Purpose: Monocytes play a pivotal role in controlling tissue inflammation and repair. They infiltrate the tissues and develop into two main subsets of macrophages in response to local signals: inflammatory (M1-) and tissue-repair (M2-) macrophages (Mφ). Here we investigated whether M1 and M2φ differentially modulate chondrogenesis of mesenchymal stem/stromal cells (MSC).
Methods: Monocytes isolated from buffy-coats were cultured for 5 days with GM-CSF or MCSF to induce respectively M1- or M2φ-polarization. MSC were isolated from the bone marrow of a total of 9 patients and expanded for two passages. MSC or skin fibroblasts (as control cells) and polarized macrophages were cultured in collagen scaffolds up to 3 weeks alone or after being mixed (at the ratio 1:1). In order to determine the reached cell number, selected constructs were generated by combining carboxyfluorescein succinimidyl ester (CFSE) labeled MSC with macrophages. To investigate the role of soluble factors in modulating MSC differentiation, conditioned media harvested from M1φ and M2φ were supplemented during the chondrogenic culture of MSC. Resulting tissues and/or isolate cells were assessed histologically (glycosaminoglycan, GAG), biochemically (GAG contents), cytofluorimetrically (percentage of cells expressing or not CD45, and CFSE) and by RT-PCR (Sox9 and collagen-II)
Results: Coculture of MSC/M2φ in scaffolds resulted in statistically significant 1.9-fold higher GAG content than what would be expected (defined as *chondro-induction*). Chondro-induction was lower (1.3±0.4) and less reproducible when coculture was performed with M1φ and did not occur with skin fibroblasts. GAG contents of constructs generated by solely macrophages were undetectable. Histological analyses of constructs confirmed the biochemical results. In the coculture there was no modulation of the chondrogenic genes. As compared to monocultures, in co-culture MSC and M2φ numbers decreased less markedly (at day 7, MSC were 84% and 42% of the initial number, M2φ were 26% of the initial number and undetectable, respectively for co-cultured and monocultured cells).
Conclusions: We have demonstrated that coculture MSC/Mφ2 results in synergistic cartilage tissue formation, which is not mediated by soluble factors alone. Further studies are envisioned to investigate whether Mφ2 modulate the survival of specific MSC sub-populations. Finally, in vivo studies are necessary to assess the clinical relevance of our findings in the context of cartilage repair.

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POSSIBLE CHONDROPROTECTIVE EFFECT OF CANAKINUMAB: AN IN VITRO STUDY ON HUMAN OSTEOARTHRITIC CHONDROCYTES

