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Thesis Title

Novel insights in the pathogenesis of Juvenile Idiopathic Arthritis: an experimental *in vitro* and *in vivo* model for studying the role of different subsets of T helper lymphocytes

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Alice: “How long is forever? “

White Rabbit: “Sometimes, just one second.”

Lewis Carrol

I want to thank the people who allowed this wonderful second.

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1. Abstract

Juvenile idiopathic arthritis (JIA) is the most frequent chronic inflammatory rheumatic condition in childhood, predisposing to a long-lasting morbidity and physical disability. JIA encompasses heterogeneous conditions that can be grouped in three major types: oligoarthritis, polyarthritis, and systemic arthritis. Although the pathogenesis of JIA is mostly unknown, the first two forms appear to be classical autoimmune conditions, induced by the breakdown of immunologic self-tolerance with a main involvement of the adaptive immunity, while the third is mainly mediated by the innate immunity. How T cell activation leads to joint damage is still a subject of many studies, that are also oriented to identify drugs able to prevent chronic inflammation by inhibiting biological key factors.

Aims of our study were to analyze how different T lymphocytes influence synovial fibroblasts (SFbs) in driving the articular cartilage destruction, and to evaluate the effect of IL-17 inhibition in this experimental model.

SFbs were obtained from healthy children and patients affected by JIA, and T helper lymphocytes subsets were isolated from blood of healthy controls, as well as from synovial fluid of active joints in patients with JIA. Cartilage invasion ability and degradation activity were studied in the presence of normal and JIA SFbs, in the presence of normal SFbs incubated with supernatants or corresponding cytokines from different T cell clones, and after the addition of the IL-17 blocker secukinumab. *In vitro* experiments were integrated with *in vivo* studies based on the inverse-wrap technique in SCID mice.

We observed that JIA SFbs, compared to healthy SFbs, produced large amounts of matrix metalloproteinases (MMPs) and invaded cartilage with subsequent damage. Similar effects were detected by incubating healthy SFbs with the supernatants of different T helper subsets. T helper 17 cells promoted the release of MMP9 by SFbs, while non-classic Th1 mostly induced an over-activation of urokinase-plasminogen-activator. The invasive and destroying ability of healthy SFbs treated with stimulated T helper 17 conditioned media and with IL-17 resulted significantly reduced after the addition of secukinumab.

In conclusion, a complex cross-talk between SFbs and different T cell clones predisposes to joint cartilage damage. Th17 lymphocytes through the release of IL-17 activate SFbs

stimulating their invasive and destructive ability via MMPs, and this can be prevented by secukinumab.

2. Introduction

Juvenile idiopathic arthritis (JIA) is the most common chronic inflammatory rheumatic condition in childhood, representing a potential cause of long-lasting morbidity and physical disability. According to the International League of Associations for Rheumatology (ILAR) JIA is characterized by a chronic arthritis lasting at least six weeks, beginning before the 16th birthday, and of unknown etiology [1-3].The diagnosis requires the exclusion of any other known causes of articular inflammation, and the 6-week period has been chosen to mark the chronicity of the disease while considering a sufficient period to rule out viral, reactive, or other arthritides that could simulate this condition.

JIA encompasses a heterogeneous clinical spectrum consisting of different entities, currently classified on the base of ILAR classification criteria in 7 distinct subtypes. In relation to the clinical features such as the number and the pattern of involved joints, the serologic markers, and the systemic manifestations assessed at the disease onset and during the first six months of illness it is possible to identify: oligoarthritis, rheumatoid factor (RF) positive polyarthritis, RF negative polyarthritis, juvenile psoriatic arthritis (JPsA), enthesitis-related arthritis (ERA), systemic-onset JIA (sJIA), and undifferentiated JIA (not fulfilling the criteria for any one category or fulfilling the criteria for more than one) (Table 1) [1].

To make these groups more homogeneous, they are mutually exclusive. Indeed, the main intention of this classification is to define homogenous groups to optimize treatment choices, and follow-up plans and to reliably estimate the disease prognosis. However, even if the ILAR classification, updated in 2001, is still widely used, it has been recently criticized. Some of the categories still result too heterogeneous, as the polyarticular RF negative subtype and the psoriatic arthritis. On the other hand, even an early onset of arthritis in very young female subjects with a high risk of uveitis can be observed in several JIA subtypes, such as the oligoarticular, polyarticular RF negative and psoriatic subgroups. Similarly, the classification within JIA of systemic-onset disease also needs to be reconsidered, since its clinical and pathophysiological characteristics appear completely different respect to the other JIA subtypes. Therefore, the PRINTO organization (Pediatric Rheumatology INternational Trial

Organization) has recently proposed a prospective study to validate a new classification able to improve the current one [4].

2.1. Epidemiology

The epidemiology of JIA widely varies across different geographic areas. In addition to that, the heterogeneity of the methodologies used in different studies (e.g., diagnostic criteria, size of the population studied, referral bias) makes the prevalence and incidence of JIA not exactly defined. In general, literature suggests the incidence in Europe and North America to range from 2 to 20/100,000 children, with a prevalence between 16 and 150/100,000 children [2] (Giancane. Rheumatology Therapy 2016). In a study from Australia the prevalence in childhood is reported to be as high as 400 in 100.000 [5]. On the other hand, the prevalence of JIA is reported to be lower in Asia than in Europe and North America.

Likewise, the distribution of different JIA subtypes varies among different countries and ethnicities highlighting that JIA is the result of complex interactions involving genetic and environmental factors [6] (Schwarz, 1997). In North America oligoarthritis is the most common subtype, while RF positive polyarthritis is the least frequent one [2]. African Americans are more likely to have RF positive polyarticular disease [6]. In Caucasian children of European descent oligoarthritis is the most common JIA subtype. ERA results to be more prevalenrt in Mexican and Asian children [7]. sJIA is mostly described in Indian and Japan children, accounting for 30-50% of all JIA cases, while in Europe and North America it represents 10% of all subtypes [8].

Females are usually more involved than males (> 2:1); however, gender distribution differs with arthritis subtypes, and ERA is more often observed in males, while sJIA shows a homogeneous distribution among males and females [2,9].

The age at the disease onset also differs among subtypes, with an early onset in oligoarthritis (median age of 4 years), and a later one in ERA (median age 11 years), and RF positive polyarthritis (median age 12 years), while sJIA can begin at any age [9].

2.2. Clinical profile

The presence of arthritis (swelling and/or the association of pain with limited range of motion in a joint) is mandatory for the diagnosis of JIA. All the joints can be affected, most often the

large ones of the lower limbs, although in the polyarticular form the small joints of hands and/or feet are often involved. The number of joints affected varies depending on the type of JIA. Enthesitis, that is the inflammation where tendons or ligaments insert into the bone, may be associated with JIA, especially with ERA category. Subcutaneous nodules are atypical features, seldom observed in association with the RF positive polyarthritis. Systemic signs such as fever, fatigue and rash dominate the sJIA presentation, while in this category arthritis can develop days to weeks later. First signs of arthritis can be subtle, and articular pain may be mild making JIA hidden in younger children, unable to properly refer their symptoms, until the increasing morning stiffness, and /or the worsening of their articular functional limitation are evident. Older children and adolescents on the contrary, usually complain of a functional limitation depending on the location of the arthritis and experienced difficulty in writing, inability to brush their hair or to open a bottle. Joint stiffness is most marked in the morning or after a prolonged period of inactivity.

2.2.1. Oligoarthritis

Oligoarthritis involves four or fewer joints in the first six months of the disease. It is further subdivided into persistent and extended subtypes when, after this initial period, the number of affected joints is no more than four or exceeds four, respectively [1,10].

In fact, in approximately 25% of children arthritis, after the first six months the disease, spreads to four or more joints [11].

The oligoarticular category, that represents 50-60% of all cases, typically involves females younger than six years of age [2]. Arthritis is predominantly localized in the large joints of the lower limbs.

Patients show general good conditions, and articular symptoms may be insidious. Inflamed joints appear swollen, but not very painful or tender [12,13].

Iridocyclitis, rather than arthritis, represents the main potential disability feature in these children [14,15]. This chronic anterior uveitis is typically pauci/asymptomatic (white eye uveitis), making regular ocular screening paramount. The ocular disease course is recurrent or persistent, and eye involvement is bilateral in two thirds of cases. Uveitis can precede arthritis in 10% of patients; ocular and articular inflammation are independent.

Laboratory inflammatory markers are usually normal, or slightly increased with the acute phases of the illness together with a mild anaemia and leucocytosis [13,16]. The antinuclear antibodies, ANA, positivity is reported in 70-80% of children and is found to be a uveitis risk factor [14].

Oligoarthritis has been associated with HLA genes (HLA-A, HLA-B, and HLA-DR), supporting the main role of the adaptive immune response in the pathogenesis of this condition. Genes implicated in regulating cytokine responses (e.g., those encoding tumor necrosis factor (TNF)-α, Macrophage migration inhibitory factor (MIF), inteleukin (IL)-6, and IL-1α) and cellular activation predispose to the disease onset and influence the aggressivity of its course.

The disease is characterized by a good prognosis in general, and remission is achieved frequently especially in the monoarticular forms. However, the extension of the arthritis to a higher number of joints and uveitis are two potential, severe complications. Hip, cervical spine, ankle, or wrist involvement increased inflammatory markers and radiological signs of articular damage are poor prognostic factors.

2.2.2. Polyarthritis

In polyarticular JIA arthritis affects five or more joints during the first six months of disease [1]. This category is further classified into two subgroups, according to rheumatoid factor (RF) positivity the RF positive or RF negative polyarthritis subgroups.

RF negative category is more common, representing 11-30% of all JIA patients, while RF positive category represents 2-10% of the cases [17, 18, 19].

Females are more frequently involved in both subgroups. A biphasic pattern can be observed in RF negative polyarthritis onset, with a first peak between two and four years and a second one between six and twelve years. In the RF positive subgroup, the disease onset is usually in later childhood and adolescence. The higher degree of inflammation with respect to the oligoarthritic group may express with systemic features such as low-grade fever, weight loss, moderate hepatosplenomegaly, and lymphadenopathy, and a subsequent growth retardation [18].

The arthritis generally involves the small joints of the hands and wrists; the small joints of feet can be sometime observed. Although quite unusual in other subgroups, cervical spine, elbow, hip, and shoulder are not infrequently interested by articular inflammation, as well as temporomandibular joints [17, 20].

Within the RF negative polyarthritis category three different presentations can be recognized.

The first one shares many features with the oligoarticular condition, such as the early disease onset, the female predominance, the asymmetric distribution of arthritis, the high uveitis risk, the presence of ANA, and an association with HLA-DRB1*0801.

The second one mostly affects young female children, a symmetrical distribution of arthritis involving large and small joints, is often ANA negative, and appears to be like RF negative rheumatoid arthritis in adults. The prognosis is variable.

The third is the most aggressive, with a limited response to treatments, a poor prognosis and commonly complications. It is characterized by a dry polyarthritis: a rare form of diffuse arthritis without clears signs of synovial effusions or thickening and without notable abnormalities in the laboratory assessment.

RF-positive polyarticular JIA resembles adult rheumatoid arthritis sometimes also showing subcutaneous nodules, as those observed in rheumatoid arthritis, and which course parallels serum RF levels [18].

Laboratory tests in polyarthritis, especially in the RF positive subtype, can show a mild/moderate increase of inflammatory markers, transaminase levels, and leukocyte count, with an inflammatory anaemia [12].

ANA positivity is observed in 50-80% of patients with a RF negative polyarthritis, and in 55% of those who are RF positive, but in this last category ANA are not associated with an increased uveitis risk. Patients with RF positivity also commonly have positive anti-CCP (Cyclic Citrullinated Peptide) antibodies.

Anti-CCP and RF are the most suitable predictors of severe joint damage [21]. RF positive polyarthritis has long been identified to represent the true paediatric version of adult rheumatoid arthritis, and genetic analyses seem to confirm this [22].

Polyarticular JIA has been observed to be associated with HLA-DR4.

A high rate of joint damage is expected to be associated with a polyarthritis course. The prognosis for the RF positive subtype is more severe with a high risk of rapid progression to extensive joint involvement and early joint erosion with secondary deformities, particularly in the hands and feet.

2.2.3. Juvenile psoriatic arthritis

In this form of JIA, the presence of arthritis concomitantly with psoriatic lesions or two of the following: dactylitis; nail pitting or onycholysis; psoriasis in a first-degree relative confirms the diagnosis. The association of skin and articular manifestations is observed in 10% of the pediatric patients, since in the rest of the cases, psoriasis precedes or follows joint involvement. As a result, some patients are first classified into other subtypes of JIA.

Psoriatic arthritis is twice as common in girls. Family history of skin psoriasis is reported in more than 50% of the cases.

Arthritis distribution varies from a symmetrical small-joint type to an asymmetrical lower-extremity large-joint presentation up to a polyarticular extension like seropositive rheumatoid arthritis. Some adolescent patients may express features of ERA such as sacroiliitis, enthesitis, or spondylitis. In the early-onset form, the clinical picture is similar to oligoarthritis with a predominant female involvement, and ANA positivity.

