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FRI0063 RESVERATROL ATTENUATES SYNOVIAL HYPERPLASIA IN AN ACUTE ANTIGEN-INDUCED ARTHRITIS MODEL BY AUGMENTING AUTOPHAGY AND DECREASING ANGIOGENESIS

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Background: Previously, we have demonstrated that dietary supplementation with resveratrol lowers synovial hyperplasia, inflammatory markers and oxidative damage in an acute antigen-induced arthritis (AIA) model.
Objectives: In this work, we investigated whether resveratrol can also regulate this abnormal proliferation of synovial tissue in an acute AIA model by inducing cell death pathways and by modifying the angiogenesis in the synovial membrane.
Methods: Animals were randomly divided into 3 groups: control, AIA, and resveratrol-treated AIA group. Resveratrol (12,5 mg/kg/day) was given orally 8 weeks before AIA induction until sacrifice day (48 h after intra-articular injection). Control and AIA animals were administered 100 μl of water. Resveratrol effects on autophagy and apoptosis were evaluated by LC3 and active caspase-3 expression (confocal and immunohistochemistry, respectively). Angiopoietin 1 (Ang-1), vascular endothelial growth factor (VEGF), and the nuclear factor NF-*kappa*-B p65 subunit (p65) were also determined by immunohistochemistry and cartilage degradation with Safranin-O.
Results: Resveratrol significantly reduced the histological score of synovial tissue. Results showed a significant higher expression of LC3 signals in the AIA synovial membranes, compared with control samples, in which the presence of vesicles was easily observed. Interestingly, the synovial tissues from the resveratrol group showed a significantly ($p \leq 0.001$) higher signal for LC3, compared with the AIA samples. Active caspase-3 expression was up-regulated at the same level in the synovial membranes of AIA group than in resveratrol-treated AIA group; however, in resveratrol-treated AIA group active caspase-3 signal was mainly located in the inflammatory cells. Resveratrol consumption significantly attenuated Ang-1 signal, whereas expression of VEGF showed a non significant reduction. Resveratrol administration also mitigated, even not significantly, p65 expression that was significantly higher in the AIA animals than those from the control animals. In addition, resveratrol decreased articular cartilage degradation.
Conclusions: These data suggest that resveratrol is able to modulate synovial hyperplasia by increasing autophagic cell death and limiting angiogenic response in an acute AIA model, which could also modulate the inflammatory and destructive processes for rheumatoid arthritis.
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FRI0064 CHEMOKINE RECEPTOR 6 MODULATES ARTHRITIS IN A T CELL DEPENDENT MANNER

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Background: Rheumatoid arthritis (RA) is an inflammatory autoimmune disease, characterized by synovial infiltration of various cells. Chemokines are involved in the recruitment of different cell types into the synovial membrane. Accumulation of CCR6 expressing mononuclear cells can be found in joints of RA patients. CCR6 expression has also been reported on $CD4^+$ T cells, in particular regulatory as well as Th17 cells. In addition, a subset of regulatory T cells, namely $CD25^-Foxp3^+$ T cells, can upregulate CCR6 and RANKL and thereby can promote osteoclastogenesis.
Objectives: In this study, we investigated the role of CCR6 in the pathogenesis of arthritis using different arthritis models.
Methods: Clinical as well as histological signs of arthritis were investigated in the collagen-induced arthritis (CIA), K/BxN serum transfer arthritis and in the human tumor necrosis factor (hTNFtg) arthritis model, comparing *wt* and *CCR6-/-* mice. We analyzed the phenotype of lymph node cells by flow cytometry and cytokine concentrations in serum. Anti-collagen antibodies and cytokines were measured by enzyme-linked immunosorbent assay.
Results: The K/BxN serum transfer arthritis and hTNFtg arthritis model are known to be T cell independent. Since CCR6 is an important component of the innate immune system we compared the development of arthritis in both models. We did not detect any significant differences in clinical signs of inflammation or histological severity of arthritis between *wt* and *CCR6-/-* mice. In addition, bone volume was similar between *wt* and *CCR6-/-* mice. To investigate the role of CCR6 as part of the adaptive immune system in the development of arthritis we induced CIA in *wt* and *CCR6-/-* mice, which is known to be T cell dependent. *CCR6-/-* mice were almost completely protected from CIA. Indeed, analyses of T cell subsets by flow cytometry revealed a significant reduction of $CD25^-Foxp3^+$ T cells.
Conclusions: CCR6 is necessary for the generation of pathogenic $CD25^-Foxp3^+$ T cells in CIA, suggesting an important function of CCR6 on T cells in the development of autoimmune arthritis.
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FRI0065 HELMINTHES BASED NOVEL COMPOUND, TUFTSIN-PHOSPHORYLCHOLINE (TPC) AMELIORATES ESTABLISHED MURINE ARTHRITIS

