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European Journal of Immunology

Running title: Th17 and non-classic Th1 in cartilage degradation

Th17 lymphocyte-dependent degradation of joint cartilage by synovial fibroblasts in a humanized mouse model of arthritis and reversal by secukinumab

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Keywords: Juvenile idiopathic arthritis, cartilage degradation, T helper lymphocytes, synovial fibroblasts.

LIST OF ABBREVIATIONS

SFbs: synovial fibroblasts; Th: T helper lymphocytes; cl: classic; ncl: non-classic; sec: secukinumab; CM: conditioned medium; RA: rheumatoid arthritis; JIA: juvenile idiopathic arthritis; MMP: matrix metallo-proteinases; TIMP: tissue inhibitor of metallo-proteinases; uPA: urokinase plasminogen activator; uPAR: urokinase plasminogen activator receptor; PAI-1: plasminogen activator inhibitor; SCID: Severe Combined Immunodeficiency; TNF: tumor necrosis factor; IFN: interferon; IL: interleukin; FITC: fluorescein isothiocyanate; RT PCR: real time PCR.

ABSTRACT

Received: 26/05/2020

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/eji.202048773.

How T-helper (Th) lymphocyte subpopulations identified in synovial fluid from patients with juvenile idiopathic arthritis (JIA) (Th17, classic Th1, or non-classic Th1) drive joint damage is of great interest for the possible use of biological drugs that inhibit the specific cytokines. Our objective was to clarify the role of such Th subpopulations in the pathogenesis of articular cartilage destruction by synovial fibroblasts (SFbs), and the effect of Th17 blockage in an animal model. SFbs were isolated from healthy subjects and patients with JIA, and peripheral blood Th lymphocytes subsets were obtained from healthy subjects. Fragments of human cartilage from healthy subjects in a collagen matrix containing JIA or normal SFbs grafted under-skin in SCID mice were used to measure cartilage degradation under the effects of Th-supernatants. JIA SFbs over-express MMP9 and MMP2 and Th17 induce both MMPs in normal SFbs, while non-classic Th1 up-regulate uPA activity. *In vitro* invasive phenotype of normal SFbs is stimulated with conditioned medium of Th17 and non-classic-Th1. In the *in vivo* "inverse wrap" model, normal SFbs stimulated with supernatants of Th17-lymphocytes and non-classic Th1 produced a cartilage invasion and degradation similar to JIA SFbs. Secukinumab inhibits the cartilage damage triggered by factors produced by Th17.

INTRODUCTION

In Rheumatoid Arthritis (RA) and Juvenile Idiopathic Arthritis (JIA) the synovial lining of the joints undergoes villous hypertrophy and hyperplasia, resulting in pannus formation, a tumor-like spread of inflamed synovial tissue accounting for joint cartilage degradation (**1-3**). Granulocytes, monocytes/macrophages and lymphocyte subpopulations trigger or maintain inflammation also upon activation of resident cells [endothelial cell, synovial fibroblasts (SFbs), osteoclasts] (**1, 4**). In RA and JIA, which is the most frequent chronic rheumatic disease of childhood, different subsets of CD4+T cells play a pivotal role (**5**).

Immunological functions provide the main criterion to classify subpopulations of effector CD4+ T-lymphocytes. Th1 cells protect the host from intracellular pathogens (**6**), Th2 cells are involved in protection from helminths (**7, 8**) and Th17 cells defend from extracellular bacterial and fungal infections. Th17 express the CD161 antigen and produce IL-17A (**6**). In several autoimmune and inflammatory diseases (including JIA and RA), Th-lymphocytes trigger the pathogenic chain leading to disease manifestations (**9**). The synovial fluid (SF) of JIA is rich inTh17 cells and more than half of such IL-17 producing cells were found within the interferon-gamma (IFNγ)-producing subpopulation (**10**). We observed an enrichment of CD4+ CD161+ T-cells in the SF of involved joints of children with oligoarticular-JIA, compared with their peripheral blood (**11**). Such Th lymphocytes included the Th17 cells and also the "non-classic Th1" cells, so called since they produce IFN-γ rather than Th17, but express CD161, IL-23R and RORC2. We have also shown that Th17 present in the SF of JIA shift to non-classic Th1 under stimulation of IL-12, an inflammatory cytokine highly produced in the affected joints (**11, 12**). Therefore, although the role of lymphocyte subsets in the pathogenesis of chronic joint inflammation is still a matter of debate, it is likely that CD4+ T cells may control joint inflammation through the activity of the cytokines produced on resident articular cells, including SFbs. Many studies on JIA and RA have shown that SFbs may degrade cartilage by several classes of matrix-destroying enzymes (**1-3, 13, 14**). In the present work we have studied the role of different Th subsets in SFbs-induced cartilage degradation.

RESULTS

In vivo cartilage degradation by JIA SFbs in the SCID Mouse model

Cartilage degradation was studied in SCID mice through the "inverse wrap" implantation technique. Figure 1 shows the results of human cartilage degradation by normal SFbs and JIA SFbs 60 days after implantation. While normal SFbs produced a weak cartilage infiltration associated with a similarly weak infiltration of CD45+ inflammatory cells (Figure 1 panel A and Figure 1 Panel B, upper part), a consistent cartilage destruction was observed only in the presence of JIA SFbs, matched with an intense infiltration of CD45+ cells (Figure 1 panel A, and panel B, lower part). These data are in agreement with the observations that, in JIA, SFbs infiltrate and destroy joint cartilage (**1**).