Skin involvement usually manifests as psoriatic plaques localized on the extensor surfaces of knees and elbows, scalp, umbilicus and back of the forearms. Generalized forms may be present. Psoriasis nail involvement includes dystrophy, onycholysis, and subungual hyperkeratosis.

One typical feature is the inflammatory involvement of the distal interphalangeal joints, the other one is the “sausage-like” finger or toe, also known as dactylitis, characterized by a diffuse swelling of an entire digit where flexor tenosynovitis is common [17].

Young female, ANA-positive patients have a high risk to develop anterior uveitis like oligoarticular JIA children.

The inflammatory syndrome is moderate or absent. ANA can be detected in low or moderate titers in a high proportion of patients. HLA B27 positivity is present in 30% of the cases.

The course is variable, since joint involvement is heterogeneous, and studies are lacking in this area.

2.2.4. Enthesitis-related arthritis

ERA, as defined by ILAR classification, represents a controversial entity, showing characteristics of both JIA and adult spondyloarthropathy [1, 12, 23]. ERA represents a heterogeneous group of arthritis due to the diversity of clinical pictures. A distinguishing sign is enthesitis, that is the inflammation of the attachment sites of the ligaments, tendons or fascia to the bones. Enthesopathy in a single site is reported in 47% of the cases, in three different sites in 18% of children [24]. The most affected tendon is the Achilles, followed by metatarsal and calcaneal insertions of the plantar fascia, and patellar insertion of the quadriceps tendon. Other sites are less commonly involved: the greater trochanter, iliac crest, pubic symphysis, ischial tuberosity, and costochondral junctions. Enthesitis is associated with severe pain. Evocative symptoms are heel pain, plantar pain, tilting buttock pain and sternal pain. Enthesitis is specific to this form and is of major diagnostic importance; however, it is not necessarily required for diagnosis according to the ILAR classification. Other clinical characteristics of ERA are:

• Hip arthritis, often present in the early phase of the disease

• Tarsitis

• Axial involvement (sacroiliac joints and joints of the spine, especially in the lumbar region)

Male older than six years of age with an asymmetric oligoarticular arthritis of lower extremities, such as in the ankle and knee, are typically affected. A quarter of patients, however, have polyarticular involvement. Hip involvement is the most significant difference from oligoarthritis.

Uveitis is associated with this form, but unlike the silent and often bilateral uveitis of oligoarticular JIA, it is unilateral and strongly symptomatic with redness, pain and photophobia.

HLA-B27 positivity is a suggestive, even if not specific, laboratory sign. It is reported in 65-80 % of patients, and its presence is associated with a poor prognosis suggesting a rapidly erosive course [23]. ANA are usually absent.

Other genetic factors linked to cytokine expression such as polymorphisms in/or near the TNF, IL-1 and IL-23 loci have been observed to influence the perpetuation of inflammation.

This form has a low risk of sequelae, even if remission is observed in less than 20% of untreated children 5 years after diagnosis [20].

2.2.5. Systemic-onset JIA

sJIA accounts for 10-20% of JIA cases. The association of arthritis with intermittent fever for at least 2 weeks plus one of the following defines the diagnosis: typical rash, lymphadenopathy, hepatosplenomegaly or serous effusion. In this disease subtype systemic symptoms dominate the clinical picture, female and males are affected with the same frequency, and the disease onset may occur at any time during childhood.

The fever has a typical profile, with peaks once or twice a day, with an evening tendency, with a return to normal and sometimes hypothermia between peaks. During fever, severe myalgia and arthralgia may exist, with a septic appearance. The general condition is preserved between the peaks. In association with the fever a macular salmon rash is evident at the level of the trunk and the root of the limbs. Sometimes it appears in the form of an itchy urticaria with Koebner's phenomenon. Arthritis can be mono, oligo or polyarticular. It mainly affects the ankles, knees and wrists but can affect virtually all joints including the cervical spine. It can be rapidly erosive with secondary ankylosis. Arthritis is not necessarily present at the onset of the disease, which is a major difficulty in diagnosis. Chronic arthritis develops in a varying percentage of patients. Pericarditis is the most frequent serositis, sometimes accompanied by pleuritis.

There is a severe biological inflammatory syndrome with high CRP, fibrinogen, ESR, transaminase, leukocytes, and platelets levels. Other markers of inflammation are serum Amyloid Protein A, total haemolytic complement level, hypergammaglobulinemia, and hypoalbuminemia. Gradually inflammatory anemia sets in. Autoantibodies (ANA and RF) are typically absent.

sJIA is more likely to develop macrophage activation syndrome (MAS). Hyperferritinemia greater than 600 ng/mL, as well as a decrease in the percentage of glycosylated ferritin, should raise fears of this complication.

Unlike the other subgroups, JIA has no sex bias, no increased risk to develop uveitis, no association with HLA genes, or ANA positivity. This subtype is mainly linked to a predominant abnormality of the innate immune system.

sJIA may have different types of evolution at later stages: approximately 40% of patients show a monocyclic course, <10% a polycyclic course, and more than half of children have a persistent course. Most patients with polycyclic or persistent sJIA have a mitigation of the systemic features, whereas arthritis becomes progressive and severe, inducing destructive changes and showing a limited response to treatments.

2.2.6. Undifferentiated JIA

This category is the least common type of JIA: 10 to 15% of inflammatory arthritis are grouped in this category. It includes all inflammatory arthritides that do not fit into any of the other subtypes or meet the criteria of at least two or more of the other subtypes. As time progresses, and the possible appearance of new signs or symptoms, some cases may be reclassified in another category.

2.3. Diagnosis

Due to the insidious course of the disease, and the absence of specific clinical-radiological-laboratory data, the definitive diagnosis can require time to be established. The diagnosis is clinical, and one of exclusion, so conditions with similar clinical pictures need first to be ruled out, and laboratory and radiological features can be supportive for that [12].

2.3.1. Laboratory

Laboratory tests are helpful both in the early phases of the disease, supporting the exclusion of other diagnoses and assisting in JIA subgroup definition, and during the follow-up for monitoring inflammatory state, potential complications, response to treatment, and side effects related to drugs.

ESR, CRP, and complete blood count are usually adequate to detect an inflammatory condition. Additional exams such as fibrinogen, ferritin, and serum amyloid A can be required

in severe inflammatory conditions as often observed in sJIA. When a MAS is suspected also triglycerides dosage and coagulation prophile need to be studied.

Kidney and liver function should be investigated at the disease onset and periodically in case of pharmacological treatments.

ANA positivity can help in the early phases of the disease to confirm the diagnosis and to give an estimation of the risk of eye involvement.

RF and CCP are indicated in older children and adolescents suspected to develop a polyarthritis, while HLA-B27 testing is applied in presence of a picture suggestive of ERA.

Synovial fluid analysis is another important laboratory tool for the diagnosis of patients with JIA, mostly useful during the initial workup investigations for excluding infectious arthritis.

2.3.2. Imaging

Although the diagnosis of JIA is based on clinical features, and no unifying international recommendations are available for imaging in JIA, this can play a helpful role in many cases for differential diagnosis, in the study of deep joints such as hip or shoulders, in structurally complex ones such as ankle or temporomandibular joint [25]. Imaging can support the detection and the definition of the extension of joint inflammation, can precisely distinguish between tenosynovitis and arthritis, and can allow the evaluation of treatment response.

Some of the major challenges are related to the choice of imaging modality, the right timing to perform it, the correct identification of the patient to be studied, as well as the lack of standardized scoring systems for all imaging modalities.

Standard radiography has long been traditional imaging for JIA. In the acute phase, it detects oedema of the periarticular soft tissues and enlargement of the joint spaces indicating an effusion.

In the late phase, it highlights osteopenia, periosteal appositions with advance of certain bone points, lines of growth arrest (Harris lines: transverse compact lines located in the metaphysis and parallel to the epiphyseal plate) and joint destruction. These lesions are staged according to the radiological classification of Steinbrocker:

- stage I: osteoporosis, infiltration of soft parts and periosteal appositions

- stage II: joint pinching
- stage III: erosion
- stage IV: fusion.

When performed at the onset of disease, the standard X-ray is mainly used to rule out the differential diagnoses of JIA (fracture, tumor, osteitis, etc.). At the late stage, it highlights complications such as asymmetry in the length of the lower limbs, periarticular or diffuse osteopenia, ankylosis of the tarsus, carpus or processes of the cervical spine. However, it has no role in the diagnosis or in the monitoring of the disease as it does not detect the signs preceding erosion.

Ultrasound is an easy and accessible way to assess a joint. It can detect synovitis early and is very useful for differentiating between synovitis and periarticular oedema (especially in the ankle) and for guiding intra-articular injection. However, this is an operator-dependent exam with limitations, and the interpretation of the findings, especially in early disease or in presence of subtle findings, is challenging. Comparison with the contra-lateral non-inflamed side may clarify the evaluation. However, some peculiar features linked to the growing skeleton can be misdiagnosed with pathological findings such as articular feeding vassels, or unossified cartilage.

Ultrasound is suitable for interventional procedures, for both diagnostic and treatment aims. It allows the display of the inflamed area, and the accurate placement of the needle tip

However, ultrasound cannot adequately visualize bone changes.

Studies are underway to validate an ultrasound evaluation score for JIA [26, 27].

MRI allows the detection of all relevant anatomical systems in inflamed joints. MRI has an important place in joint assessment. In the presence of an atypical joint picture, it is used to rule out mechanical damage to the joint (such as a meniscal problem in the knee which may be the cause of an effusion or a tumor). In inflammatory arthritis it can early detect synovitis, even before obvious clinical signs. In addition, it can reveal bone marrow oedema, a predictive sign of bone erosion, and thus can guide the initiation or adaptation of treatment (Breton. Semin Arthritis Rheum. 2012). It also has the advantage of detecting non-bony lesions such as tenosynovitis or enthesitis. This is the gold standard for TMJ and sacroiliac joints [28]. Although it is effective in all the remaining joints, it is sometimes replaced by

ultrasound, due to the constraints of its realization in children (immobilization during the examination, sedation, injection of contrast medium).

The standard MRI protocol in arthritis, defined by OMERACT includes a:

T1 spin-echo, SE, sequence
T2 fat-suppressed sequence or a short tau inversion recovery, STIR, and
T1 fat-suppressed sequence pre- and post-contrast.

MRI is the only modality able to objective bone marrow oedema and the most sensitive to detect bone erosions. Contrast administration allows a better visualization of synovitis. Furthermore, MRI shows a high sensitivity for erosions than standard radiography [29].

Computed tomography may find a place in the assessment of the facet joints, as in the cervical spine, and its use is currently accepted to study temporomandibular joint; however, the employment of this tool in JIA is limited by the relatively high radiation doses.

2.3.3. Histopathology

The hallmark of arthritis in patients with JIA is the inflammatory involvement of the synovial lining of the joint, that may progressively induce cartilage and bone erosion. The inflammatory synovitis is associated with an increased vascularization, oedema accumulation in the synovial tissue with hyperplasia of synoviocytes, and infiltration by immune and inflammatory cells such as T and B lymphocytes, dendritic cells, plasma cells, and macrophages, leading to synovial lining and villous hyperplasi*a*. The infiltration of T cells stimulates the production of pro-inflammatory cytokines [30]. The proliferation of synoviocytes and the recruitment of inflammatory cells from the peripheral circulation may contribute to pannus formation, typically expressed in RF positive polyarticular disease. Pannus is a sort of tumor-like expansion of inflamed synovial tissue which can degrade articular cartilage.

The effects of chronic inflammation have been well documented in RA, while little is known in paediatric age, especially about synovial pathology in early stages [31].

The growth of new blood vessels during the early stage of the disease predisposes to the perpetuation of the inflammatory condition in the synovium [32]; a higher angiogenesis expansion is related with a higher risk of disease extension.

In aggressive arthritis an increased numbers of mature vessels have been observed, as well as a continuous angiogenesis process as demonstrated by the expression of αVβ3 typically expressed on early undifferentiated vessels.

The immunobiological characteristics differ among JIA subgroups already at the disease onset, and the delineation of the cellular infiltrates in the synovium seems to be able to predict clinical outcome. Subgroups with a more extensive arthritis show higher infiltration by CD3, CD4, CD20 cells, and a greater angiogenetic process.

The Krenn histopathological synovitis score is a feasible and standardized haematoxylin and eosin evaluation method, used for the differential diagnosis of joint diseases. It includes the analysis of three histologic features of synovitis allowing the discrimination between low- and high-grade synovitis [33]: lining layer hyperplasia, inflammatory infiltrate, and activation of resident cells (stroma). Scores has the lowest value of zero and the maximum of nine (corresponding to the most severe inflammation).

Immunohistochemistry may allow the study of synovial cell signature and it can be used to score synovitis, and recently immunohistochemical markers has integrated Krenn score, improving sensitivity and specificity [34].

2.4. Disease Activity Measurements

Different measures can be evaluated for the assessment of disease activity such as the number of active joints, parent/patient visual analogue scale, physician visual analogue scale, laboratory data (ESR, anaemia, thrombocyte count, etc.). None of them individually seem to be totally accurate, while composite disease activity scores appear to be more reliable instruments. They pool different single measures in one tool, integrating multiple characteristics of the disease into a continuous scale or on one summary number. Juvenile Arthritis Disease Activity Score (JADAS) represents the first composite disease activity score for JIA and one of the most widely used instruments in daily practice together with the

Childhood Health Assessment Questionnaire, CHAQ, that allows the global estimation of physical, social, and mental functioning.