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Purpose: Canakinumab is a human IgGκ monoclonal antibody targeting Interleukin (IL)-1β; its action involves the neutralization of IL-1β signaling, resulting in suppression of inflammation in patients with autoimmune disorders. IL-1β plays an important role also in the destruction of cartilage and it is the most important cytokine in the pathogenesis of osteoarthritis (OA). In response to IL-1β, chondrocytes secrete other proinflammatory cytokines, neutral metalloproteinases (MMPs), nitric oxide (NO); furthermore IL-1β inhibits chondrocytes proliferation and induces apoptosis. The aim of our study was to evaluate the possible *in vitro* effect of canakinumab on cultures of human OA chondrocytes cultivated in the presence or absence of tumor necrosis factor (TNF)-α.
Methods: Human articular cartilage was obtained from the femoral heads of 5 patients (range 63-71 years) with OA according to ACR criteria undergoing surgery for total hip prostheses. The chondrocytes were isolated from the articular cartilage using sequential enzymatic digestion. The primary cultures so obtained were seeded in 6-well plates until confluence. Cells were then incubated with two concentrations (1μg/ml and 10μg/ml) of canakinumab alone or in combination with TNF-α (10 ng/ml) for 48h. In these conditions we evaluated cell viability, release of proteoglycans (PG) and NO in culture medium, inducible nitric oxide synthase (iNOS) and MMP-1, 3, 13 expression, the percentage of apoptosis and necrosis. After 24h we performed IL-1β dosage (ELISA). Finally, we used a transmission electron microscope (TEM) for morphological assessment. Data are expressed as the mean ± standard deviation of triplicate values for each experiment. Statistical analysis was performed using an analysis of variance followed by Bonferroni multiple comparison tests.
Results: Canakinumab alone at the two concentrations studied hasn't cytotoxic effect, while TNF-α caused a significant decrease of the percentage of viable cell ($P < 0.001$). Both concentrations of canakinumab tested significantly restored the cell viability in TNF-α stimulated chondrocytes. As reported in Fig 1A, canakinumab alone at the two concentrations studied didn't modify significantly IL-1β levels in the culture medium, while TNF-α produced a significant increase of IL-1β. Both concentrations of canakinumab tested significantly reduced IL-1β production (Fig 1A). No significant modification of PG levels was observed in the chondrocyte cultures treated with canakinumab alone. The presence of TNF-α determined a significant decrease ($P < 0.001$) in PG levels and canakinumab significantly increased the PG levels in cells cultured with TNF-α in a dose-dependent manner. Incubation of chondrocytes with canakinumab alone didn't affect nitrite release. The presence of TNF-α induced a significant increase ($P < 0.001$) in NO production. However, after co-incubation with TNF-α and canakinumab, at different concentrations, the levels of NO decreased significantly (1μg/ml, $P < 0.01$; 10μg/ml, $P < 0.01$). The data of the NO levels were confirmed by the immunocytochemistry assay for iNOS. TNF-α stimulated chondrocytes displayed a significant increase of MMP-1, 3, 13 gene expression; a significant reduction was shown when cells were co-treated with TNF-α and canakinumab (Fig 1B). Our experiments confirmed the pro-apoptotic effect of TNF-α and demonstrated a protective effect of canakinumab at two concentrations examined. The results concerning biochemical data were further confirmed by the morphological findings obtained by TEM.
Conclusions: It is generally accepted that IL-1β and TNF-α are the pivotal cytokines involved in OA physiopathology. Hence, the neutralization of these cytokines appears to be a logical development for OA therapy. In the present study we showed, for the first time, that canakinumab counteracts the negative effect of TNF-α on OA chondrocyte cultures, probably inhibiting IL-1β signaling.

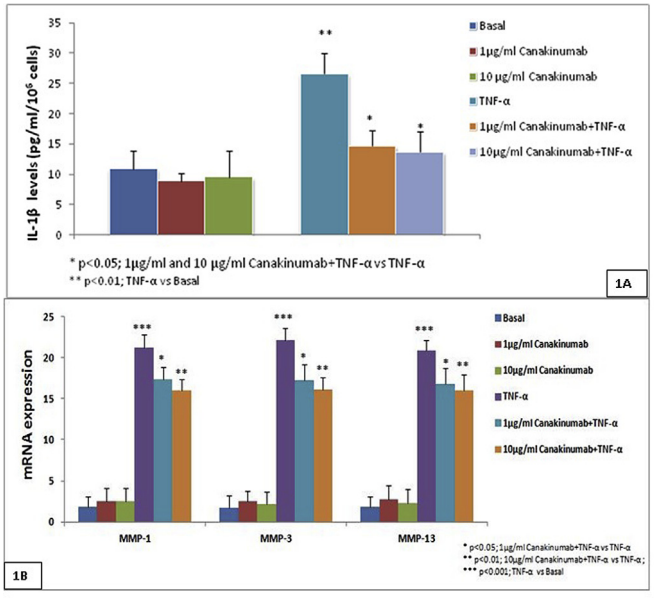

Figure 1. **A)** IL-1β levels in the culture medium; **B)**MMP-1, 3, 13 gene expression.