Analysis of protease systems and invasive phenotype of JIA versus normal SFbs

The urokinase-plasminogen activator (uPA) and matrix-metallo-proteinases (MMPs) are the main enzymes involved in invasion and degradation of articular cartilage in rheumatic diseases (**1-3**). To characterize the protease pattern of JIA SFbs, we analyzed the cell-associated fibrinolytic system (uPA, uPAR) and collagenases (MMP2, MMP9) expression in JIA SFbs compared to normal SFbs. Figure 2, panel A shows the results of Real-Time PCR of uPA, uPAR, MMP2 and MMP9 in normal and JIA SFbs. MMP2 and MMP9 were significantly up-regulated in JIA SFbs with respect to normal SFbs, accounting for an increased cell surface-associated fibrinolysis and pro-MMPs activation. Among all proteases, MMP9 was strongly over-expressed in JIA SFbs (more than 250 folds relative expression with respect to normal SFbs). To detect the MMP-activity, we have performed gelatin-zymography with 48h-conditioned media (CMs) from healthy SFbs and JIA SFbs. As shown in Figure 2 panel B, only MMP9 activity was significantly increased in the JIA SFbs CM

compared to control SFbs. Similar results were obtained in the collagen degradation assay *in vitro* in the presence of normal SFbs and JIA SFbs co-polymerized with Matrigel-2% FITC Collagen (Figure 2, panel C). To show a correlation between MMPs activity and invasion properties of JIA SFbs, we have performed Matrigel invasion assay using Boyden chambers. JIA SFbs have shown a higher invasiveness compared to normal SFbs, that was inhibited by Ilomastat, a matrix metalloproteinase inhibitor, incorporated within the polymerized Matrigel (Figure 2 panel D). Taken together, these data suggest that MMP9 activity is higher in JIA SFbs than in normal SFbs, accounting for an increase of cell invasion and collagen-degradation properties.

Effects of CD4+ T-cell clones conditioned media (CMs) and cytokines on protease systems and invasion of normal SFbs

We have analyzed the effects of CD4+ T-cell clones CMs on matrix-degrading enzymes of normal SFbs. We have previously shown that CMs of CD4+ T-cell clones do not affect proliferation and viability of SFbs (**15**).

The individual protease activities depend on the balance between enzymes and their specific inhibitors and can be assessed through specific tests. Therefore, we used the densitometric quantification of the zymograms for MMP2 and MMP9 and a colorimetric test for uPA (Figure 3 panel A and B). It is evident that activated Th17 lymphocyte supernatant cause a significant increase in the activity of MMP1 and MMP9 in normal SFbs, while activated non-classical Th1 CMs induce the expression of a strong uPA activity.

Accordingly, in an additional assay based on the Boyden-chamber Matrigel migration, we observed an increase of invasion of normal SFbs treated with CMs from stimulated Th17 T

cell clones. Interestingly, also CMs from stimulated non classic-Th1 clones had the same effect (Figure 3 panel C). When the effects of single cytokines were investigated, we found that IL-17A induced in normal SFbs an up-regulation of uPA and uPAR, as well as of MMP2 and MMP9, while the TNF-α/IFN-γ combined treatment over-expressed uPAR and lead to a down-regulation of uPA and MMP2 (Supplemental Figure 1A). No significant up-regulation was observed in MMP9 levels. We have also analyzed the effect of IL-17A and TNF-α/IFN-γ treatment on MMPs activity and SFbs invasion. By gelatin zymography we found an increase of MMP9 activity in the supernatants of SFbs after treatment with IL-17A (Supplemental Figure 1, panel B). Again, we have observed that IL-17 significantly increases invasion of SFbs, compared to minor increase stimulated by TNF-α/IFN-γ combined treatment (Supplemental Figure 1, panel C). The reduction of invasion in the presence of Ilomastat, a broad spectrum non-pecific MMPs inhibithor, demonstrated that the Th17-induced SFbs predisposition to invasiveness is MMP-dependent.

Taken together, these data show that the treatment with stimulated Th17 and non-classic-Th1 CMs, induces an activated phenotype of normal SFbs, modulating matrix-destroying enzyme production and cell invasion, similarly to JIA SFbs.

Effects of CD4+ T cell clones conditioned supernatants on cartilage degradation *in vivo*.