About the impact of JIA in children's quality of life in recent years an increasing attention on the evaluation of this aspect has emerged with the incorporation of patient-reported or parent-reported outcomes in the assessment of the health status of these patients [35]

2.5. Complications

2.5.1. Articular complications

Persistent joint inflammation results in erosion of the cartilage leading to pinching of the intra-articular space. This erosion is early in the RF positive polyarticular forms and in some systemic forms, but it is also detected after a prolonged time in all cases with inadequately controlled arthritis. These erosions lead to joint destruction with secondary ankylosis and variable functional limitation depending on the location. Subluxations, dislocations and flexion/extension deformities can occur in all joints, but they are most often encountered in the hands and feet. Radial deviation at the wrist is typical. Atlanto-axial subluxation involves a vital risk and surgical arthrodesis must be scheduled in the event of signs of compression of the marrow (paraesthesia, osteotendinous hyperreflexia). In the jaw, damage to the temporomandibular joint leads to limitation of the mouth opening and micro-retrognathia (bird's face).

2.5.2. Osteopenia

Chronic inflammation affects the bone through several factors related to the disease itself and to the treatments used, including corticosteroids.

Pro-inflammatory cytokines interact with the RANK / RANKL system thereby activating osteoclastogenesis; particularly the deleterious role of IL-6 on bone metabolism has been demonstrated [36]. Decreased physical activity secondary to joint pain leads to amyotrophy, contributing to the worsening of osteopenia.

Osteopenia can be localized near the affected joint (by local action of inflammatory cytokines in the joint and immobilization) or generalized. In children, when bone fragility results in fractures, it is called osteoporosis (Z-score <-2 in association with a fracture). In children with long active JIA, and especially if they are receiving or have received prolonged corticosteroid therapy, consider screening for vertebral compression which may go unnoticed.

2.5.3. Localized growth disorders

Blood flow secondary to inflammation stimulates localized growth resulting in enlargement of the affected joint and asymmetry compared to the contralateral limb. In other cases, chronic inflammation will result in premature epiphyseal fusion with local growth arrest and consequent deformity; at the level of the knee, blockage of the epiphyseal plate is responsible for varum or valgum deformities and limb dysmetria.

2.5.4. Generalized growth disorders

Despite all the therapeutic advances, 10% of patients will eventually experience stunted growth [37]. The cause is multifactorial: blockage of the somatotropic axis by pro-inflammatory cytokines (IL-1, IL-6 and TNFα) and early epiphyseal welding, prolonged corticosteroid therapy and nutritional factors (decreased appetite in the context of chronic inflammation). Controlling inflammation and consequently stopping corticosteroid therapy improves the chances of catching up growth.

2.5.5. Uveitis

Anterior non-granulomatous uveitis (iridocyclitis) is one of the most severe extra-articular manifestations of JIA, reported in 15-67% of the cases. Usually, it is a chronic anterior uveitis (68.3%), less frequently an acute one (16.2%), or a recurrent disease (12%); in 3.5% of the cases a panuveitis is observed [38].

JIA female patients with early ANA-positive oligoarthritis onset are at high risk for this complication, that is usually insidious and asymptomatic.

Acute, unilateral or bilateral symptomatic uveitis with pain, photophobia, redness, and change in vision may develop is 10-20% of ERA patients, especially in those with HLA B27 positivity, in these patients sometimes a hypopion is detected [17, 38].

A delay in diagnosis and treatment can lead to severe complications such as cataract, iridocrystalline synechia, band keratopathy, glaucoma or blindness.

Since this anterior uveitis is often silent, systematic slit lamp screening is required and its frequency will be determined by risk factors.

In general, screening every 3 months is recommended for high-risk cases: oligoarticular, polyarticular forms with negative RF, undifferentiated and psoriatic arthritis with:

- ANA positivity
- early onset of JIA (before the age of 7)
- disease course for less than 4 years.

Screening every 6 months is recommended for moderate risk cases: oligoarticular, polyarticular forms with negative RF, undifferentiated and psoriatic arthritis with:

- Negative ANA
- late onset of JIA (after age 7)
- disease course for more than 4 years.

Annual screening is recommended for low-risk cases: systemic, polyarticular RF positive and ERA forms.

The initial treatment is based on local ocular corticosteroid drops, while selected cases may require systemic or intraocular glucocorticoids injections.

In case of prolonged treatment or unresponsiveness, immunomodulant or immunosuppressive agents need to be added. Surgery is reserved for serious complications [39].

2.5.6. MAS

It is an acquired form of hemophagocytic lymphohistiocytosis. It can complicate all forms of JIA but the risk is major in the systemic form where it occurs in about 10% of patients. A

hypothesis put forward to explain this predisposition would be the presence of heterozygous mutations or polymorphisms in the genes involved in the cytolytic pathway. Dysfunction of cytotoxic natural killer (NK) cell activity, in combination with persistent activation of the TLR pathway and permanent IL-6 hypersecretion in a child with sJIA, exaggerates the response of macrophages to inflammatory stimuli. A triggering factor such as an infection increases the stimulation of macrophages through INFγ, thus amplifying the inflammatory response which degenerates into a cytokine storm [40]. It is a fatal complication in almost 30% of cases. MAS is characterized by plateau fever, cytopenia, disruption of liver enzymes, coagulopathy, and neurological signs. In a child with sJIA, it is precipitated by infection but also by a drug (sulfasalazine, aspirin and even biotherapies, and formerly gold salts). In half of the cases, the bone marrow shows hemophagocytic activity of the macrophages. In 2016, an international consortium defined the diagnostic criteria for MAS occurring in a patient with sJIA [41].

Despite the presence of fever as a mandatory diagnostic criterion, cases of fever-free MAS have been reported in patients receiving Tocilizumab or Canakinumab as treatment for their sJIA. Faced with any change in the typical fever curve of sJIA with an increase in ferritin to more than 600 ng/ml, one should fear MAS and carry out daily check-ups to detect it and treat it adequately.

2.5.7. Amyloidosis

Secondary amyloidosis is a very rare but feared complication of JIA. It mainly concerns the systemic form. It occurs after years of uncontrolled inflammation and initially manifests as proteinuria. In a report of adults with amyloidosis secondary to JIA the prognosis was poor with high mortality linked to secondary renal failure. However, with the major therapeutic advances of this century, one would expect a better case in future adults with JIA.

2.6. Treatment

Treatment of children with JIA aims to totally control the inflammatory process and to improve the quality of life with a normal physical and psychosocial development.

The heterogeneity among JIA subtypes, often translates into different responses to treatment.

When patients have no clinical signs nor laboratory abnormalities which can be attributed to JIA for a period of six months they can be considered in remission (complete absence of disease) [42].

Treatment is also aimed to prevent other non-articular complications, such as iridocyclitis, MAS, and growth retardation. Patients and their caregivers should be informed in detail about the treatment program, and their compliance evaluated. Additionally, treatment-related side effects need to be carefully considered and monitored.

2.6.1. Nonpharmacologic Treatments

A multidisciplinary approach is required for an optimal management of patients affected by JIA. A trained staff including not only physicians such as paediatric rheumatologists and ophtalmologists, but also nurses, physical, occupational, and psychosocial specialists can offer an optimal and standardized care to these young patients [2].

Parents should be included in this supportive network and involved in the efforts oriented in minimizing time away from school.

A psychiatric counseling is pillar for patients and their families to offer a support to the potential effects of the disease such as depression and/or anxiety related to the emotional distress and chronic pain.

More than half of patients with JIA in adulthood show a disability or limit their full capacity. The aim of physiotherapy is to improve joint efficiency, control the pain, prevent joint limitation and deformity ensuring an adequate range of motion and a proper muscle tone. Passive and active exercises, aerobic conditioning, assistive device such as walkers or wheelchairs, splinting, are used to obtain those objectives.

Occupational therapy helps to support motor skills favouring self-reliance and independence, also allowing a sense of satisfaction to children. Occupational therapy is primarily focused on maintaining the longest functional capacity of all joints, and when articular dysfunctions are already present it should indicate adaptive proceedings, ensuring the independent functioning of the patient.

Also, an adequate nutrition, and calcium and Vitamin D supplementation should always be evaluated [43].

2.6.2. Pharmacologic Treatment

2.6.2.1. Nonsteroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) are used as first line approach, but in oligoarthritis they may be able to obtain disease remission. Ibuprofen (30-40 mg/kg/day divided 3 to 4 times daily, maximum = 2,400 mg/day), and Naproxen (10-15 mg/kg/day divided twice daily, maximum = 1,000 mg/day); are the most used molecules. NSAIDs allow an anti-inflammatory and an analgesic affect. They can occasionally induce abdominal pain, thrombocytopenia and headache. Long-term treatment may cause renal toxicity. Before treatment initiation and then at least twice-yearly blood tests including complete blood count, liver and renal function tests are recommended in case of chronic daily use [12, 44]

NSAIDs usually provide only a symptomatic effect on pain and stiffness without an adequate control of the inflammatory process, making the association with an adjunctive therapy often necessary.

2.6.2.2. Local glucocorticoid treatments

Intra-articular glucocorticoid injection is indicated in case of localized disease and should be considered as a first-line approach in presence of moderate disease activity or high disease activity involving less than four joints or in children who failed a NSAIDs treatment [12, 44].

Triamcinolone hexacetonide, thanks to its low solubility, has a slow absorption and a prolonged action. The recommended dosage is 10 to 20 mg/dose for larger joints, 2 to 6 mg/dose for smaller ones. Other molecules such as methylprednisolone acetate, or triamcinolone acetonide will be favored for the injection of small joints (more soluble molecule with less risk of extravasation) or in case of infiltration of tenosynovitis to minimize the risk of local atrophy associated with triamcinolone hexacetonide.

The effect of one injection lasts for at least several months; it is possible to repeat the procedure after a few months. Skin hypopigmentation and subcutaneous atrophy are common side effects.

Local ocular corticosteroid drops are used in combination with mydriatics in the treatment of anterior uveitis. Frequent relapses and /or dependence beyond 6 months should call for the installation or adaptation of systemic treatment.

2.6.2.3. Systemic glucocorticoids

Oral corticosteroid therapy (or sometimes intravenous in severe cases) is used mainly in sJIA. The most widely used molecule is prednisone, starting at a dose of 1 to 2 mg/kg depending on the severity of the clinical presentation and with a gradual decrease upon control of biological inflammation. Methylprednisolone boluses are reserved for severe cases with damage to a vital organ or MAS. Oral corticosteroid therapy can also be used in other forms, such as at low doses while waiting for the start of the disease-modifying treatment [12].

2.6.2.4. Nonbiologic disease-modifying antirheumatic drugs

Nonbiologic disease-modifying antirheumatic drugs, DMARDs, are classically considered first-line disease modifying therapy medications. They slow the disease progression, allowing the prevention of long-term morbidity, and the need of anti- inflammatory drugs [12].

Methotrexate

This is the "gold standard" treatment. It is prescribed at a dose of 15 mg/m^2 body surface area orally once a week; exceptionally, the dose may be increased to 20 mg/m^2. Its effectiveness does not begin to show on average until 6 weeks after start of treatment. In case of oral failure, subcutaneous administration improves the response. The main toxicity is hepatic, hence the need to monitor the liver function. It can cause digestive intolerance and sometimes even premature vomiting, thwarted by the combination of folic acid in treatment.

Sulfasalazine

This is a 5-aminosalicyclic acid analog able to interfere with various inflammatory pathways, and leukotriene and prostaglandin production. Sulfasalazine is mainly used in ERA, at a dose of 50 mg/kg/day in 4 doses. Adverse effects are represented by rash, leukopenia, hepatic cytolysis as well as digestive intolerance. It is contraindicated in sJIA due to the increased risk of MAS.

Other nonbiologic disease-modifying antirheumatic agents

In case of methotrexate intolerance or toxicity Leflunomide, that is a pyrimidine synthesis inhibitor, may represent an alternative treatment. It has been successfully used for JIA treatment, showing an equivalent effect to methotrexate [45]. Leflunomide however is officially indicated for the treatment of rheumatoid arthritis in adults, and its use in young patients is not recommended because its teratogenicity and long half-life.

Cyclosporine, hydroxychloroquine, azathioprine, thalidomide, tacrolimus, or cyclophosphamide have been used for the treatment of JIA; however, these drugs are not listed in international guidelines, and for them there are no clear recommendations. Cyclosporine has shown a positive effect in patients with sJIA-associated MAS.

Combination of different nonbiologic DMARDs is not recommended for the risk of additive immunosuppression and adverse effects.

2.6.2.5. Biological immunomodulators

Biological agents are treatments resulting from a biotechnological process; they changed the history of the disease. More and more, we rely less on corticosteroids and are moving towards biotherapy earlier. Biotherapy works against cytokines which are at the centre of the inflammatory reaction. They carry a theoretical risk of susceptibility to infection. Monitoring should watch out for undesirable effects such as disturbance of the hepatic assessment or cytopenia (neutropenia and thrombocytopenia).