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BRAZILIN BLOCKS THE INDUCTION OF NFKB1/P50 IN HUMAN CHONDROCYTES AND SYNOVIOCYTES

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Purpose: Brazilin is one major constituent of Caesalpinia sappan and is known to possess anti-bacterial, anti-oxidative, and anti-inflammatory activities. Osteoarthritis (OA) creates a disequilibrium between the catabolic and anabolic activities of chondrocytes in remodeling the

cartilage extracellular matrix (ECM). Pro-inflammatory signaling contributes to the degradation of the cartilage matrix. Under OA conditions, synoviocytes produce a range of inflammatory mediators, including IL-1β. Upon binding to chondrocytes, these mediators activate specific signal transduction cascades, such as the nuclear factor-kappa-B (NF-κB) pathway, which acts as one central regulator of the catabolic processes that suppresses the synthesis of ECM components, and induces the expression of matrix metalloproteinases and enzymes of the ADAMTS family, leading to perpetuated cartilage breakdown.
This study aimed to evaluate the effects of brazilin in osteoarthritic chondrocytes and synoviocytes with particular focus on the NF-κB pathway.
Methods: Brazilin was isolated from Caesalpinia sappan extract (CSE) and identified using HPLC and NMR methods. Chondrocytes and synoviocytes were isolated from OA patients undergoing total knee replacement surgery. Cells were either left untreated for control samples, were treated with 10 ng/ml IL-1β, or were pre-treated with 10 μg/ml brazilin prior to the addition of 10 ng/ml IL-1β. Genes associated with NF-κB-mediated signal transduction were examined in primary human chondrocytes using an NF-κB signaling array and in silico pathway analysis. The induction and inhibition of NFKB1, its gene product p50, and the precursor p105 were investigated in primary human chondrocytes and synoviocytes using RT-qPCR and Western blotting.
Results: Nine NF-κB pathway-related genes (BIRC3, CCL2, CSF1, CSF3, IL1B, IL8, NFKB1, NFKB2 and TNFAIP3) were found to be significantly up-regulated by IL-1β-stimulation and significantly down-regulated after pre-incubation with brazilin. Pathway analysis revealed NFKB1 as one major gene regulating the anti-inflammatory activities of brazilin. RT-qPCR assays confirmed that the up-regulation of NFKB1 mRNA in IL-1β-stimulated primary chondrocytes and synoviocytes was significantly reduced by pre-treatment with brazilin. Western blotting showed that IL-1β treatment increased p105 protein in chondrocytes with a peak after 1 h followed by a steady decline, while increasing constantly the amount of p50 over 24 h. Brazilin suppressed the IL-1β-mediated induction of NFKB1/p50 in chondrocytes and synoviocytes.
Conclusions: The present study suggests that brazilin effectively blocks the induction of NFKB1/p50 in cytokine-stimulated primary human chondrocytes and synoviocytes, pointing towards a chondroprotective potential of brazilin, which may be beneficial for reducing cartilage breakdown in OA.

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EFFECT OF BONE MARROW SURGICAL APPROACH AND RAPIDLY DEGRADING PRESOLIDIFIED SUBCHONDRAL CHITOSAN/BLOOD IMPLANT ON RESURFACING OF CHONDRAL DEFECTS IN A SHEEP MODEL