To translate our observations *in vivo,* a synthetic gelatin sponge containing a piece of linearly cut cartilage (about 3.5 mm^3) was soaked with healthy subject-derived SFbs suspended in conditioned media (CMs) of unstimulated or stimulated T cell clones (classic-Th1, non-classic-Th1, Th17) and inserted under the skin of anesthetized SCID mice (Charles River). Weekly peri-engraftment injections of 50µl of the same CMs were performed over a period

of 60 days. Animals were then sacrificed and the implants were recovered. We have observed a consistent degradation of cartilage in the presence of stimulated Th17 and non-classic-Th1 CM compared to CM from unstimulated cells and to normal control (untreated SFbs) (Figure 4, panel A). We have also analyzed the infiltration of inflammatory cells by CD45 staining (Figure 4, panel B). Under the effect of classic and non-classic-Th1 CM we have observed infiltration of CD45+ cells, while in presence of stimulated-Th17 CM we have observed clusters of SFbs deeply penetrating into the cartilage, associated with the presence of very few CD45+ cells. These evidences are in agreement with our previous observations that culture supernatants of both classic and non-classic-Th1, but not of Th17 clones, induce CD106 (VCAM-1) up-regulation in SFbs. This effect, mediated by tumor necrosis factor (TNF)-α, was crucial for the retention of circulating leukocytes in inflamed joints (**15**). Therefore, Th17 clones, unlike Th1, directly induce SFbs activation enhancing their invasive and destructive capacity.

***In vitro* and *in vivo* activity of secukinumab**

Finally, we wanted to better define whether the effects on SFbs induced by Th17 CM, were really mediated by IL-17A. To this aim we used an anti-IL17A mAb (secukinumab) on *in vitro* and *in vivo* experiments. First of all, we verified that secukinumab does not interfere with the proliferation of SFbs (Figure 5, panel A). Then, we assessed if secukinumab was able to produce its effects in our *in vitro* system. Since IL-17A has been reported to activate RA SFbs and to induce release of IL-8 (**16**), we measured its expression by normal SFbs incubated with the CM of Th17 lymphocytes, in the absence and presence of secukinumab (Figure 5, panel B). Only activated Th17 (Th17+) induced a strong expression of IL-8 which

was specifically blocked in the presence of secukinumab, thus indicating the efficiency of the used antibody.

Then we performed invasion experiments and demonstrated that secukinumab efficiently reduced the invasion of Matrigel-coated filters induced by activated Th17 (Th+) (Figure 5, panel C). The same panel shows that the CM of activated non-classic Th1 (Th1 ncl +) stimulates the invasiveness of the SFbs, which however is not reversed by to the antibody. Secukinumab inhibited MMP9 over-production in normal SFbs induced by activated Th17 (Figure 5, panel D). Based on these findings we carried out an *in vivo* experiment, according to the procedure used in Figure 4, but using only activated and non-activated Th17 lymphocytes, in the presence and absence of secukinumab (Figure 6) showing that the antibody, administered once a week for two months together with the CMs, abolishes SFbs-dependent cartilage degradation stimulated by Th17+ CM.

DISCUSSION

We have evaluated the role of classic and non-classic-Th1 and Th17 lymphocytes in cartilage degradation. In the "inverse wrap" model, SFbs from oligoarticular-JIA patients produced a remarkable cartilage degradation (from 12% to 23%). H&E histology and IHC for CD45 expression indicated that the cartilage infiltration clusters had a mixed composition, as composed of SFbs and leukocytes, suggesting that cartilage degradation is supported by the proteolytic activity of SFbs and of leukocyte infiltrates. *In vitro* evidences indicated

overexpression of MMP9 and of uPAR in JIA SFbs compared to those of healthy subjects, accompanied by higher collagenolysis and matrigel degradation in invasion assays. Therefore, we studied whether the different cytokine mixtures produced by CD4+ subpopulations could induce in SFbs of healthy subjects a phenotype similar to that of JIA SFbs. Following incubation of healthy subjects SFbs with "control" and "stimulated" media of classic and non-classic-Th1 and of Th17, we have studied the resulting protease features. We found that Th17 lymphocytes produced the most important variations in target SFbs: a strong over-expression of MMP9 and a lower increase of MMP2, while activated non classic Th1 lymphocytes up-regulated uPA activity. The cell associated uPAR/uPA system and secreted MMPs are the most important proteolytic systems involved in joint pathologies (**17**). We have previously shown that SFbs of RA patients express all the components of the fibrinolytic system, exhibiting the typical pattern of invasive tumor-like cells (**3**). A previous study (**18**) has described an over-release of soluble uPAR into the blood of RA patients, reaching values that reflect the erosive activity in RA. By the "inverse wrap" technology, we have obtained the reduction of cartilage degradation by systemic delivery of uPAR-antisense oligonucleotides, which resulted into the loss of function of RA SFbs-associated fibrinolytic system (**14**). Another study has demonstrated that antibodies against uPA reduce disease progression in mouse arthritis models (**19**).

The proteolytic efficacy of MMPs has been described in many pathological processes, such as tumor infiltration and metastasis, osteoarthritis (OA), and RA (**20, 21**). Many MMPs are expressed in chondrocytes and SFbs. Some of them are implicated in the regulation of cartilage turnover and plasticity under normal and pathological conditions (OA) (**22**). The collagenases are produced by SFbs (MMP1) and chondrocytes (MMP13), while gelatinases

(MMP2 and MMP9) and stromelysins (MMP3) are prevalently produced by SFbs (**22**). All the aforementioned proteases have been shown to be increased in the joints of OA and RA.