Before starting treatment, latent tuberculosis should be ruled out by performing an intradermal test. In the long term, the data concerning the risk of neoplasia, initially feared, are reassuring.

TNF-α antagonists

Among the 5 anti-TNF agents available, 4 are used in paediatrics. The fifth, Certolizumab Pegol is currently in a phase III study for JIA.

All anti-TNF agents are theoretically similar in effectiveness and there is no preference in the choice of treatment except in cases of uveitis associated with JIA where only Adalimumab is approved.

Anti-TNF confer better efficacy when combined with MTX due to the action of MTX which inhibits the formation of autoantibodies against them.

Anti-interleukins

Several molecules that antagonize interleukins such as IL-1 and IL-6 have marketing authorization for JIA. They are generally used as a second line (anakinra in particular situations is used as first line treatment for SJIA, before resorting to corticosteroid therapy).

Abatacept (CTLA4-Ig)

It is a fusion protein that binds the extracellular domain of cytotoxic T lymphocyte antigen 4 (CTLA4) to the Fc fraction of immunoglobulin IgG1. It has a modulating effect on the co-stimulation of T lymphocytes.

Abatacept (Orencia®) has been shown to be effective in polyarticular forms refractory to MTX and even anti-TNF.

Two forms are available, intravenously from 6 years of age (10 mg / kg with a maximum of 1g every two weeks for the first 3 infusions then every 4 weeks) and subcutaneously from 2 years (50 mg for a weight <25 kg, 87.5 mg for a weight between 25 and 50 kg and 125 mg for a weight> 50 kg).

JAK inhibitors

Janus Kinases are part of a family of tyrosine kinases which through STAT phosphorylation activate an inflammatory cascade with stimulation of interleukins and INFγ. Among the Janus Kinase inhibitors, Tofacitinib, a selective inhibitor of JAK3, obtained authorization by the Food and Drug Administration for use in polyarticular JIA (or systemic with polyarticular evolution) from 2 years of age. The oral dose is titrated according to weight, with a maximum of 5 mg for a weight greater than 40 kg.

Rituximab

This anti-CD20 monoclonal antibody is reserved for refractory cases of JIA. It has been used off-label in polyarticular forms (especially RF positive) with satisfactory results.

Biosimilars

Biosimilars are "generics" of biotherapies. Studies in children are missing although some have received Marketing Authorization by analogy to the original products.

2.7. Prognosis

Oligoarticular disease usually has a very good prognosis, with a high percentage of children obtaining a permanent remission, especially those with a monoarticular involvement. On the other hand, one quarter of the cases may progress to persistent polyarticular disease.

Polyarthritis shows less frequently a prolonged remission. Symmetrical joint involvement, early wrist or hip inflammation, RF positivity, and prolonged active systemic disease are all factors related with poor long-term outcomes. However, children, compared with RF-positive adults affected by rheumatoid arthritis, are at less risk for some severe complications such as lung involvement or vasculitis.

Children with systemic-onset disease often have a very heavy systemic inflammatory condition with a high risk to develop MAS, and a variable response to medical therapies. The course of sJIA is highly variable, and a percentage of patients develop a severe polyarticular course resistant to medical treatments, with disease persisting into adulthood.

In recent years the prognosis of JIA has changed with the availability of novel pharmacological treatments able to inhibit the biological mechanisms involved in the persistence of inflammation.

Prompt and accurate diagnosis and early treatments started in the so called “window of opportunity” are crucial to prevent joint damage and preserve adequate functionality. Some studies highlighted the existence of a “window of opportunity” in early phase of the disease, during which prompt treatment predispose to higher rates of remission also improving long-term outcomes [46].

2.8. Pathogenesis

JIA is an umbrella term encompassing heterogeneous conditions that can be grouped in 3 major presentations: oligoarthritis, polyarthritis, and systemic onset diseases. Although the pathogenesis of JIA is mostly unknown, the first two forms of JIA appear as classical autoimmune conditions, induced by a breakdown of immunologic self-tolerance with a main involvement of the adaptive immunity, while in the systemic subtype the dysregulation of

innate immune pathways plays a central role, with increased levels of interleukins (IL)-1β, IL-6, and IL-18.

At the end, a final activation of monocytes and neutrophils represent the common pathological pathway inducing joint inflammation and damage.

2.8.1. Systemic onset disease

With respect to other forms of JIA, sJIA shows distinct systemic clinical features, such as the elevation of inflammatory markers, no association with HLA, no prominent involvement of autoreactive T cells, and absence of autoantibodies.

Recognition of some polymorphisms in cytokine genes and their receptors confirms a genetic predisposition. Genetic associations observed in familial hemophagocytic lympohistiocytosis have also been shown in patients with sJIA-associated MAS. The identification of pathogen-associated molecular patterns by TLRs present on innate immune cells, or of endogenous ligands triggers the activation of innate immune pathways, and induces the transcription of factor NFκB, leading to the up-regulation of genes encoding proinflammatory cytokines (especially IL-1 and IL-6, but also IL-18 and TNF). IL-1β plays a key role in the pathogenesis and activated peripheral blood mononuclear cells are responsible for the large production of IL-1β in these patients. IL-6 and IL-18 together with phagocyte-specific S100-proteins (S100A8, S100A9 and S100A12) are also strictly correlated with disease activity and complications.

The cytokines drive systemic signs, promote osteoclast-mediated bone resorption and the apoptosis of osteoblasts, while they inhibit chondrocyte proteoglycan synthesis. IL-1 and IL-18 also provide the perpetuation of the inflammatory responses.

An increase in circulating innate immune cells such as neutrophils, monocytes, and immature myelomonocytic precursors has been observed in active disease and an expanded expression of genes linked to the activation of the monocyte/macrophage lineage has been demonstrated by microarray studies [47, 48].

The identification of the central role played by cytokines in sJIA has been successfully translated into therapeutic approaches, and great results have been obtained inhibiting IL-1 and IL-6.

2.8.2. Poly and oligoarthritis

Genetics play a contributing role in the occurrence of JIA. Studies of monozygotic twins show a concordance of JIA of 25%, a risk clearly higher than the prevalence in the general population [49]. Likewise, the multiplex study of families with members suffering from JIA confirms a risk for siblings multiplied by 11.6 (range 4.9–27.5) compared to the general population [50].

Apart from the aggregation of familial cases of JIA, there is also a familial predisposition to other autoimmune diseases suggesting a common genetic susceptibility to different autoimmune phenotypes. The STAT4 and PTPN22 variants are examples of predisposition to multiple autoimmune diseases [51].

Except for a few publications of cases of monogenic disease due to mutations in the LACC1 gene [52] (Kallinich. Pediatr Rheumatol Online J. 2016), JIA is the result of a complex multigenic predisposition. The association of certain alleles of the HLA system with JIA is well known (HLA-A2 and HLA-B27 of the HLA class I system and HLA-DRB1 and HLA-DP of the HLA class II system). Since the function of molecules in the HLA system is to present antigens to CD4 and CD8 T lymphocytes, this association suggests the determining role of T lymphocytes in the development of JIA.

There are many environmental factors involved in the occurrence of JIA, many of them related to the microbiome. Among the protective factors, we note breastfeeding through bifidobacteria and immunological factors (soluble IgA, IgG, IL-10 and defensins), and early exposure to microbial diversity and increase in bifidobacteria commensals (hygiene theory) [53].

Risk factors include early exposure to antibiotics with a higher risk with repeated exposure [54]. Antibiotics are thought to alter the intestinal microbiome, leading to activation of pro-inflammatory cytokines and self-reactive T lymphocytes, contributing to the installation of autoimmunity [55]. Likewise, delivery by caesarean section constitutes a risk in relation to a lower microbial diversity compared to delivery by vaginal route. Infections have long been suggested as important in the pathogenesis of JIA: Borrelia in some cases of oligoarticular

JIA and Salmonella in some cases of enteropathy related to arthritis. Other examples of arthritis secondary to viruses (EBV, parvovirus B19) and bacteria (Mycoplasma, Campylobacter, Shigella and Streptococcus pyogenes) have been mentioned [56]. Other factors could also be incriminated such as diet, sun exposure and vitamin D level, passive smoking, socio-economic level, pollutants.

Inflamed synovium is exposed to leukocyte infiltration, and T-cell infiltrates are predominantly represented by B lymphocytes, dendritic cells, and macrophages. Mostly, CD4+, T-helper (Th) cells seem to play a key role in the pathogenesis of this condition [57, 58]. Th lymphocytes are crucial in starting and perpetuating diverse immune responses. These cells show a high plasticity upon receiving extrinsic signals. These lymphocytes are activated via T cell receptor engagement in association with cytokines and costimulatory molecules released by innate immune cells and then differentiate in functionally different Th cells.

Different subsets of lymphocytes could be distinguished on the basis of their characteristic immunological profile function, cytokines production and distinctive transcription factor expression [59-61]

Classically, Th1 lymphocytes are characterized by the expression of the transcription factor T-bet, the production of gamma interferon (IFN-γ), and their main role is the regulation of cellular immunity, and the defence from intracellular infections.

Th2 cells exhibit the transcription factor GATA-3, are able to release type 2 cytokines (IL-4, IL-13, IL-5, IL-9) and play a role against helminths [62].

A third subset of CD4+ T cells are the regulatory T cells, Tregs, characterized by the expression of FoxP3, with a main role in regulating the immune response, maintaining self-tolerance and homeostasis [63].

In recent years another lineage of helper T cells has been recognized: the Th17 subset. These cells can be distinguished by the expression of the transcription factor ROR-γt [64-66]. They produce IL-17A, IL-17F and IL-22, show the lectin receptor CD161 as typical surface marker, and their role in the immune response is mainly to protect against extracellular bacterial and

fungal infections [67-69]. They also play a significant role in mucosal immunity and in autoimmune conditions [70].

In humans, Th17 lymphocytes derive from a CD161+ naive CD4 (+) T cell of umbilical cord blood and neonatal thymus [69].

A network of inflammatory cytokines such as IL-1, IL-6, Il-23, and TGF-β released by professional antigen-presenting cells support the evolution of Th17 cells from naïve Th cells [71] (Dong. Nat Rev Immunol. 2008. Then, the IL-6-JAK-STAT3 axis translate in the upregulation of transcription factors RORγt and RORα, inducing the typical signature cytokines IL-17A, IL-17F, and IL-22 [70, 71]. These cytokines favour mucosal host defence, and recruit neutrophils.

Th17 cells are implied in the pathogenesis of many autoimmune, inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, and psoriasis [73]. In rheumatoid arthritis it has been postulated that self-reactive (also called arthritogenic) Th cells evolve in effector Th17 cells, infiltrate the synovium and initiate joint inflammation [74]. Activated antigen-presenting cells expose self-peptide to self-reactive T cells, and release a large amount of IL-6, IL-1, IL-23, and TNFα, that, together with the surrounding tissue-derived TGF-β, trigger the differentiation to effector Th17 cells. Synoviocytes further recruit Th17 thanks to cytokines (such as IL-1β, IL-17, and TNFα) produced from both activated synoviocytes and Th17 cells [74]. Indeed, sinovial fibroblasts have been shown to be involved in cartilage destruction through cytokines and matrix-degrading enzymes release [75].

Studies in animal models of autoimmune arthritis have shown the pivotal function played by Th17 cells in the pathogenesis of arthritis and their role has been suggested also for juvenile arthritis [76].

The synovial fluid from active joints of oligoarticular JIA children has emerged to be enriched of CD161 + T cells Th17 cells [75].

However, compared to Th1 cells, Th17 appear clearly less represented at inflammatory sites, and indeed inflamed joints are rather infiltrated by lymphocytes with an intermediated phenotype between Th1 and Th17 cells. The reason for this is the high plasticity of pure Th17 cells that, in presence of IL-12 and TNF-α, rapidly evolve into a hybrid phenotype, called

non-classic Th1, sharing many features with Th1 cells [77]. The presence of non classic Th1 in the synovial fluid of inflamed joints of children affected by JIA correlate with the markers of disease activity [75,78].

Non classic Th1 still express specific molecules of pure Th17 cells from which they derive, such as CD161, RORC, CCR6, IL4I1, and IL-17 receptor and share with them T-cell receptor clonality (thus supporting their origin from Th17). However, non classic Th1 lose the ability to produce Il-17 and acquire the those to produce IFN-γ and GM-CSF [79, 80].

Synovial fibroblasts, granulocytes, and macrophages are involved in joint damage acting as the effector arm of the imbalanced immune response acting through the release of different proteases such as cell-associated plasminogen activation system, matrix-metallo proteinases (MMPs), acid-hydrolases and cathepsins [81]. MMPs are a group of enzymes exerting different catalytic actions, based on which they are categorized in different subgroups such as gelatinases MMP2 and MMP9, collagenases, or matrilysins. The cell-associated plasminogen activation system encompasses the serine-proteinase urokinase plasminogen activator (uPA) and its receptor (uPAR). The activation of uPA induces a series of proteolytic events also including MMPs, leading to the degradation of the macromolecules of the extracellular matrix [81]. The final protease effect is related to the balance between enzymes and their specific inhibitors.