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Background: In areas where helminthes infections are common, autoimmune diseases are rare. Treatment with helminthes or their ova, improved clinical findings of several autoimmune diseases. Based on the helminthes biological activity, a novel chimeric be-specific molecule was synthetized of tuftsin-phosphoryl-choline (PC)-TPC.
Objectives: To study TPC treatment in established collagen induced arthritis (CIA) mice, and the mechanism of activity.
Methods: Arthritis was induced in DBA male mice by immunization with collagen emulsified in TB-mycobacteria at the tail base. Boost was given 3 weeks later. Treatment with TPC started when the clinical score was 2. Cytokines were measure in culture-fluid of splenocytes in-vitro. T regulatory cells and B regulatory cells were measured by FACS. TPC effect on TLR4 expression was studied using HEKTM-mTLR4 cells system and its inhibitor. M1 shift to M2 was performed in RAW macrophages differentiated to M1 by PMA followed by LPS. TPC was added and IL-6, TNFalpha, IL-10 were tested by ELISA.
Results: Starting TPC treatment of CIA mice after disease establishment, had a significant lower arthritis score in comparison with control vehicle subjected mice (i.e. TPC-6.8±0.8 vs vehicle-13.8±0.45; $p<0.0001$). Joints staining revealed normal joint structure in TPC treated mice, whereas, control mice treated with PBS, PC or tuftsin had severe inflamed joints. Likewise, TPC enhanced anti-inflammatory response by enhanced IL-10 secretion, reduced pro-inflammatory cytokines secretion (IL-1β, IL-17, IL-6, and TNF-α) ($p<0.001$). Furthermore, TPC induced expansion of splenic CD4+CD25+FOXP3+ T regulatory cells (Tregs) and IL-10+CD5+CD1d+ B regulatory cells (Bregs). The mechanism underling the TPC related immunomodulatory activity was attributed to its bi-specific activity: a) Shift of Raw cells macrophages from pro-inflammatory macrophages M1 to anti-inflammatory M2 secreting anti- IL-10 ($p<0.001$), through the tuftsin part of TPC. b) TPC inhibited significantly TLR4 expression by HEKTM-mTLR4 cells ($p<0.02$) *via* the phosphorylcholine end. Our data indicated that TPC significantly ameliorated established CIA by anti-inflammatory immunomodulatory activity.
Conclusions: Our data may lead to a novel bi-specific self small molecule for therapy of patients with advanced RA.
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FRI0066 GASTRIN-RELEASING PEPTIDE AND ITS RECEPTOR INCREASE ARTHRITIS FIBROBLAST-LIKE SYNOVIOCYTE INVASIVENESS OVER THE PI3K/AKT PATHWAY

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Background: Rheumatoid arthritis (RA) is an autoimmune disease where the chronic inflammation and subsequent cartilage and bone erosion lead to joint destruction. The fibroblast-like synoviocytes (FLS) have a central role in disease pathogenesis and *in vitro* FLS invasiveness correlates with articular damage in RA patients. Gastrin-releasing peptide (GRP) plays an important role in the immune and inflammatory response. GRP is found in synovial fluid of RA patients and its receptor (GRPR) is found in synovial membrane of murine arthritis. RC-3095 in an antagonist of GRPR.
Objectives: To evaluate the role of GRP and GRPR on invasive behavior of mice FLS and to evaluate the GRP-induced signaling on PI3K/AKT pathway.
Methods: FLS were isolated from joints of DBA/1J mice with collagen-induced arthritis. Expression of GRPR in FLS was investigated by immunocytochemistry and western blot (WB). Proliferation of FLS treated with GRP (0.1 μM – 10 μM)

(n=3) and RC-3095 (0.05 μM – 10 μM) (n=4) was assessed by Sulforhodamine B assay in 24h, 48h and 72h. Invasion assay with FLS was performed using a Matrigel-coated transwell system over 24h in two different experimental arms: first, FLS were treated with GRP (10 μM), RC-3095 (1 μM) or GRP+RC-3095 (GRP 10 μM and RC-3095 1 μM) (n=6) and after, FLS were treated with GRP (10 μM), Ly294002 (10 μM), or GRP+Ly294002 (GRP 10 μM and Ly294002 10 μM) (n=4). Akt phosphorylation was assessed by WB.