While the CM of activated non classic Th1 induced an over-activity of uPA in normal SFbs (Figure 3), the CM of activated Th17 (Figure 3), as well as IL-17A alone (Supplemental Figure 1) up-regulated *in vitro* the expression and activity of MMP9 and MMP2 and stimulated SFbs invasion that was inhibited by Ilomastat. We have therefore used the "inverse wrap" model in nude mice to verify the effect on cartilage degradation of SFbs incorporated in the collagen sponge around the joint cartilage specimens. We have previously shown that culture supernatants of both classic and non-classic-Th1, but not of Th17 clones, induced CD106 (VCAM-1) up-regulation on SFbs in a TNF-α-dependent manner. Such a feature determines retention of circulating leukocytes in the synovial lining of inflamed joints as it probably happens in RA and JIA patients, whose SFbs express higher levels of CD106 than those from healthy donors (**15**). All such premises suggested a predictable scenario of what we could expect from *in vivo* experiments: a cartilage infiltration of a mixed population of SFbs and leukocytes with conditioned mediums of classic and non-classic-Th1 lymphocytes, and an infiltration of an almost pure population of SFbs with conditioned medium of Th17 lymphocytes. We have experimentally proved such expectations. These results, while confirming the crucial role of TNF-α/CD106 (VCAM-1) axis in leukocyte recruitment and cartilage degradation, suggest a differential production of serine-proteases (uPA) and MMPs (non-classic-Th1 for uPA and Th17 for MMPs) and identify a pathogenic sequence that we have tried to control by the use of secukinumab. Indeed, the anti-IL17A antibody has proven to be very effective in preventing cartilage degradation induced by activated Th17 CMs. Actually, several data indicate that in the

natural history of JIA pure Th17 become pathogenic during their passage the Th17/Th1 and to the non-classic Th1 phenotype (**10, 11, 23**) and therefore in consideration of the powerful destructive potential of IL17A-dependent MMP9/MMP2 production by SFbs, secukinumab treatment could play a crucial role at the very beginning or in the early stages of the disease. Moreover, our results define a key role also of activated non-classic Th1 cells in mediating cartilage degradation and SFbs invasion, independent of IL-17A, suggesting that additional cytokines produced by this cell subset could induce similar effects on SFbs. Further, the identification of immune cell subpopulations that induce protease over-production, pave the way for the use of biological therapeutics capable of blocking protease activity such as MMP9.

MATERIALS AND METHODS

Patients and samples

SFbs were derived as previously described (**14**, **15**) from 8 oligoarticular-JIA patients (age mean: 9 years; range: 6–15 years; 4 of them were treated with NSAIDs and 3 with methotrexate) and 6 healthy controls (age mean: 8 years; range: 5–10 years), after informed written consent and with the approval of Ethics Committee of Anna Meyer Children Hospital, Florence, Italy. Synovial fluid samples were collected during therapeutic arthrocentesis from the knee joint aspirates, while normal synovial tissue was obtained from the knee joints of six healthy subjects undergoing orthopaedic surgery for knee traumatic events. Donors did not suffer from any chronic inflammatory diseases and did not take any drug, therefore they were called healthy and their SFbs were called “normal” or “control”. Normal human cartilage was obtained from the non-arthritic knee joints of patients undergoing routine surgery at the

Department of Orthopedics (Azienda Ospedaliero-Universitaria Careggi, Florence, Italy). For patients under the age of 18 years, written consent was obtained by parents or guardians. All the procedures have been conducted according to the principles expressed in the Declaration of Helsinki.

Synovial fibroblasts isolation and culture

SFbs were isolated as previously described (**14, 15**). See details in Supplementary M&M.

T-cells isolation and characterization and preparation of conditioned supernatants

T cells were isolated and characterized as previously described (**11, 15, 24**). See details in Supplementary M&M.

SFbs treatment with T-cell clones conditioned supernatants and cytokines

See details in Supplementary M&M.

Quantitative Real-time PCR analysis

See details see Supplementary M&M.

Matrix-metallo-proteinases (MMPs) Gelatin Zymography, and quantification of uPA activity

Zymographies were performed as previously described (**25**). A colorimetric assay was used to measure uPA activity. See details in Supplementary M&M.

Collagen degradation assay

Collagen degradation assay was performed as reported (**26**). For details see Supplementary M&M.

Invasion assay in Boyden chambers

Invasion was studied in Boyden chambers. See details in Supplementary M&M.

***In vivo* experiments (SCID mouse model) and histology**

The "inverse wrap" technology in the SCID mouse model was used, as previously described (**14, 27**) and is detailed in Supplemental M&M. All *in vivo* procedures were approved by the ethical committee of Animal Welfare Office of Italian Work Ministry and conformed to the legal mandates and Italian guidelines for the care and maintenance of laboratory animals.

Statistics

Results are expressed as the mean ± S.E. Statistical comparisons between two samples were performed using the Student's t-test, unpaired or paired and between different groups were performed using the ANOVA test.

AUTHOR CONTRIBUTIONS

FM and LM conceived, designed the study and performed the experiments. AL developed the methodology. AC, AB, FB and DB performed *in vivo* studies and histologic analysis. MC, AM and MCR acquired the data. FM, LM, AL and GF analyzed and interpreted the data. TG

and RC recruited JIA patients. FL, LC, FA, RC and MDR wrote, reviewed and revised the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

This work was supported by grant from the Italian Ministry of Health (Ricerca Finalizzata 2010, project code: RF-2010-2314610), by Ente Cassa di Risparmio of Florence and partly funded by Novartis. We would like to thank Dr. Frank Kolbinger of Novartis in providing Secukinumab and Dr. Laura Calosi of the Department of Experimental and Clinical Medicine for histological analysis.