A growing interest is currently developing in the field of oncology around T lymphocyte regulation mediated by the so-called immune checkpoints, whose manipulation represents a potential powerful tool against cancer. Immune checkpoints are critical molecules with an inhibitory or stimulatory function, involved in the regulatory pathways of the immune system. The development of the revolutionary anti-cancer therapy with immune checkpoint blockade made James Allison and Tasuku Honjo to win the 2018 Nobel Prize in Medicine. Programmed death-1 (PD-1) and its ligands (PD-Ls) were first identified in 1992, since that time several other immune checkpoints have been recognized [82].

The United States Food and Drug Administration has approved anti-PD1 and anti-PD-L1 antibodies; despite a successful action against cancer, an increasing number of autoimmune complications have been reported in association with these treatments [83].

This aspect is now drawing the attention of rheumatologists, raising the question of how defects in immune checkpoints can contribute to the pathogenesis of rheumatological diseases.

PD-1, or CD279, is a cell surface receptor mostly expressed on T cells, B cells, myeloid cells, natural killer cells, and thymus cells; it belongs to CD28/B7 family of receptors.

PD-1/PD-L signaling pathway acts as a negative regulator on T cell receptor-mediated lymphocyte proliferation, cytokine release, and CD28-mediated costimulation [84, 85].

The T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT, also called WUCAM, Vstm3, VSIG9) is a co-inhibitory receptor belonging to Ig superfamily mainly expressed on T cells; it is involved in a complex regulatory network involving multiple checkpoint inhibitory receptors [86]. Several studies have investigated the role of these molecules both in humans and in animal models of rheumatoid arthritis, however their role in the pathogenesis is still unclear [87].

In the paediatric rheumatology filed the study of the potential role of immune checkpoints in the pathogenesis of JIA is still a pioneering topic [88].

3. AIM OF THE STUDY

The present study is focused on:

1. the definition of the respective role of Th1 and Th17 cells in cartilage degradation and synovial pannus vascularization in JIA; and

2. the evaluation of the possible role of secukinumab in interfering with human cartilage degradation and synovial pannus vascularization in an in vitro and in vivo model.

4. MATERIALS AND METHODS

4.1. Patients and samples

SFbs were obtained according to previously described processes from eight patients with a mean age of nine years (range six–fifteen years) affected by oligoarthritis, three of whom received methotrexate, and four NSAIDs, and from six healthy controls with a mean age mean of 8 years (range: five–ten years) [89,90].

Inflamed synovial fluid samples were taken from JIA patients during routine knee arthrocentesis, while healthy synovial tissue was obtained from children who underwent orthopaedic surgery of the knee after traumatic events. Healthy controls had no chronic inflammatory diseases nor recent pharmacological treatments; their SFbs were defined "control" or "normal". Normal human cartilage was acquired from the non-arthritic knees of children undergoing routine surgery.

The approval of the Ethics Committee of Anna Meyer Children Hospital, Florence, Italy, and informed written consent by parents or guardians were obtained. All the procedures have been done according to the Declaration of Helsinki.

4.2. Isolation and culture of synovial fibroblasts

SFbs were isolated following the protocol previously described [89, 90].

Synovial tissue obtained from normal controls was minced into small pieces, then plated with FBM-2 (Fibroblast Growth Medium, Microtech, Napoli, Italy) in culture dishes, and added with 10% FBS for expansion. SFbs from JIA were selected by centrifugation of SF obtained from active joints of JIA children. SFbs were then characterized and used within the seventh passage in culture.

4.3. Isolation and characterization of T-cells and production of supernatants

Isolation and characterization of T cells were obtained as previously described [78, 90].

Selected T-cell clones with the following phenotypes: Th17 (CD161+IL-17+IFNγ-), nonclassic and classic Th1 (CD161+IL17-IFNγ+ and CD161-IL17-IFNγ+, respectively), were obtained from peripheral blood of healthy donors and polyclonally stimulated with anti-CD3 plus anti-CD28 mAb (human T-Cell Activation/Expansion Kit, Miltenyi Biotec), for 72 h to obtain culture conditioned media (CM), that were stored at −30°C. TNF-α, IFN-γ, and IL-17 levels were evaluated in the supernatants of T-cell clones by CBA flex set assay following the manufacturer instruction (BD Bioscience).

4.4. Exposition of synovial fibroblasts to T-cell clones conditioned supernatants and cytokines

SFbs from healthy children were plated in T25 flasks and grown to approximately 80% confluency, then incubated for 48 h in medium alone or in association with unstimulated or CD3/CD28-stimulated CM from Th1 (classic and nonclassic) or Th17 clones or with recombinant TNF-α (10 ng/mL), IFN-γ (5 ng/mL), and their combination. SFbs were then recovered by trypsinization and used for real time (RT) PCR analyses, for invasion and collagen degradation assays. Media from pre-treated SFbs cultures as indicated above were collected for zymographic analyses of gelatinase activity.

4.5. Quantitative Real-time PCR analysis

Total RNA was prepared utilizing Trizol reagent, Invitrogen, agarose gel checked for integrity, and reverse transcribed with iScript cDNA Synthesis Kit as manufacturer's instructions. mRNA expression of selected genes was determined by RT PCR using the primers (IDT, TemaRicerca) under various conditions. We used a Quantitative RT-PCR with an Applied Biosystem 7500 Fast RT-PCR System (Applied Biosystem) employing a SYBR green-based detection with the following default PCR settings: 40 cycles of 95°C for 15 s and of 60°C for 60 s. The "Delta-delta method" was used to compare relative gene expression results with 18S ribosomal RNA as the housekeeping gene. The data were then normalized to results obtained in untreated SFbs.

4.6. Matrix metalloproteinases Gelatin Zymography, and uPA activity measurement

Zymographies were done as described [91]. Urokinase plasminogen activator, uPA, activity was measured by a colorimetric assay.

SFbs (25×10^3) were seeded in RPMI 1640 with 10% FCS. Cells were than washed three times with serum-free medium at semi confluence and incubated in 0.2% FCS medium until confluence, detached, and finally counted. Aliquots of 30 μL of supernatants from normal SFbs before and after treatment with different Th1-CD161+ sub- sets, and from JIA SFbs cultures have been subjected to electrophoresed through 10% Novex Zymogram Gelatin Gels, Invitrogen, and developed following the manufacturer's instructions. Gelatinolytic activity attributable to MMPs appeared in gels as a transparent band *versus* a blue background of stained gelatine. Bands were quantified by ImageJ software. Urokinase plasminogen activator (uPA) activity was quantified on aliquots of culture medium by a colorimetric assay, Abcam.

4.7. Collagen degradation assay

Collagen degradation assay was conducted as as previously reported [92].

A Matrigel layer with 2% FITC-labelled collagen monomers was used. SFbs from healthy donors and JIA SFbs suspensions were copolymerized with Matrigel containing 2% FITC-labelled collagen. Migration was observed for 40 h. At the end of incubation, solid-phase collagen containing the cells was pelleted, whereas by spectrofluorometry FITC released into the supernatant was studied. Results have been expressed as percentages respect to complete collagenase digestion of cell-free collagen lattices (100%).

4.8. Invasion assay in Boyden chambers

Boyden chambers were used to analyse the invasion. The upper and lower compartments were separated by 8 μm pore size polycarbonate membranes coated with Matrigel (50 μg/filter) (BD Biosciences).

In chemoinvasion experiments, normal SFbs (2X104 cells) were suspended in 200 μL FMB-2 plus 2% FBS and placed in upper compartment, while in the lower one 200 μL of T-cell clones CM or unconditioned FBM plus 2% FBS (used as control) were placed.

In spontaneous invasion experiments, 2X104 normal subjects-derived SFbs or JIA derived SFbs were suspended in 200 μL of FBM-2 plus 2% FBS and placed in the upper compartment of Boyden chambers. Fresh FBM-2 plus 2% FBS was also put in the lower area. In both experiments, invasion was performed for 6 h at 37°C in 5% CO2, then filters were recovered and fixed in methanol. Non-invading cells on the upper area of the filter were separated with a cotton swab while invasive cells that appeared adherent on the lower filter surface were stained and counted utilizing a light microscope. Results were expressed as number (± SD) of migrated cells.

4.9. In vivo experiments: inverse wrap implantation technique in a SCID mouse model and histological analysis

The "inverse wrap" technology in the SCID mouse model was used, as previously described (Figure 1) [89, 93].

Four-week-old SCID mice from a germ-free breeding colony (Charles River) were used for the experiment. Synthetic gelatin-sponge containing a piece of cartilage was soaked with almost 5X105 JIA derived SFbs or healthy SFbs suspended in sterile saline. Four of these sponges containing cartilage and SFbs were placed under the skin of anesthetized mice.

Mice were divided in 8 groups including three mice each on the base of the treatment received:

Group 1, controls with implants that did not undergo treatment for 60 days, used to analyse basal cartilage degradation in the absence of JIA or healthy-SFbs stimulation.

Group 2, mice implanted with JIA SFbs that did not undergo treatment for 60 days.

Group 3, mice implanted with normal SFbs and weekly treated with subcutaneous injections around the transplant for 60 days, with 50 μL of CM of no stimulated classic-Th1.

Group 4, treated according to the same schedule of group 3 but with medium of activated classic Th1 cells.

Groups 5 and 6, mice treated with weekly injections of medium from unstimulated and

stimulated nonclassic Th1, respectively.

Groups 7 and 8, mice treated with weekly injections of medium from unstimulated and stimulated Th17 cells.

Experiments were repeated three times with cartilages from three different donors. After sixty days, the mice were killed, and the implants were removed, embedded in TissueTek embedding medium (Miles, Elkhart, IN, USA), snapfrozen, and conserved at -70°C.

Each cartilage specimen was hematoxylin/eosin, and CD45 IHC stained and evaluated by Image-G, measuring the percent of degradation.

All in *vivo* procedures adopted were approved by the ethical committee of Animal Welfare Office of Italian Work Ministry and resulted to be conform to the legal mandates and Italian guidelines for the care and maintenance of laboratory animals.

4.10. 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.

5. RESULTS

5.1. Study of protease systems and invasive ability of JIA and normal SFbs

The fibrinolytic system (uPA, uPAR) and collagenases (MMP2, MMP9), that represent the main proteases system implied in the invasion and degradation of joint cartilage in JIA have been analysed comparing normal *versus* JIA SFbs.

As shown in Figure 2, according to the results of Real-Time PCR, MMP2 and mostly MMP9 appeared clearly up-regulated in JIA SFbs compared with normal SFbs, inducing cell surface-associated fibrinolysis and pro-MMPs activation.

MMP-activity has been detected by gelatin- zymography with 48h-conditioned media (CM) obtained from healthy and JIA SFbs: MMP9 activity was strongly increased in association with JIA SFbs compared to healthy SFbs. Similar, SFbs obtained from JIA patients and co-polymerized with Matrigel-2% FITC showed a high grade of collagen degradation assay in *vitro*. We also observed JIA SFbs to exert a high invasive ability in Boyden migration chambers, that was possible to prevent, incorporating the polymerized Matrigel with Ilomastat, a matrix metalloproteinase inhibitor.

All this data explain how MMP9 activity is increased in JIA SFbs with respect to normal SFbs, and how it mediates cartilage invasion and degradation.

5.2. Analysis of cartilage degradation in the SCID Mouse model

The "inverse wrap" implantation technique in SCID mice was used to study cartilage degradation. In Figure 3 the results related to the human cartilage degradation mediated by normal and JIA SFbs, documented 60 days after implantation are reported. Normal SFbs are associated with a poor cartilage infiltration and a mild infiltration of CD45+ inflammatory cells, while in the presence of JIA SFbs a clear cartilage degradation in association with an intense infiltration of CD45+ cells was detected.

5.3. Analysis of different CD4+ T-cell clones conditioned media and cytokines on protease systems and invasion ability of healthy SFbs

We have analyzed the influence of different CD4+ T-cell clones CM and cytokines on normal SFbs in terms of cartilage degradation ability (Figures 4 and 5).

It has already been documented how CM of CD4+ T-cell clones do not modulate proliferation and viability of SFbs [90].

Densitometric analysis of the zymogram for MMP2 and MMP9 and a colorimetric test for uPA have shown that exposition of normal SFbs to activated Th17 lymphocyte supernatant

increases the activity of MMP1 and MMP9, and those exposed to activated non- classical Th1 CM causes the expression of a strong uPA activity.

An increase of invasion of normal SFbs was observed at the Boyden-chamber Matrigel migration when exposed to CM from activated Th17 T cell clones. A similar effect was documented in non classic-Th1 clones.

Analyzing the effects of single cytokines, we observed that IL-17A induced an up-regulation in normal SFbs of MMP2, MMP9, uPA and uPAR, while the TNF-α/IFN-γ combined conditioning activates an overexpression of uPAR and causes a down-regulation of MMP2 and uPA while no significant effects were observed in MMP9.

MMP9 activity appeared to be increased at Gelatin zymography assay in the supernatants of healthy SFbs treated with IL-17A.

