ACTIVITY OF MONOMERIC SECOND MITOCHONDRIAL ACTIVATOR OF CASPASES (SMAC) MIMETIC COMPOUNDS ON HUMAN FIBROBLAST-LIKE SYNOVIOCYTES FROM RHEUMATOID ARTHRITIS PATIENTS

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LIST OF ABBREVIATIONS

DMEM – Dulbecco's modified Eagle medium
FBS – fetal bovine serum
FLS – fibroblast-like synoviocyte
IAP – inhibitor of apoptosis proteins
OA – osteoarthritis
RA – rheumatoid arthritis
SMAC – second mitochondrial activators of caspases
SMC – SMAC mimetic compounds

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1. INTRODUCTION

1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that may affect up to 1% of the general population [1]. The aetiology of RA is unknown; nevertheless, it is commonly accepted that genetic and environmental factors together with the activation of the immune system, may lead to this autoimmune condition and the production of pro-inflammatory cytokines. Overall, these events lead to synovial hyperplasia, angiogenesis and mononuclear cell infiltrates that eventually induce the progressive destruction of cartilage and bone. Although RA has a prevalent localization in the joints, it may be associated with systemic complications that may affect potentially any organ of the body, which is the reason why it is associated with progressive disability and early mortality that are responsible for a severe socioeconomic burden for the patient and for the society [1].

The pathogenic mechanisms underlying the initiation and the progression of RA are still to be clarified. However, it is evident that uncontrolled proliferation of fibroblast-like synoviocytes (FLSs) plays a pivotal role in the pathogenesis of RA-associated synovial hyperplasia, in particular in the early phase of the disease [2].

1.2 Pathogenesis of rheumatoid arthritis

The pathogenesis of RA has been recently reviewed by McInnes et al. [1]. Advances in the understanding of many pathogenic mechanisms underlying the onset and the progression of RA have been made. Nevertheless, there is a lack in the understanding of the mechanisms that trigger RA-related autoimmunity in the early phase of the disease, and of the reasons why the inflammatory process is peculiarly localized in the joints.

RA pathogenesis involves the interplay of both genetic and environmental factors that act together on an altered immune system (figure 1). The importance of a genetic background in RA is supported by the observation that concordance rates in monozygotic twins are 15% to 30% vs. a rate of 5% in dizygotic twins [3]. The involvement of immune-regulatory factors is supported by genome-wide analyses confirming the association of RA with human leukocyte antigen (HLA) loci such as the HLA-DRB1, at least in patients displaying a positivity for rheumatoid factor (RF) or anti-citrullinated protein antibodies (ACPA). In particular, the susceptibility to RA is conferred by the presence of the so-called "shared epitope" QKRAA in the HLA-DRB1 region [4]. The role of the "shared epitope" is supposed to consist in the promotion of autoreactive adaptive immune responses by different mechanisms, such as the selection of a predisposing autoreactive T-cell repertoire, the alteration of the affinity of the peptides, the alteration of the antigen presentation process or the molecular mimicry with microbial peptides. Furthermore, independent of its role in antigen recognition, the "shared epitope" may have a direct pro-inflammatory signalling function [1]. Many other risk alleles have been identified in RA patients, especially those with a positivity for ACPA. All these alleles are linked to immune-regulation, such as TNFR-associated factor 1 (TRAF1)-C5 and c-REL, involved in nuclear factor kappaB (NF-κB)-dependent signalling or PTPN22 and CTLA4 that play a role in T-cell stimulation, activation and functional differentiation [1]. Genetic risk factors are less characterised in ACPA-negative patients and involve HLA-DRB1*03, interferon (IFN) regulatory factors (e.g. IFN response factor 5) or lectin-binding proteins such as C-type lectin domain family 4 member A [5]. These different molecular subsets explain the worse prognosis experienced by ACPA-positive RA patients [1].