CONFLICT OF INTEREST DISCLOSURE

All authors declare no conflicts of interest.

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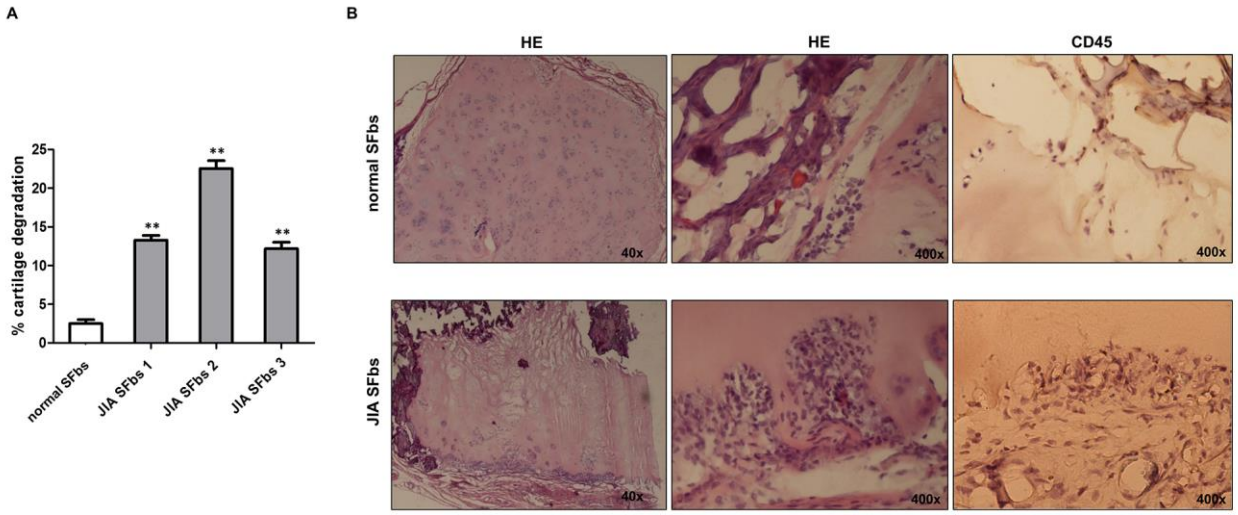

Figure 1. ***In vivo*** **Cartilage degradation by JIA-SFbs in the SCID mouse model.** Human cartilage degradation by normal and JIA SFbs in SCID mouse model 60 days after implantantion. (**A)**. Histogram represents the percentage (mean of four different sponges of three different experiments ± SD) of cartilage degradation measured by Image J software, in the presence of normal or JIA SFbs. (**B**). Hematoxylin-Eosin and CD45 IHC. The microphotographs are representative of four replicate in three different mice (12 sections for each condition). Original magnification x 40 and x 400. ** $p < 0.01$ compared to normal SFbs.