IL-17 also promotes the invasion of SFbs, that instead appeared less influenced by TNF-α/IFN-γ combined treatment. The reduction of invasion in the presence of Ilomastat, that inhibits MMPs, demonstrates that Th17- related SFbs invasiveness is mediated by MMPs.

CM from stimulated Th17 and non-classic-Th1 activates normal SFbs, triggering matrix-lysing enzyme production and cell invasion.

5.4. Analysis of CD4+ T cell clones conditioned media on cartilage degradation *in vivo*

We translated our results in vivo by the inverse wrap implantation technique, as shown in Figure 6.

A consistent cartilage degradation was documented in pieces treated with stimulated Th17 and non-classic-Th1 CM respect to CM from unstimulated cells and to healthy control (untreated SFbs). CD45 staining showed infiltration of inflammatory cells in pieces treated with classic and non-classic-Th1 CM, while those treated with stimulated-Th17 CM were characterized by SFbs penetrating deeply into the cartilage, and only few CD45+ cells.

5.5. Secukinumab effect on *in vitro* and *in vivo* experiments

In the final part of our experiments, we wanted to demonstrate that the effect of Th17 on SFbs is mediated by IL-17A. Secukinumab, that is a fully human monoclonal antibody, mAb, has been used for *in vitro* and *in vivo* experiments (Figure 7).

We ruled out the interference of secukinumab in the proliferation of SFbs.

We quantified IL-8, that is known to be released by RA SFbs exposed to IL-17A, after incubation of healthy SFbs with the CM of Th17 cells, in the presence and absence of secukinumab [94].

IL-8 increased in association with activated Th17, while the addition of secukinumab blocked of this production.

In the *in vitro* experiments we demonstrated that secukinumab is able to inhibit SFbs invasion of Matrigel-coated membranes promoted by activated Th17, but not that induced by activated non-classic Th1. MMP9 production related to activated Th17 could also be blocked with Secukinumab).

The *in vivo* and *in vitro* experiments with the inverse wrap technique have been repeated using only CM from activated and non-activated Th17 cells, that were than weekly administered for a period of two months in addition or not with secukinumab. In mice treated with secukinumab the SFbs-dependent cartilage degradation mediated by CM from activated Th17 was abolished.

6. DISCUSSION

Several cell types such as T and B lymphocytes, synovial fibroblasts, monocytes/macrophages, granulocytes, and osteoclasts are involved in joint tissue damage in chronic autoimmune arthritis, playing different, integrating functions. Monocytes/macrophages and granulocytes cooperate in triggering and amplifying inflammation, while activated osteoclasts induce bone resorption leading to bone erosion and predisposing to local and systemic osteoporosis, but Th cells are the real drivers and activators of the immunological orchestra at the origin of the autoimmune process. The CD4 Th population embraces several effector subsets with distinct surface markers, transcriptional regulators, effector molecules, cytokines and different functional roles. Additionally, these cells have shown to be plastic, able to modify their phenotype in response to specific stimuli in a context-dependent manner, switching their characteristics in relation to the new environment.

In JIA Th1-skewed cells infiltrate the synovial membrane in response to the chemokine CXCL10, resulting the most represented lymphocytes at the inflamed site and, on the basis of this observation, they have been hyphotesized to be the main effectors of the pathogenetic mechanisms [95].

More recently, a new subset of CD4+ T helper cells, called Th17 cells, has been identified. These cells are involved in host protection against extracellular bacteria. However, growing experimental data has demonstrated their prominent role also in many autoimmune diseases, including adult and childhood arthritis [76]. The synovial fluid from active joints of children affected by JIA is enriched of cells producing IL-17A, and these cells express high levels of ROR-γT, their typical transcription factor. Th17s themselves appeared to be increased, however they are less represented when compared to Th1 [97].

A possible explanation of this seems to be related with their attitude to switch to another, more aggressive phenotype. Th17 cells infiltrating the inflamed areas evolve to an intermediate phenotype called Th17/Th1 that expresses the ability to produce IFN-γ but still can produce IL-17. In a second phase these cells rapidly loose this ability, maintaining only the capacity to secrete IFN-γ [64, 78, 79].

Since these Th1 lymphocytes still show some Th17 characteristics such as the expression of ROR-γT, CD161, and CCR6 they are defined as non-classic Th1 cells [75, 79, 98].
How these different Th subsets are involved in the pathogenesis of JIA is still unclear, especially in which way they orchestrate the engagement of tissue resident cells and recruit circulating leukocytes in the inflammatory process.

We first compared the role of healthy and JIA synovial fibroblasts in cartilage aggression, showing that JIA SFbs are responsible for a consistent cartilage damage associated with a concomitant inflammatory infiltration. A mixed population of CD45 positive leukocytes and of fibroblasts was present at the histological examination of cartilage, suggesting that both these cell types are involved in cartilage degradation: fibroblasts in a direct way, leukocytes in an indirect one, amplifying the inflammatory process.

Cartilage degradation is mainly linked to the fibrinolytic system (uPAR/uPA/PAI-1) and that of the MMPs/TIMPs [99].

Many studies have documented the involvement of different components of the plasminogen activation system in the pathophysiology of chronic arthritis, especially of uPA mediating bone and cartilage destruction in inflammatory conditions [100].

Serratì et al showed how the inhibition of uPA and uPAR decreases the cartilage invasion in *in vitro* and *in vivo* experiments [89], and others showed that antibodies against the catalytic site of uPA reduce the disease progression in mouse arthritis models [99].

MMPs are powerful proteolytic enzymes able to act on the whole range of extracellular matrix proteins, mediating cellular invasiveness in different conditions such as rheumatoid arthritis, tumor metastases, and osteoarthritis [101,102].

Resident joint cells, such as SFbs and chondrocytes, express different MMP, some of which are crucial for the regulation of cartilage plasticity and turnover in physiologic and pathologic conditions [103]. SFbs are able to produce collagenase MMP1, gelatinases MMP2 and MMP9, stromelysin MMP3, and chondrocytes collagenase MMP13, primary involved in articular non-collagen matrix components degradation [103]. A previous study has documented in patients affected by rheumatoid arthritis an increase in plasma levels of soluble uPAR, with values that parallel the erosive activity in joints [104].

In vitro studies conducted in the first part of our study demonstrated that JIA SFbs release higher levels of MMP9 and uPAR than SFbs obtained from healthy subjects. JIA SFbs are also able to invade the cartilage as observed in the spontaneous matrigel invasion tests, and this action is mainly MMP9-mediated, as revealed by the inhibitory effect obtained adding Ilomastat, that is a MMP-9 inhibitor.

As our initial data suggested a different behaviour of JIA SFbs compared to normal ones, we focused the subsequent experiments in analysing the effect of CM and cytokines from different CD4+ subsets on normal SFbs.

We *in vitro* analysed the effects on protease system incubating normal SFbs with stimulated and unstimulated media of classic and non-classic-Th1 and of main selected cytokines that were TNF-a and IFN-γ for classical and non- classical Th1, IL-17A and IL-17F for Th17. CM obtained from activated non classic Th1 triggered an over-activity of uPA in healthy SFbs, while the CM of activated Th17 triggered *in vitro* the expression and the activity of MMP9 and MMP2 and stimulated SFbs invasion. The invasion ability of SFbs could be inhibited by Ilomastat, demonstrating the key role played by this protease.

IL-17 emerged as the cytokine responsible of the up-regulation, both in terms of expression and activity, of all the main protease enzymes, finally promoting SFbs invasion, and cartilage degradation.

To confirm these data, we proceeded with an *in vivo* experiment based on the inverse wrap technique on a SCID mouse, evaluating the effect of different conditioned media obtained from subsets of activated and non-activated CD4+ lymphocytes. Resident articular SFbs resulted to be involved in the inflammatory process by activated Th17 cells that promoted their stimulation and their capability to infiltrate and destroy joint cartilage. We also showed that this passage is mediated by the action of IL-17, selectively inhibiting it with the monoclonal anti-IL17A antibody, secukinumab. This was previously demonstrated in the *in vitro* experiment as well.

On the other hand, classic and non-classic Th1 induced the retention of circulating leukocytes in the inflamed joints, triggering the expression of CD106 (VCAM-1) on SFbs thanks to the action of TNF-α, as already observed by Maggi et al [90].

Therefore, different CD4+ Th lymphocytes play different roles in the pathogenesis of JIA influencing SFbs in distinct ways including the protease production that is mainly represented by uPA in relation with non-classic-Th1 and by MMPs in relation with Th17.

In conclusion, these results reveal the correlation between cartilage degradation with protease system and identifies the role of different JIA-associated Th subpopulations (non-classic Th1 and Th17) in the pathogenesis of immune-mediated synovial degradation in JIA.

Classic and non-classic-Th1 promote CD106 (VCAM-1) expression in SFbs through TNF-α, thus attracting circulating leukocytes in the inflamed joints; activated non-classic Th1 lymphocytes in an uPA/uPAR-dependent manner promote cartilage degradation, and Th17 lymphocytes through the release of IL-17 activate SFbs, stimulating their invasive and destructive ability via MMP9/MMP2.

Understanding the cross-talks between SFbs and the cytokines produced by different subsets of CD4+ T cells in the pathogenesis of joint cartilage damage may represent the base to create new targeted therapeutic strategies for children affected by JIA and other immune-mediated articular inflammations. This has particular importance considering the failures obtained with anti-protease therapy in cancer clinical trials: the direct inhibition of MMPs has in fact yielded unsatisfactory or even counterproductive results [105]. The identification of immune cell subpopulations that induce protease over-production, pave the way for the identification of biological therapeutics capable of blocking their activity.

7. References

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8. Tables and Figures

Table 1. Main features of JIA categories (ILAR classification).

Subtype	Arthritis	Uveitis	Other features	Laboratory data
Systemic	Arthritis usually presents later, often weeks after the disease onset and it can be preceded by arthralgias. All joints can be involved; ankles and knee toghether with wrist are usually the first to be interested. Almosta half of the patients develop a chronic arthritis with a progressive course.	Abstent	Intermittent fever, adenopathy often generalized, evanescent eruption in trunk in associacion with fever Hepato and splenomegaly Serositis.	Severe increase in inflammatory markers, inclusive of ferritin, fibrinogen. Increase of WBC, platelets.
Oligoarthritis	Monoarticular arthritis is very frequent. Arthritis usually affects large joints of the lower extremities.	80% Chronic anterior uveitis, typically pauci/asymptomatic (white eye uveitis), tipically ANA-associated		ANA
Polyarthritis	RF negative subtype: similar to oligoarticular JIA with a more extended arthritis also involving small joints, TMJ, cervical spine. RF positivesubtyper: erosive arthritis with a symmetric pattern, mostly affecting small joints of the hands.	50%	RF positive: Evolution similar to adult Rheumatoid arthritis	Increased inflammatory markers ANA RF CCP
Enthesitis-related arthritis	Asymmetric arthritis with a typical involvement of the joints of lower limbs, but also sacroiliac joints.	10-20%. Acute, symptomatic anterior uveitis (red eye); occasionally associated IBD	Enthesitis (; Achilles tendon, patellar tendon,…) Association with IBD	HLA-B27
Psoriatic arthritis	Expression variable: from a symmetrical small-joint type to an asymmetrical lower-extremity large-joint presentation. Some adolescents may express features of ERA	10-15%	Psoriasis, onycholysis, nail pitting, dactylitis	ANA Increased/normal inflammatory markers Mild anemia

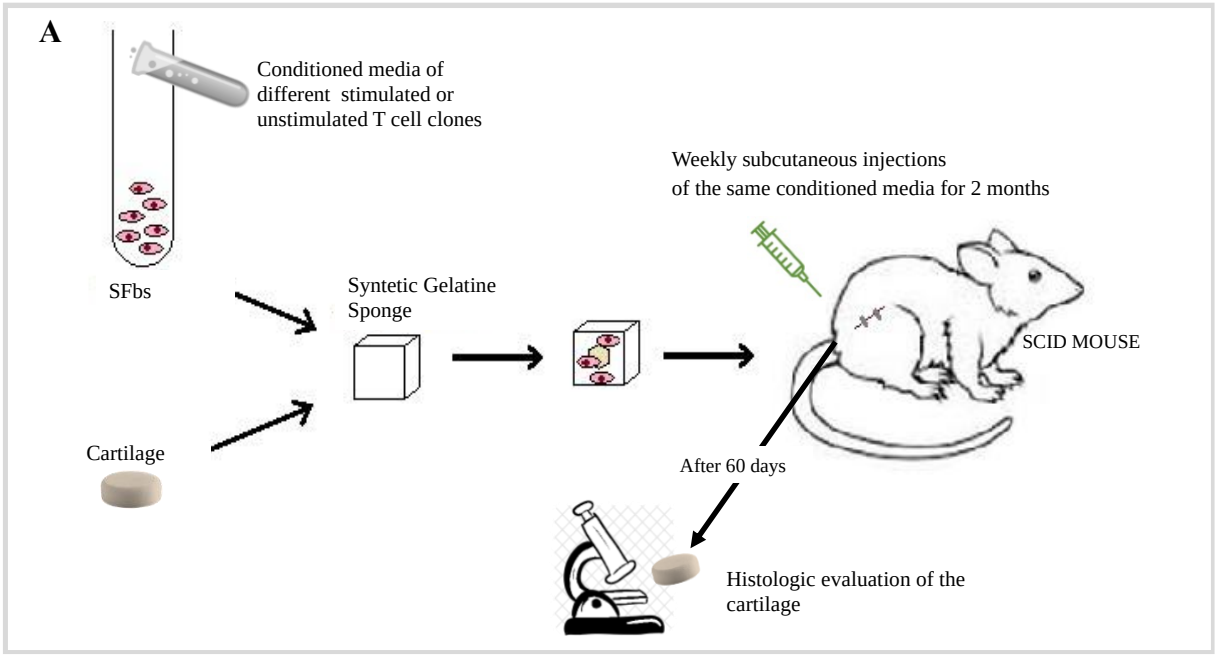
ESR: Erythrocyte sedimentation rate. CRP: C-reactive protein. ANA: Anti-nuclear antibody. IBD: Inflammatory bowel disease. TMJ: temporomandibular joint.