Results: GRPR protein was detected in FLS both by immunocytochemistry and WB. GRP and RC-3095 treatments did not affect FLS proliferation. Exposure to GRP increased FLS invasion (5356±1027) by nearly two-fold compared with untreated cells (2845±532) ($p<0.02$), while the treatment with RC-3095 reduced FLS invasion (1723±271) compared with untreated cells. Treatment with GRP+RC-3095 (2670±499) reversed the invasiveness effect of GRP ($p<0.0001$). GRP also increased phosphorylated AKT expression in FLS by 30% ($p<0.001$). When Ly294002 was added with GRP (7772±1200), it prevented the GRP-induced increased cell invasiveness (11036±953.6) ($p<0.001$) (Figure 1).

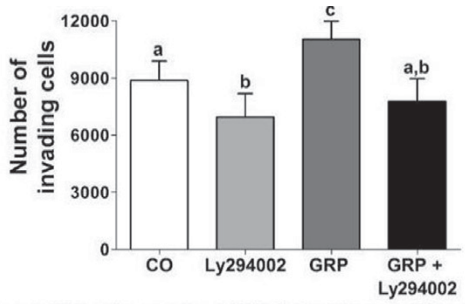

Figure 1. FLS invasion assay (n = 4). GRP significantly increased FLS invasion compared with untreated cells, whereas Ly294002 present opposite effect. Bars represent mean ± SEM. Data were analyzed by generalized estimating equation (GEE) followed by Bonferroni. a) p<0.008 versus Ly294002; p<0.026 versus GRP; b) p<0.001 versus GRP; c) p<0.001 versus GRP+Ly294002.

Conclusions: Our group demonstrated for the first time GRPR expression in FLS and that GRP are able to activate FLS invasion through AKT pathway. Finally, our results suggest that GRP/GRPR pathway could be relevant in the development of FLS-targeted therapy for RA.

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FRI0067 MICRORNA PROFILING OF MTX-TREATED FIBROBLAST-LIKE SYNOVIAL CELLS IN RHEUMATOID ARTHRITIS REVEALED A POSSIBILITY OF MICRORNA-887-3P AS NOVEL THERAPEUTIC TARGET OF RA

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Background: The hallmarks of rheumatoid arthritis (RA) is the expansive of fibroblast-like synovial cells (FLS) in affected joints, causing joint destruction. FLSs are resistant to programmed cell death resulting in aggressive, invasive phenotype like a cancer invades and the hyperplastic synovial tissue destroys cartilage and bone. Therefore inhibition of FLS proliferation and is one of the therapeutic targets of RA. MicroRNAs (miRNAs), a group of small non-coding RNAs, have been shown to regulate cell differentiation through regulation of gene expression post-transcriptionally. Recently, several studies reported anti-cancer drugs modulate miRNA expression and that have been considered as one of important mechanism of cellular action to drug[1,2].

Objectives: To investigate the changes in microRNA expression profiles in response to MTX.

Methods: RA-FLS was treated with MTX with 1nM 48 hours, that could inhibit IL-6 production from RA-FLS. To investigate differentially expressed miRNAs in response to MTX, we performed miRNA array analysis. Expression of miR-887–3p in response to MTX of RA-FLS was analyzed by quantitative real-time PCR. To investigate the functional role of miR-887–3p, RA-FLS was transfected with synthetic precursor miRNA (pre-miR)/inhibitors of miRNA (anti-miR) of miR-887–3p using Lipofectamine and then gene expression microarray was performed. The cytokine/ chemokine production was screened by Multiplex cytokine/chemokine bead assays and confirmed by ELISA. Finally, we analysed migratory activities of RA-FLS by scratch assay.