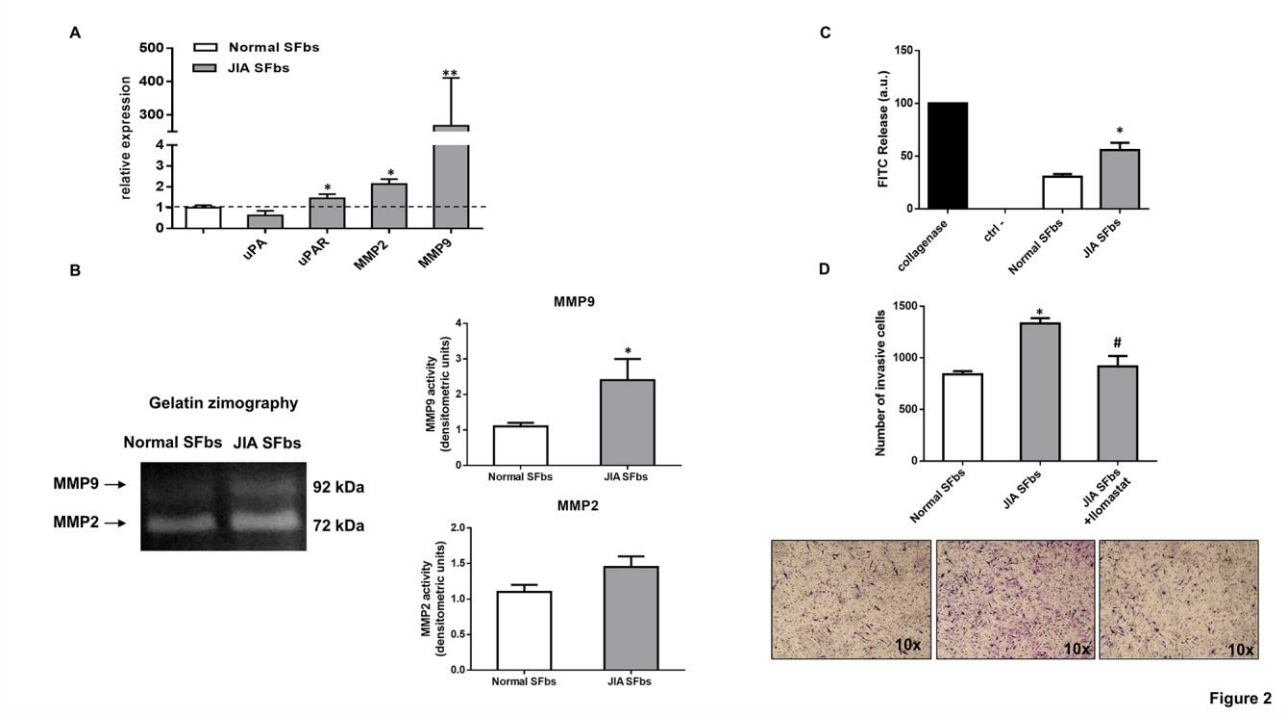

Figure 2. Protease systems and invasion of normal and JIA SFbs. (**A**). Real Time PCR for uPA, uPAR, MMP2 and MMP9. Histogram represents the mean of three different experiments ± SD. (**B**). Gelatin zymography. Histograms represent the mean ± SD of three different experiments performed in triplicate. (**C**). Collagen degradation assay. Histograms report the percent values (the mean ± SD of three different experiments). (**D**). Spontaneous matrigel invasion ± Ilomastat. Data are reported as number of migrated cells (the mean ± SD of three different experiments). * $p < 0.05$ compared to normal SFbs, # $p < 0.05$ compared to JIA SFbs.

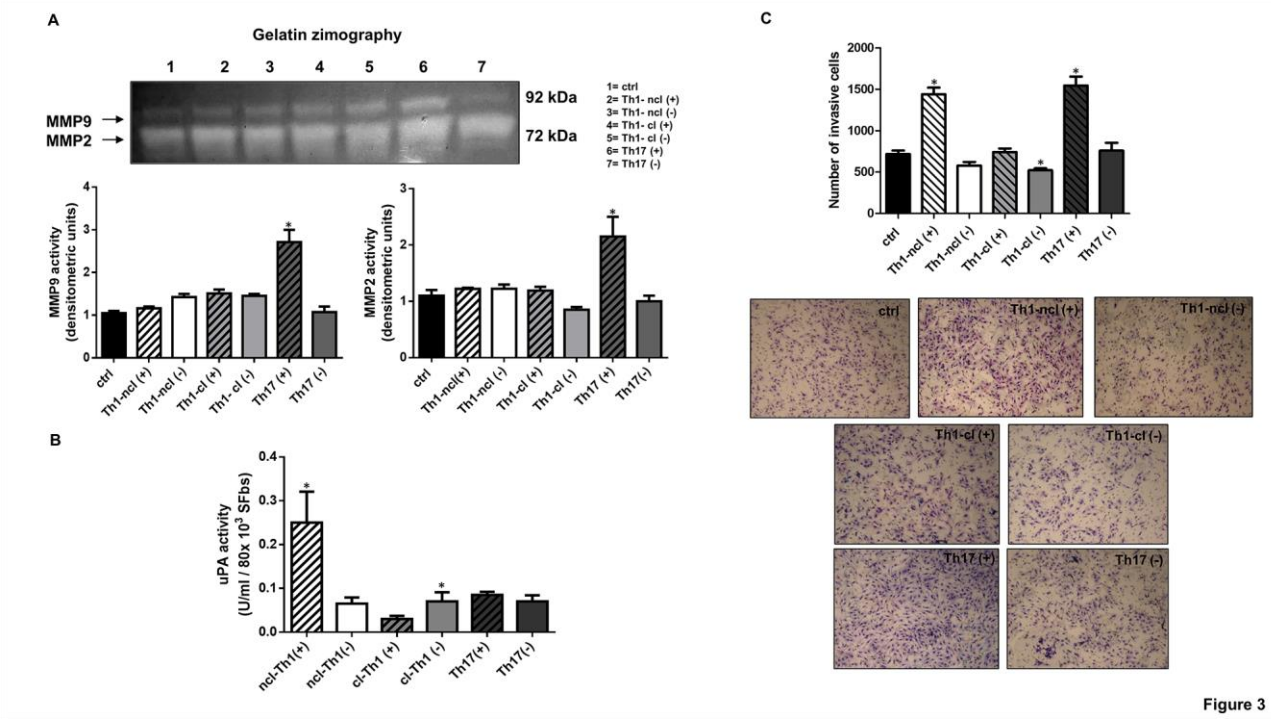

Figure 3

Figure 3. Effects of CD4+ T cell clones CM on protease systems and invasion of normal SFbs. Normal SFbs were incubated for 48h in medium alone or with unstimulated (-) or CD3/CD28-stimulated (+) CM from Th1-cl and Th1-ncl or Th17 clones. (**A**). Gelatin zymography. Histograms represents the mean ± SD of three different experiments. (**B**). uPA activity (U/ml culture medium /80 x 10^3 SFbs). (**C**). Invasion experiment. Data are reported as number of invasive cells (the mean ± SD of three different experiments). * $p < 0.05$ compared to unstimulated normal SFbs (ctrl).