Figure 1. Inverse wrap implantation technique in a SCID mouse model.

A. Scheme of the procedure: synthetic gelatin-sponge containing a piece of cartilage is soaked with JIA derived synovial fibroblasts (SFbs) or healthy SFbs suspended in sterile saline. Four of these sponges containing cartilage and SFbs are placed under the skin of anesthetized mice.

After sixty days, the mice are killed, and the implants removed. Each cartilage specimen is hematoxylin/eosin, and CD45 IHC stained and evaluated by Image-G, measuring the percent of degradation.

B. Photo of surgical implantation of the sponge under the skin of the mouse.



B

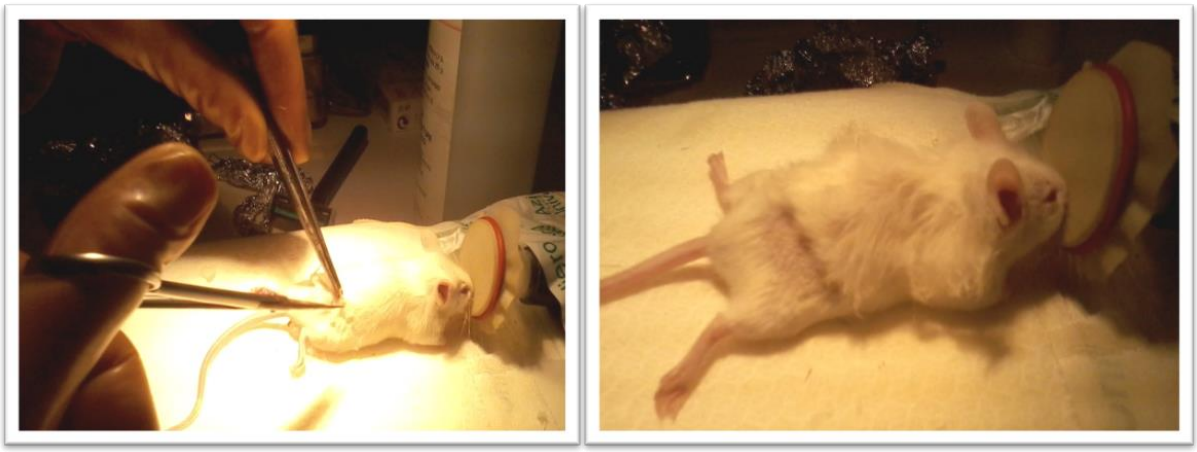
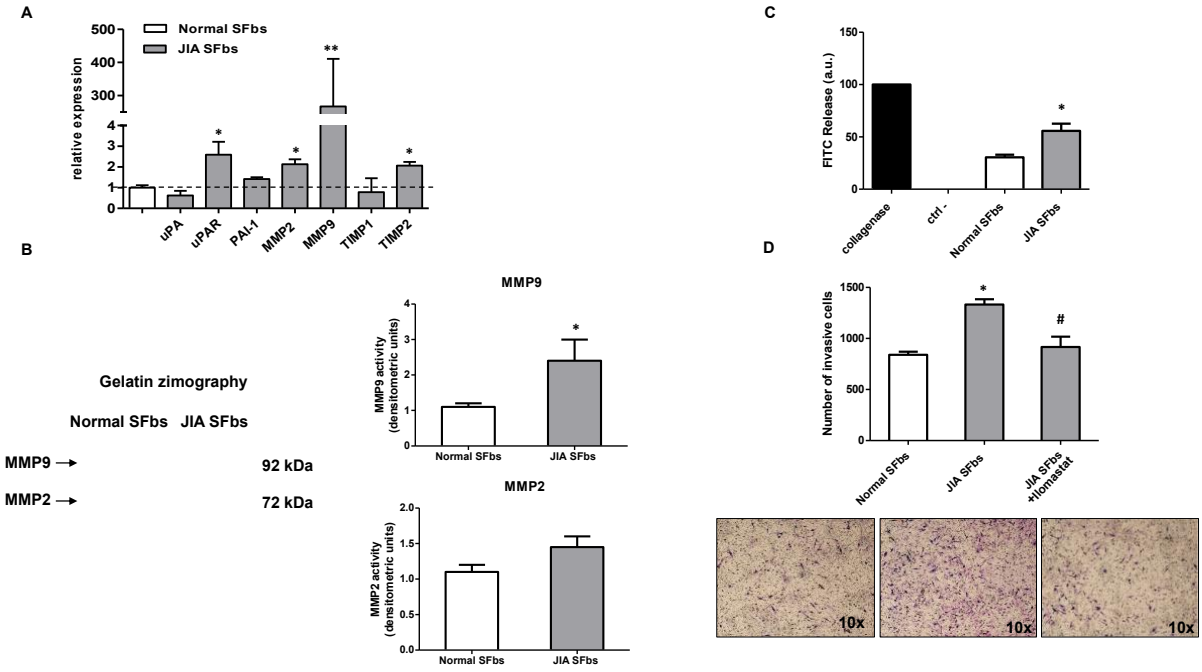


Figure 2. Protease activity and invasion properties of healthy *versus* JIA synovial fibroblasts (SFbs)



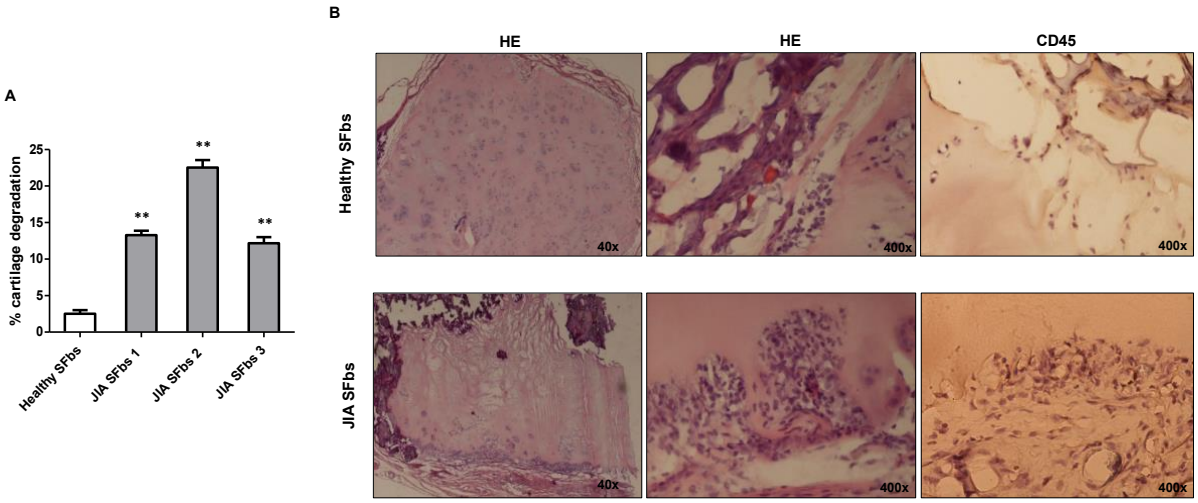
A. Real Time PCR detected the expression of MMP2, MMP9, uPA, uPAR, PAI-1, TIMP1 and TIMP2 from normal and JIA SFbs were analyzed for. Histogram reports the mean of three different experiments ± SD.

B. 24h conditioned media were collected from normal and JIA SFbs and gelatinolytic activities were evaluated by gelatin zymography. Typical zymography gel bands are shown on the left.

C. Healthy and JIA SFbs suspensions were co-polymerized with Matrigel containing 2% FITC-labelled collagen. After 40h of incubation, FITC release was measured.

D. Normal and JIA SFbs were suspended in DMEM 2% FBS and placed in the upper well of Boyden chambers in presence or absence of Ilomastat. Representative microphotographs (10X) of migrated cells are shown under the respective histogram. * Shows statistical significance ($p < .05$) compared to Normal SFbs, # shows statistical significance ($p < .05$) compared to JIA SFbs.

Figure 3. *In vivo* Cartilage degradation by JIA synovial fibroblasts (SFbs) in the SCID Mouse model.



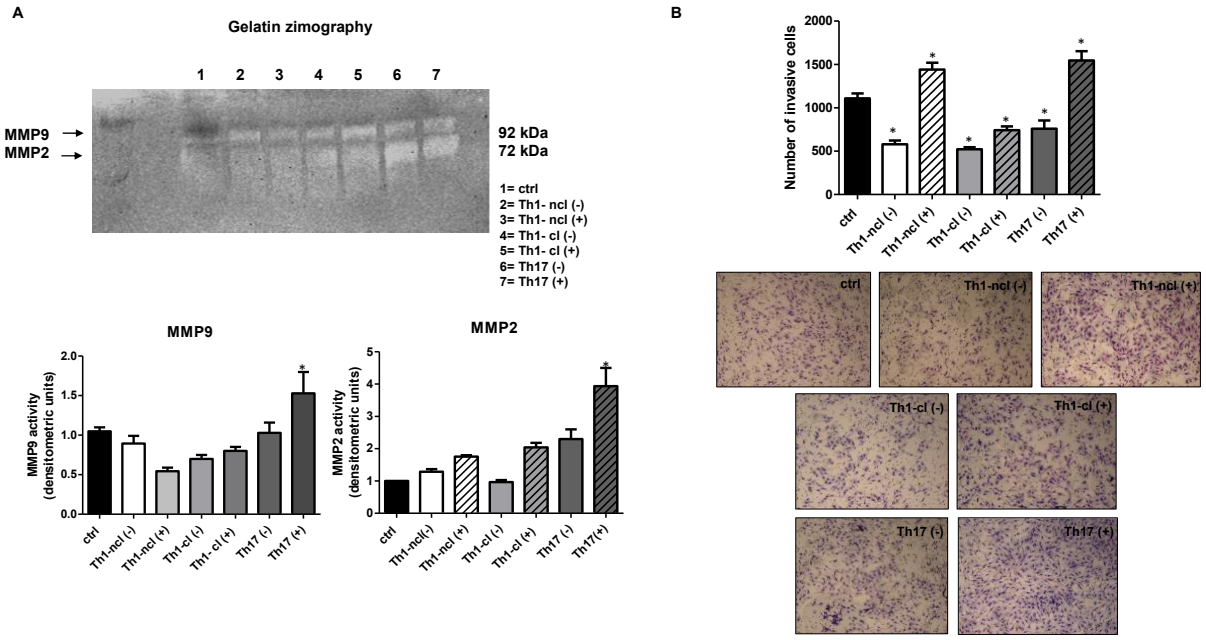
Human cartilage degradation by normal and JIA SFbs in SCID mouse model 60 days after implantantion.

A. Histogram corresponds to the percentage of cartilage degradation detected by Image J software, in each implant.

B. Hematoxylin-Eosin and CD45 stained specimens showed the degree of cartilage degradation by SFbs and inflammatory cells infiltration respectively.