Results: After 48 h of treatment with MTX, 7 miRNAs were up-regulated and 6 miRNAs were down-regulated as compared with that of untreated control. Among them quantitative real-time PCR with additional samples confirmed that miR-887–3p was up-regulated in response to MTX treatment of RA-FLS (1.79±0.46 -fold, $p<0.05$, n=7). To elucidate the functional consequence of the deregulation of miR-887–3p of RA-FLS, we performed gain-and-loss of function assays with miR-887–3p. Microarray analysis with gene ontology analysis revealed several genes correlated with cell signalling was modulated by miR-887–3p. Multiplex bead assay showed that overexpression of miR-887–3p decreased cytokine/chemokine production of RA-FLS such as TNF-a, GM-CSF, CCL4. Among these candidates, the secretion of GM-CSF was consistently and strongly decreased from RA-FLS transfected with pre-miR-887–3p. Furthermore overexpression of miR-887–3p reduced migratory activity of RA-FLS in scratch assay.

Conclusions: Our result showed that MTX altered micro RNA expression profiles in RA-FLS. MiR-887–3p might be downstream effector of MTX in suppression of its cytokine production and invasive phenotype. This knowledge may also be useful for the development of novel therapeutic strategies for RA based on other treatments able to boost the cellular reservoir of miR-877–3p.

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FRI0068 REDUCTION OF PROLIFERATION, MIGRATION AND INVASION OF RHEUMATOID SYNOVIOCYTES BY ALL-TRANS RETINOIC ACID

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Background: Fibroblast-like synoviocytes (FLS) are pivotal in inflammation and joint damage of rheumatoid arthritis (RA). These cells proliferate, become resistant to apoptosis, migrate and invade, contributing to perpetuate synovial inflammation and destruction of cartilage and bone. Current treatments of RA are focused against inflammatory factors and immune cells, however, a significant percentage of patients do not successfully respond. Combined treatments with drugs that control inflammation and with others that reverse the pathogenic phenotype of FLS could improve the prognosis of these patients. An unexplored area includes vitamin A and its metabolites. These compounds modulate differentiation, development, apoptosis and proliferation. Indeed, retinoids are being successfully used in the treatment of several cancers for their anti-proliferative and pro-differentiative actions. In addition, several studies have shown a notable reduction of cell migration and invasion in different cell types after treatment with all-trans retinoic acid (ATRA). However, it is not known if ATRA could modify the migratory and invasive ability of rheumatoid synoviocytes.

Objectives: To analyse the effect of treatment with the retinoid all-trans retinoic acid in proliferation, migration and invasion of rheumatoid synoviocytes.

Methods: FLS were obtained from 8 RA patients. Cellular proliferation was determined using the CellTiter-Glo luminescent viability assay (Promega). Migration was analysed by wound healing assay, using Ibidi inserts. Percentage of migrating cells was determined by Image J. Invasion was tested by the Boyden chamber method using inserts (Millicell) coated with Matrigel (BD Biosciences). Invasion was determined by quantification of Giemsa stained cells on the bottom side of the inserts under the microscope. Proteomic analysis was performed using LC-MALDI-TOF/TOF and 1-DE and 2-DE gels. MS and MS/MS data was searched against the UniProt/Swiss-Prot database of protein sequences.

Results: We analysed the effect of ATRA in the spontaneous proliferation of FLS from 8 RA patients. ATRA treatment significantly reduced proliferation at 48, 72 and 96 h (p=0,005; p=0,002; p=0,04; respectively). Next, we analysed the effect of ATRA on RA FLS migration and invasion. Migration of RA FLS treated with ATRA for 96 h was reduced by 46% when compared with cells treated with vehicle control (p=0,002). In addition, RA FLS invasion was also impaired after ATRA treatment, as the invaded area was 31% lower than in controls (p=0,018). To elucidate the mechanisms underlying the effects of ATRA on RA FLS a proteomic analysis was performed. We compared the proteome of FLS from 5 RA patients treated with ATRA or with vehicle using LC-MALDI analysis. The differentially identified proteins in ATRA-treated FLS *vs* control FLS were Septin, Rho GTPase activating protein (ARHGAP1) and actin related protein 2 (ARP2). Validation experiments using 1-DE and 2-DE gels were performed.

Conclusions: Overall these results reveal that retinoid treatment reduces the proliferative, migratory and invasive capacity of RA FLS, indicating that this treatment could decrease joint damage and loss of function in RA patients.

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