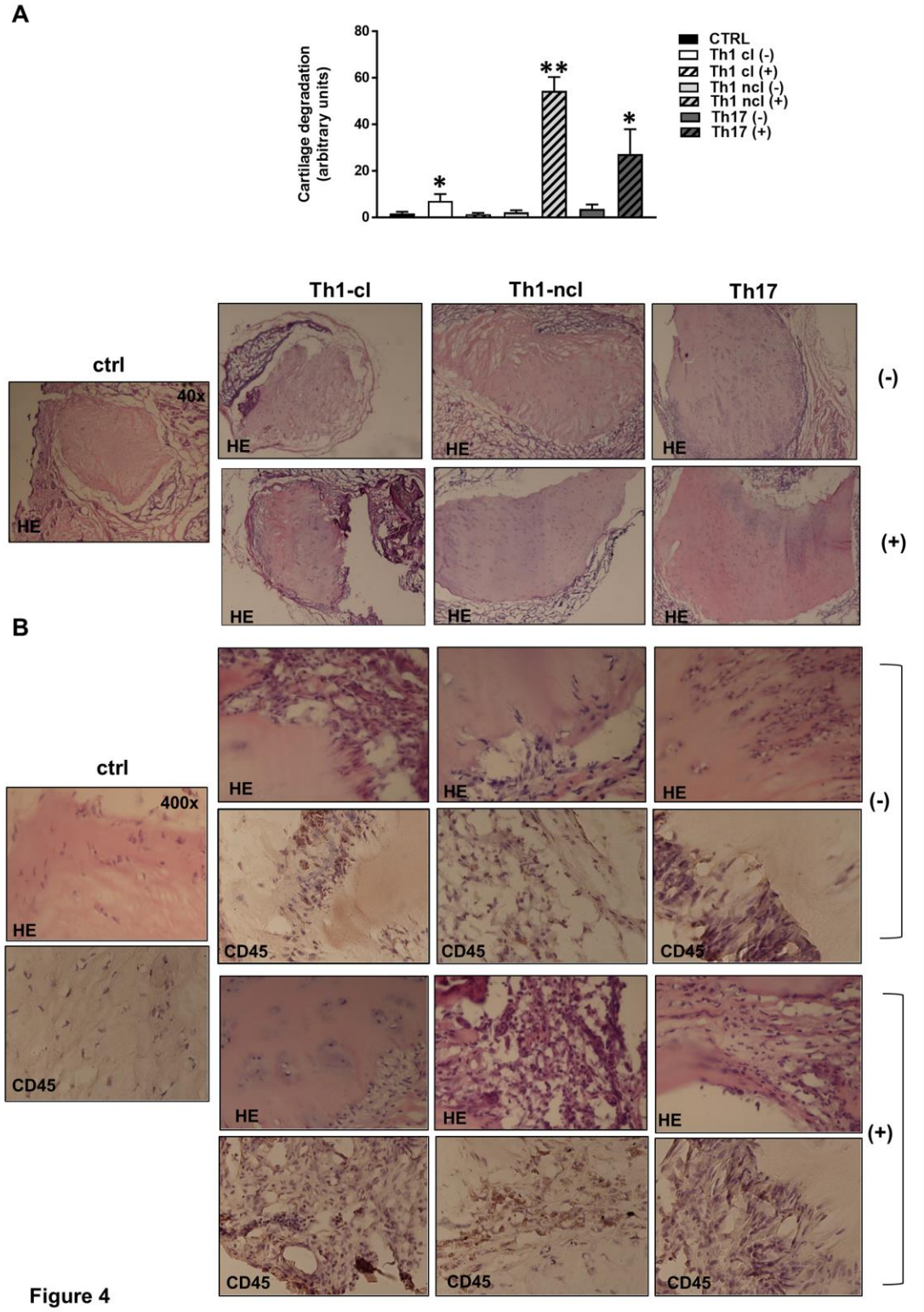

Figure 4

Figure 4. Effects of CD4+ T cell clones conditioned supernatants on cartilage degradation *in vivo*. Collagen sponges with normal SFbs suspended in CMs of unstimulated or stimulated T cell clones were inserted under the skin of anesthetized mice, and weekly peri-implant injections of the relevant medium were performed. After 60 days, the implants were recovered and subjected to H&E and IHC analysis. **(A)**. Histogram represents the mean of four different sponges ± SD of cartilage degradation, in three different experiments. (**B**). Hematoxylin-Eosin and CD45 IHC. Original magnification x 40 and x 400. * $p< 0.05$ and ** $p<0.01$ compared to control unstimulated normal SFbs.