Predisposing environmental factors can also contribute to the complexity of the pathogenesis of RA. In particular, exposure to cigarette smoking or silica may increase the risk of developing RA, especially when HLA-DR4 alleles are present [6]. Peptidyl-arginine deiminase type IV (PADI4) is responsible for post-translational modifications in pulmonary and other barrier tissues that may

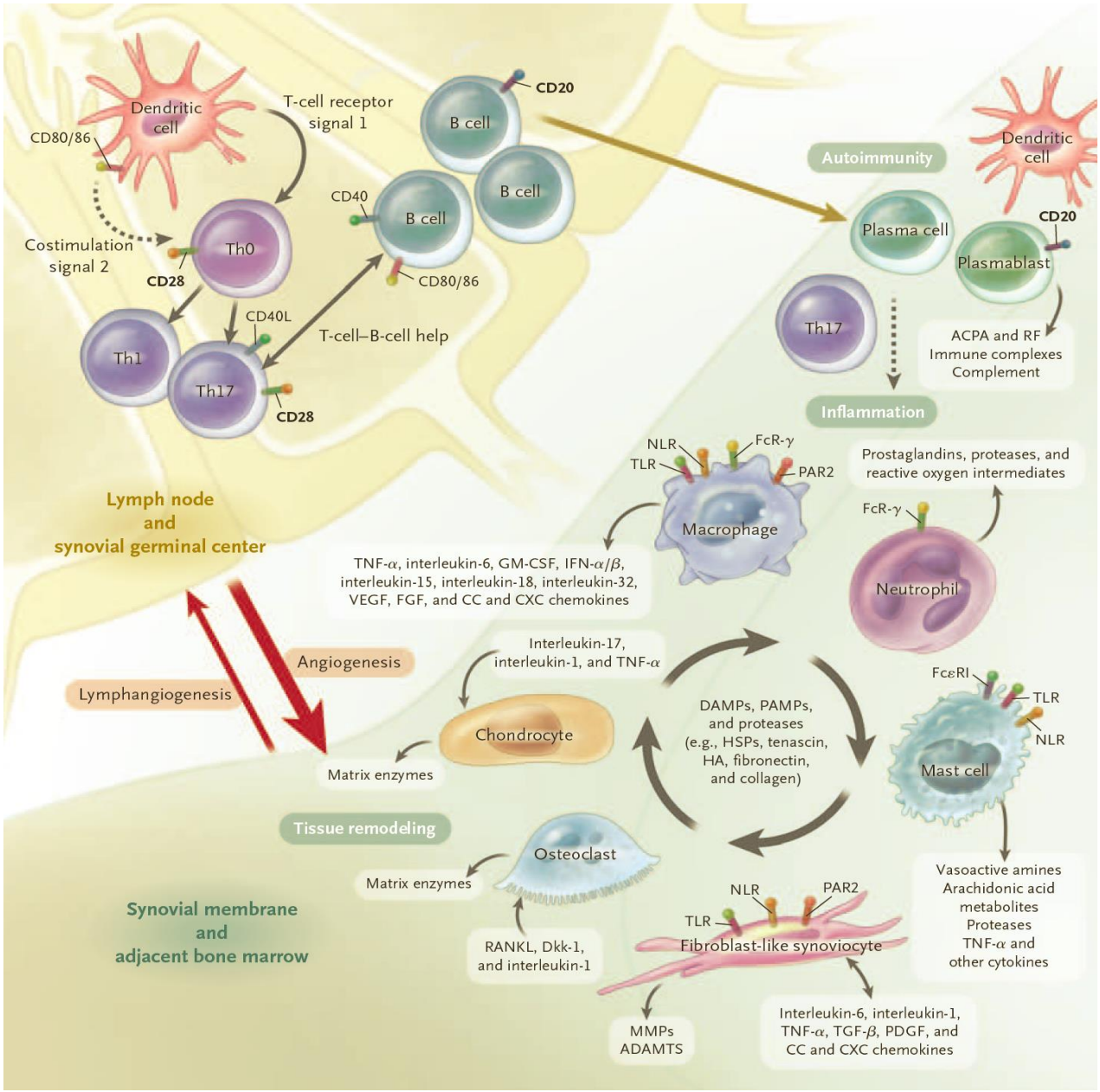


Figure 1. **The pathogenesis of rheumatoid arthritis (RA).** In the lymph node, the co-stimulation-dependent interactions among dendritic cells, T cells, and B cells occur with consequent autoimmune response to citrulline-containing self-proteins. In the synovial lining and adjacent bone marrow, adaptive and innate immune pathways are activated promoting tissue remodelling and damage. The chronic phase of RA is mediated by the interactions among leukocytes, FLSs, chondrocytes, and osteoclasts, together with the molecular products of damage. ADAMTS: a disintegrin and metalloprotease with thrombospondin-1–like domains; DAMP: damage-associated molecular pattern; Dkk-1: dickkopf-1; FcR Fc receptor; FcRI: high-affinity IgE receptor; FGF: fibroblast growth factor; GM-CSF: granulocyte–macrophage colony-stimulating factor, HA: hyaluronic acid; HSP: heat-shock protein; IFN: interferon; MMP: matrix metalloproteinase; NLR nucleotide-binding oligomerization domain–like receptor; PAMP: pathogen-associated molecular pattern; PAR2: protease-activated receptor 2; PDGF: platelet-derived growth factor; RANKL: receptor activator of nuclear factor κB ligand; TGF-β: transforming growth factor β; Th0: type 0 helper T cell; Th1: type 1 helper T cell; Th17: type 17 helper T cell; TLR: toll-like receptor; TNF-alpha: tumour necrosis factor alpha; VEGF: vascular endothelial growth factor.
[From McInnes IB and Schett G, Pathogenesis of rheumatoid arthritis, N Engl J Med 2011; 365:2205-19].

result in quantitative or qualitative alterations of the citrullination of mucosal proteins. A loss of tolerance to such neo-epitopes may elicit autoimmune responses in ACPA-positive patients [7].