** shows high statistical significance ($p < .01$) compared to Normal SFbs.

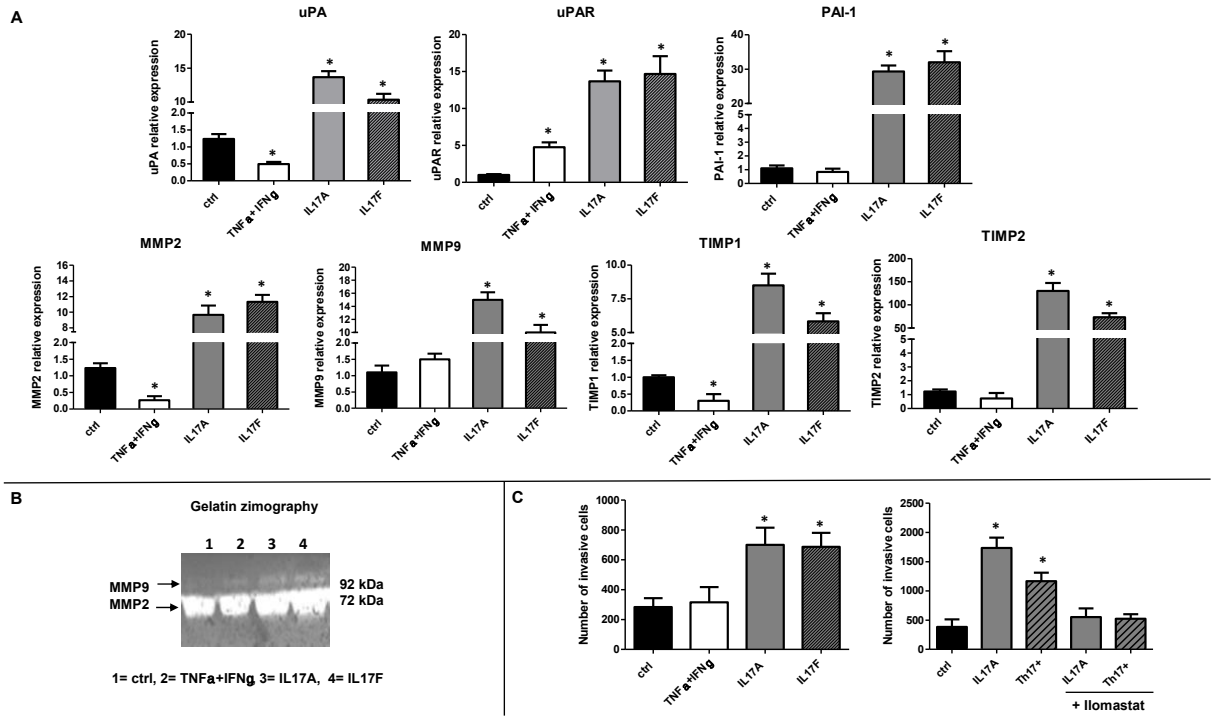
Figure 4. Effects of different CD4+ T cell clones conditioned supernatants on protease activity and invasion ability of healthy synovial fibroblasts (SFbs). SFbs were incubated for 48h in medium alone or with unstimulated (-) or CD3/CD28-stimulated (+) culture supernatants from Th1 (classic, cl-Th1, and non-classic, ncl-Th1, or Th17 clones.



A. After 48 of incubation, conditioned media obtained from healthy SFbs either untreated (ctrl) or treated with T cell clones conditioned media were subjected to gelatin zymography. Typical zymography gel bands are shown on the top.

B. Invasion assay in Boyden chambers. Healthy SFbs were placed in the upper well, while different unstimulated or CD3/CD28-stimulated culture supernatants obtained from Th1 (classic and non-classic) or Th17 clones were placed in the lower well. Data represent number of invasive cells. Representative micro-photographs of migrated cells are shown under the respective histogram. * Shows statistical significance ($p < .05$) compared to unstimulated healthy SFbs (ctrl).

Figure 5. Effects of Th1 and Th17 cytokines on protease systems and invasion ability of normal synovial fibroblasts (SFbs). SFbs were treated for 48h with IL-17A and IL-17F or with TNF-α and IFN-γ.

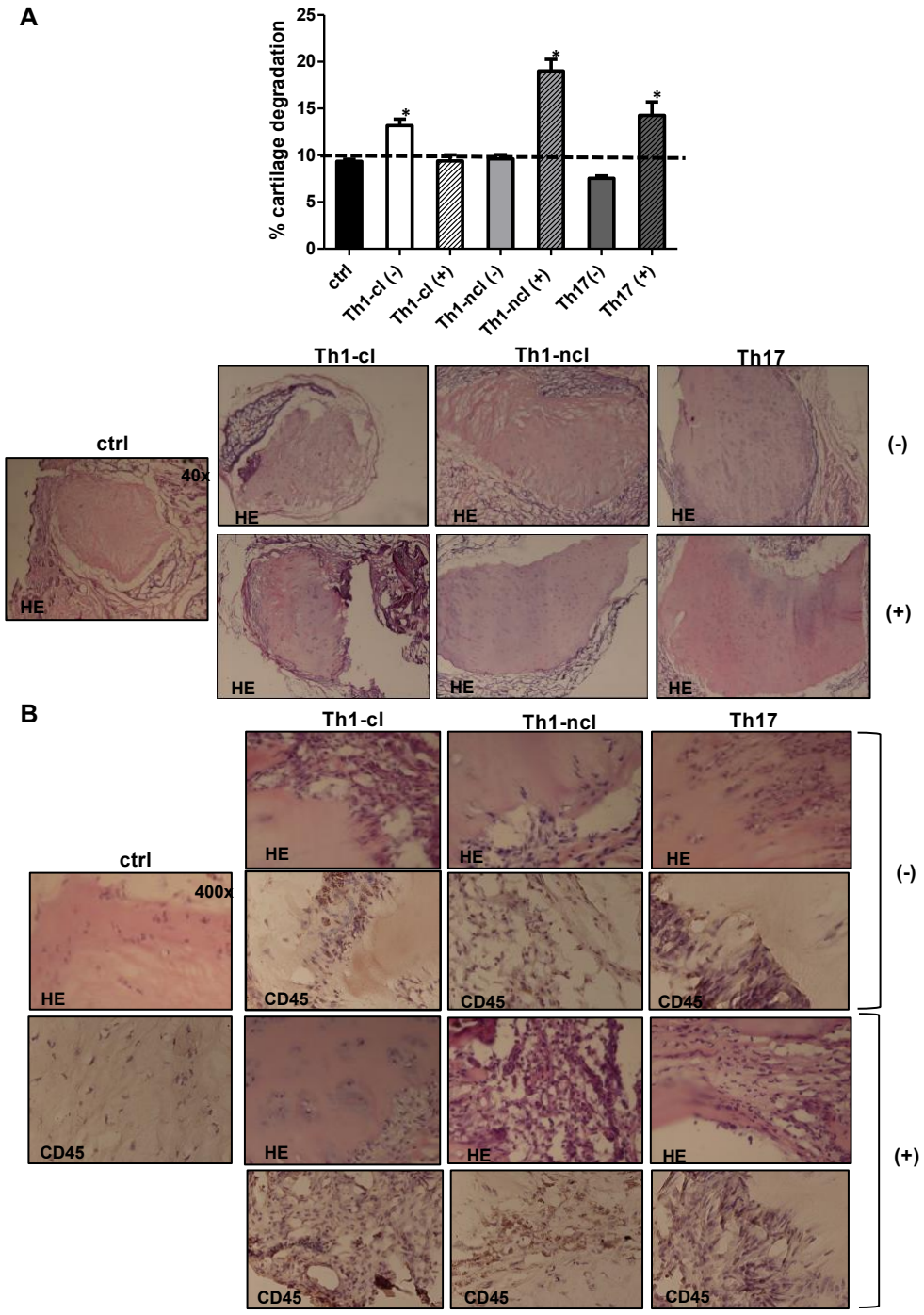


A. Real Time PCR for MMP2, MMP9, uPA, uPAR, PAI-1, TIMP1 and TIMP2. Histogram represents the mean of three different experiments ± SD.

B. Gelatin zymography of conditioned media recovered from normal unstimulated SFbs (ctrl) or stimulated 48h with TNF-α+IFN-γ or IL-17A and IL-17F.

C. Chemoinvasion experiments in Boyden chambers. Normal SFbs were placed in the upper well, while IL-17A and IL-17F or TNF-α+IFN-γ or CD3/CD28-stimulated culture supernatants from Th17 clones were placed in the lower. Ilomastat was added in the upper well together with the cell suspensions. * Shows statistical significance ($p < .05$) compared to unstimulated Normal SFbs (ctrl).

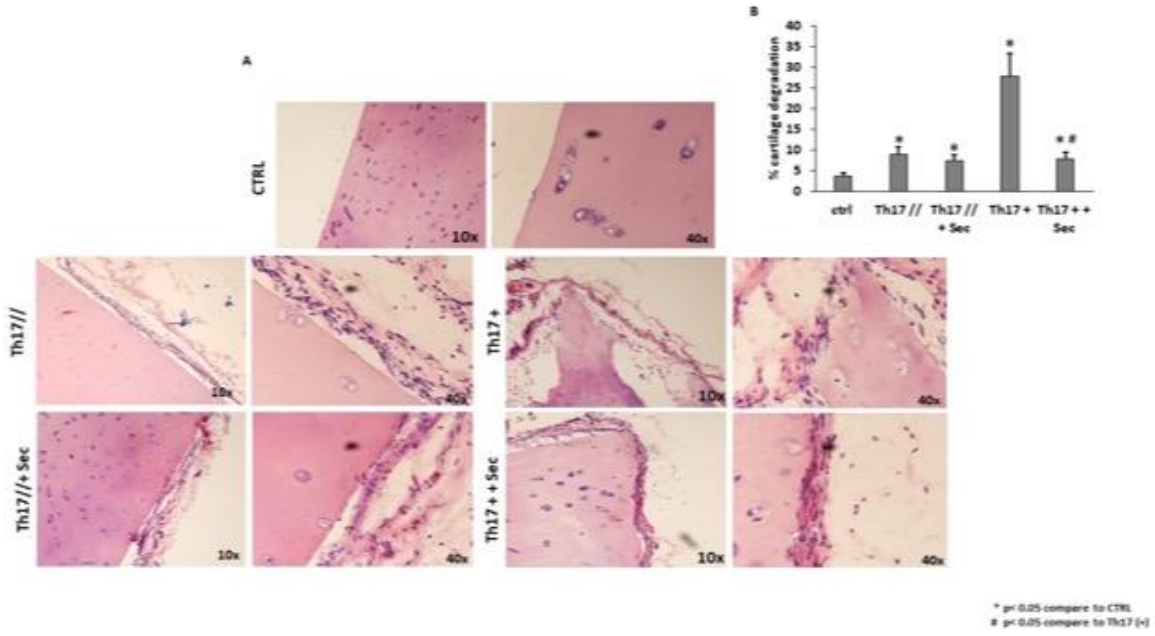
Figure 6. Effects of different stimulated and unstimulated CD4+ T cell clones conditioned supernatants on cartilage degradation *in vivo* (inverse wrap implantation technique).



A. Histogram shows the percentage of damaged cartilage in each implant, in three different experiments.

B. Hematoxylin-Eosin and CD45 stained specimens showed the degree of cartilage erosion by SFbs and inflammatory cells infiltration. ** shows high statistical significance ($p < .01$) compared to Normal SFbs.

Figure 7. Effects of secukinumab on cartilage degradation induced *in vivo* by CD4+ Th17 cell clones conditioned supernatants.



Healthy synovial fibroblasts (SFbs) suspended in conditioned media of unstimulated (//) or stimulated (+) Th17 cell clones were subcutaneously implanted in the animals. Peri-engraftment injections of 50µl of the same CM were weekly effectuated in presence or absence of secukinumab. After 2 months mice were sacrificed, the implants recovered and subjected to immunohistochemistry analysis.

A. H&E staining. Microphotographs shows the degradation of cartilage in the presence of stimulated Th17 CM compared to CM from unstimulated cells and to control (untreated SFbs).

B. Histogram indicates the mean of cartilage degradation in four different sponges ± SD. * $p<0.05$ and ** $p<0.01$ compared to control unstimulated normal SFbs. # $p<0.05$ compared to stimulated Th17-treated SFbs.

9. List of personal publications

September 2018-September 2021

1: Gamalero L, Simonini G, Ferrara G, Polizzi S, Giani T, Cimaz R. Evidence- Based Treatment for Uveitis. Isr Med Assoc J. 2019 Jul;21(7):475-479.

2: Marino A, Tirelli F, Giani T, Cimaz R. Periodic fever syndromes and the autoinflammatory diseases (AIDs). J Transl Autoimmun. 2019 Dec 17;3:100031. doi: 10.1016/j.jtauto.2019.100031.

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8: Ferrara G, Giani T, Caparello MC, Farella C, Gamalero L, Cimaz R. Anakinra for Treatment-Resistant Kawasaki Disease: Evidence from a Literature Review. Paediatr Drugs. 2020 Dec;22(6):645-652. doi: 10.1007/s40272-020-00421-3.

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11: Ferrara G, Petrillo MG, Giani T, Marrani E, Filippeschi C, Oranges T, Simonini G, Cimaz R. Clinical Use and Molecular Action of Corticosteroids in the Pediatric Age. Int J Mol Sci. 2019 Jan 21;20(2):444. doi: 10.3390/ijms20020444.

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14: Guleria S, Gamalero L, Cimaz R, Giani T. Artifactual Skin Lesions: A Trap for Rheumatologists. J Clin Rheumatol. 2021 Jan 1;27(1):e26-e27. doi: 10.1097/RHU.0000000000001219.

15: De Filippo C, Di Paola M, Giani T, Tirelli F, Cimaz R. Gut microbiota in children and altered profiles in juvenile idiopathic arthritis. J Autoimmun. 2019 Mar;98:1-12. doi: 10.1016/j.jaut.2019.01.001.

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17: Bassi A, Piccini P, Filippeschi C, Oranges T, Giani T, Neri I, Cimaz R, Martino M. Inflammatory morphea presenting as a hemifacial acquired port-wine stain. Arch Dis Child. 2019 Mar;104(3):296. doi: 10.1136/archdischild-2017-314721.

18: Maccora I, Marrani E, Ricci S, Azzari C, Simonini G, Cimaz R, Giani T. Common variable immunodeficiency presenting as sarcoidosis in a 9-year-old child. Int J Rheum Dis. 2020 Mar;23(3):448-453. doi: 10.1111/1756-185X.13775.

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