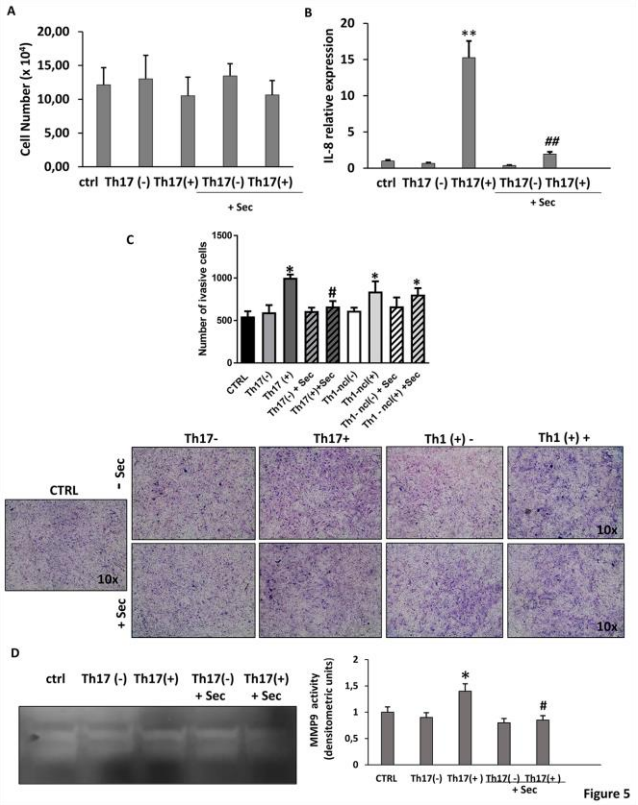

Figure 5

Figure 5. *In vitro* activity of secukinumab. (A). Cell proliferation of normal SFbs 48h treated with Th17 cells CM ± secukinumab 20 µg/ml. Results are expressed as mean of three different experiments ± SD. **(B).** Real time PCR for IL8. Values (the mean ± SD of three different experiments) are normalized to normal SFbs. **(C).** Invasion experiments. Data are

reported as number of invasive cells (the mean ± SD of three different experiments). **(D).** Gelatin zymography. Histograms represent the mean ± SD of three different experiments performed in duplicate. * $p<0.05$ compared to unstimulated normal SFbs (ctrl). # $p<0.05$ compared to Th17+ stimulated normal SFbs.

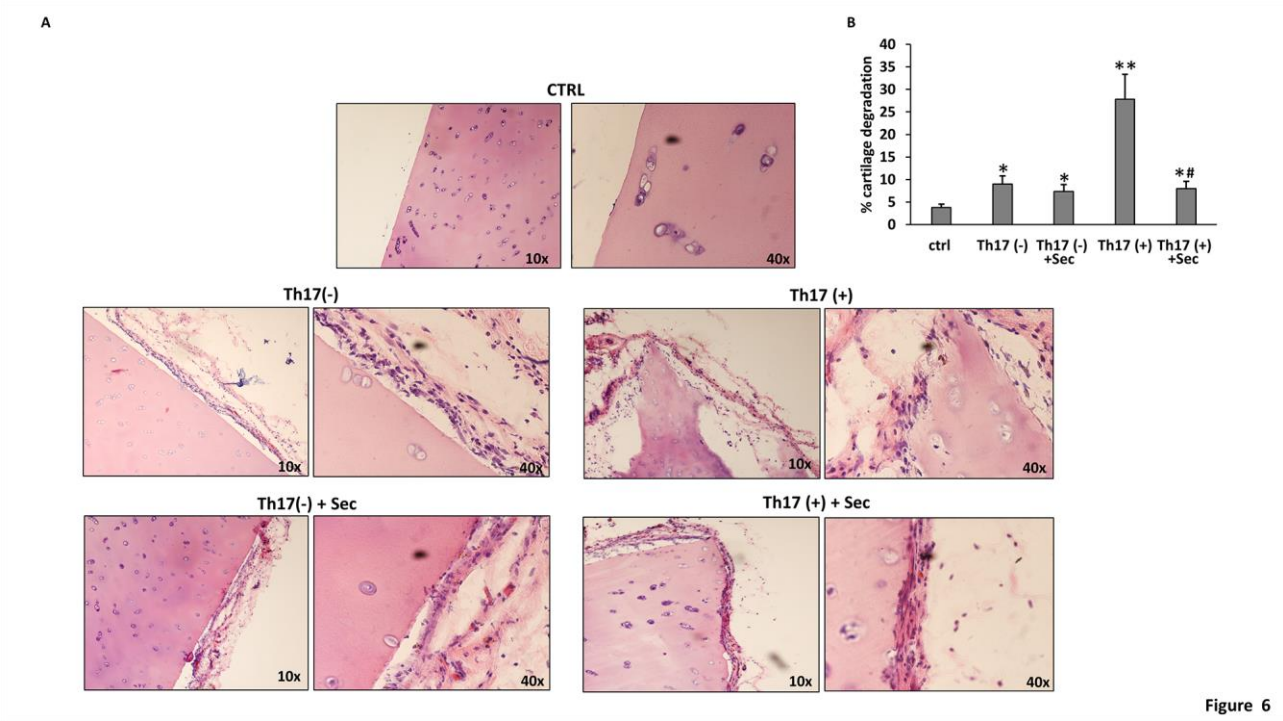

Figure 6. ***In vivo*** **activity of secukinumab.** Human cartilage degradation in SCID mouse model by SFbs treated with CD4+ Th17 cells CM ± secukinumab. **(A).** H&E staining. The microphotographs are representative of four replicate in three different mice (12 sections for each condition). Original magnification x 100 and x 400. **(B)** Histogram represents the mean of cartilage degradation in four different sponges ± SD, measured by Image J software, in three different experiments. * $p<0.05$ and ** $p<0.01$ compared to control unstimulated normal SFbs. # $p<0.05$ compared to stimulated Th17-treated SFbs.