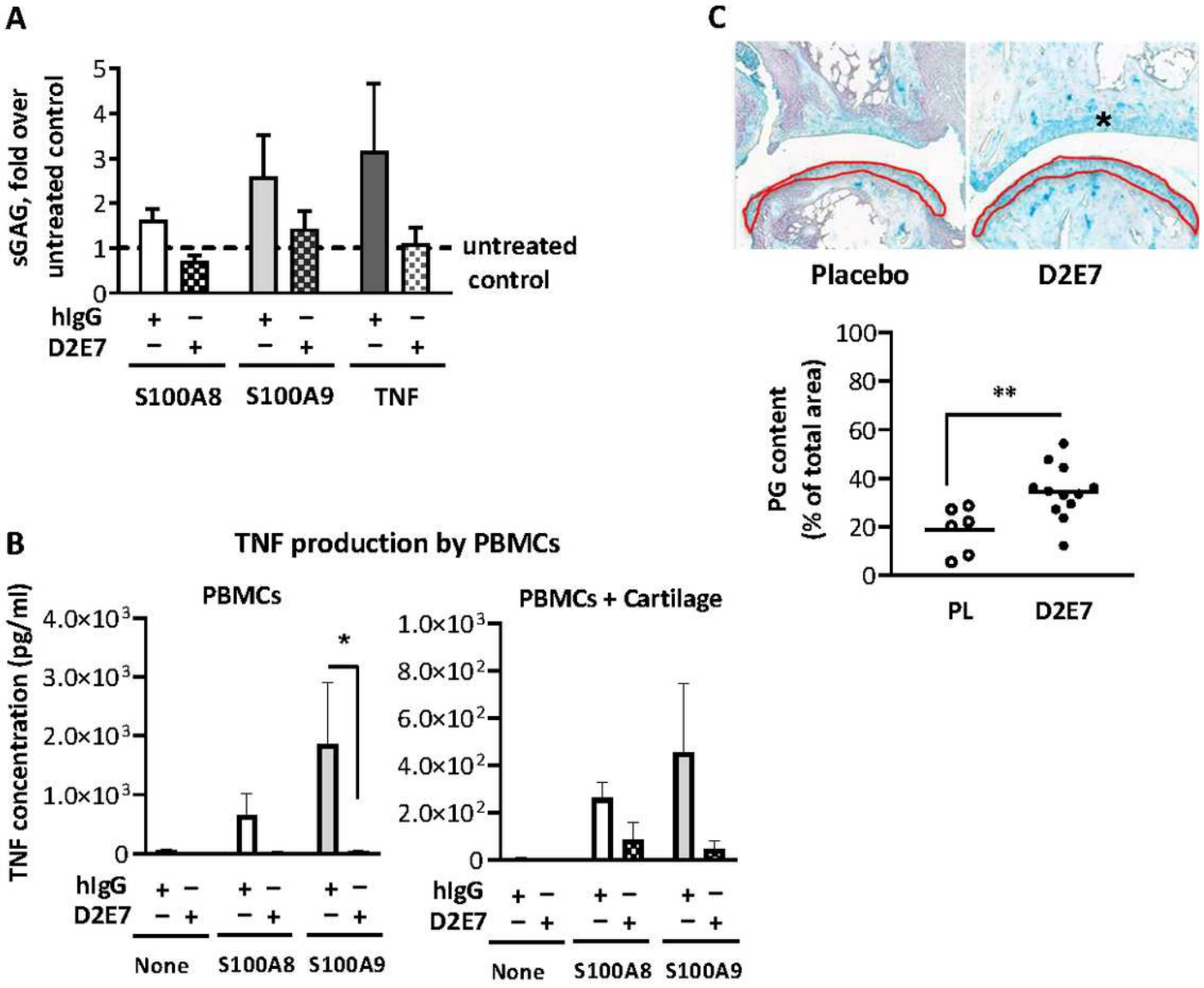

Figure 4

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