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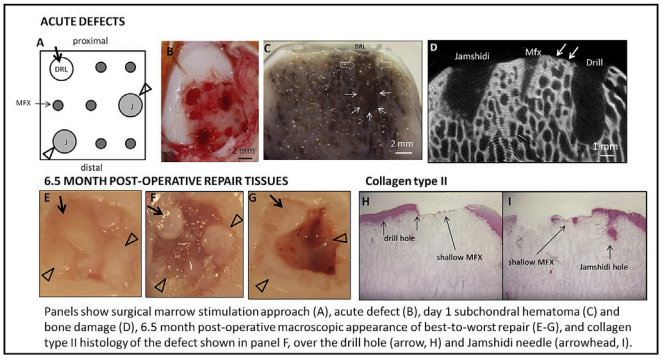
Purpose: Bone marrow stimulation for cartilage repair is partly limited by inadequate stem cell recruitment from the trabecular bone marrow to the cartilage lesion. We tested the hypothesis that the cartilage repair elicited by marrow stimulation can be improved by subchondral delivery of rapidly degrading pre-solidified chitosan/blood implants to bone defects with free communication with the trabecular bone marrow. Jamshidi needles and a drill burr were used to create 2mm diameter bone defects with cleanly removed bone to maximize cell recruitment.
Methods: Full-thickness 10x10mm cartilage defects were created in the medial femoral condyle in both knees of 11 mature sheep. Based on a pre-planned template, three 2-mm diameter bone holes were generated systematically at the corner of each defect with a Jamshidi biopsy needle and a drill burr, and the area between the holes was perforated with six smaller microfracture holes (Fig. 1A-B). 10 kDa chitosan/autologous whole blood implant was presolidified ex vivo with coagulation factor (recombinant human Factor VIIa, rhFVIIa, or Tissue Factor, TF) and inserted into each of the three 2-mm diameter holes. Contralateral control defects were treated with whole blood presolidified with rhFVIIa or TF. Day 1 (N = 1) and 6.5 month repair (N = 10) was evaluated by macroscopic scoring, and micro-CT, histomorphometry for collagen type I and II, and histological scoring (ICRS-2). Four intact age-matched condyles were micro-CT scanned for baseline bone parameters. 6.5 month cartilage repair tissues were also analyzed by biochemistry for glycosaminoglycan (GAG) and collagen (% weight per wet weight). The General Linear Model (Statistica, Statsoft, V6.2, NB, USA) was used to analyze differences due to surgical approach (drill hole vs 2 averaged Jamshidi holes per defect, N = 20, vs intact condyles N = 4) and differences due to implant condition (N = 10). $p < 0.05$ was considered significant.
Results: At day 1 post-operative, the marrow stimulation approach led to a 65% decrease in the subchondral bone volume fraction (BVF, %) compared to intact condyles, along with extensive subchondral bleeding (Fig. 1A-D). Rapidly degrading chitosan implant was retained near the surface of the bone defects at day 1, with no significant effects on bone or cartilage repair features at 6.5 months. At 6.5 months post-operative, most medial femoral condyles (but not lateral femoral condyles) developed large osteophytes. Only modest subchondral bone repair was observed in the defect area (13% increase in BVF compared to acute defects), and in 3 out of 20 defects, net bone resorption around the Jamshidi biopsy hole was observed (Fig. 1G). The larger 2mm bone holes were consistently resurfaced with collagen type II repair with higher collagen content (18% vs 15% w/w) and lower GAG (2.4% vs 4.4% w/w) than tissue outside the defect, while shallow microfracture holes were poorly resurfaced (Fig. 1F). Compared to Jamshidi biopsy holes, drill burr elicited a higher overall cartilage repair histological quality (33 ± 28 vs 49 ± 25, p = 0.041, N = 20 marrow stimulation holes, Fig. 1H-I).
Conclusions: Blood vessels sheared by Jamshidi surgical marrow stimulation can devitalize the subchondral bone plate and provoke sporadic bone necrosis. In large animal condyles, bone cleanly removed by Jamshidi biopsy or drilling is slow to repair with mineralized tissue. 2-mm diameter drill holes are resurfaced with soft repair tissue whereas shallow microfracture holes have inadequate marrow communication and are poorly resurfaced. Marrow stimulation surgical approaches need to find a balance between bone damage/blood vessel rupture and subchondral implants that create optimal inflammatory responses that elicit cell influx and osteochondral repair.



Panels show surgical marrow stimulation approach (A), acute defect (B), day 1 subchondral hematoma (C) and bone damage (D), 6.5 month post-operative macroscopic appearance of best-to-worst repair (E-G), and collagen type II histology of the defect shown in panel F, over the drill hole (arrow, H) and Jamshidi needle (arrowhead, I).

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A TISSUE ENGINEERED OSTEOCHONDRAL COMPOSITE FOR CARTILAGE REPAIR: AN IN VIVO STUDY

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Purpose: The aim of this work is to validate the efficacy of a tissue engineered osteochondral composite for the treatment of cartilage lesion produced in adult pigs. The osteochondral composite was manufactured by combining an osteo-compatible cylinder and a neo-cartilagineous tissue obtained by seeding swine articular chondrocytes into a collagen scaffold.
Methods: Articular cartilage was harvested from the trochlea of six adult pigs and chondrocytes were isolated; after the in vitro expansion, chondrocytes were seeded onto a collagen scaffold that was pre-integrated in vitro to an osteo-compatible cylinder. The seeded osteochondral scaffolds were cultured in chondrogenic medium for 3 weeks, then they were surgically implanted in osteochondral lesions performed in the trochlea of the same pigs from which the cartilage was