In addition, infectious agents have been related to the development of RA, although the underlying mechanisms are yet to be elucidated, with molecular mimicry being a possible explanation. Data suggest that autoimmunity related to RA may begin at a mucosal site long before the onset of joint inflammation. Many candidates have been proposed as the initial site of inflammation: the oral mucosa – a periodontal disease caused by Porphyromonas gingivalis, which expresses PADI4, may promote the citrullination of self-proteins –, gastrointestinal mucosa – the gastrointestinal microbiome could influence autoimmunity by means of a "bacterial signature" – and the lung mucosa – with smoking and other forms of bronchial stresses likely involved [1].

Other factors may be related to RA pathogenesis, such as a dysregulation of the hypothalamic-pituitary-adrenal axis and of the production of pro-inflammatory cytokines. The well-known greater frequency of RA in women than in men, the observation that the onset of RA often follows adverse life events and the evidence from experimental models of the presence of neuro-immunologic interactions all speak in favour of this hypothesis [8]. In fact, the central nervous system normally also regulates the immune system, contributing to its homeostasis. The consequences can be both local – several neurotransmitters are involved in RA synovitis –, and systemic – cytokine production is boosted in the hypothalamus in the case of peripheral inflammation [1].

The progression of RA is a multi-step process, which likely begins with a pre-articular phase, in which autoantibodies such as RF or ACPA are detectable in the sera of patients, years before the onset of an overt arthritis. The citrullination likely induces a loss of tolerance to self-proteins in the so-called transitional phase of RA [1]. Nevertheless, it is not clear how a systemic dysregulation of the immune system eventually results in a localised inflammation inside the joints. Likely, this is due to microvascular, neurologic, biomechanical and micro-trauma mechanisms or other tissue-

specific pathways. In the clinical phase, synovitis is initiated. Finally, positive feedback loops perpetuate the inflammation and contribute to the development of systemic disorders, overall contributing the complexity of RA presentation [1]. Both the innate and the adaptive branches of the immune system are involved in the initiation of RA. In fact, in the joints, peripheral blood monocytes are attracted by pro-inflammatory stimuli into the synovial membrane, where they differentiate into macrophages and undergo activation, secreting pro-inflammatory cytokines such as tumour necrosis factor (TNF)-alpha, interleukin-1 (IL-1) and IL-6, reactive oxygen species and nitrogen intermediates and exert their function via phagocytosis and antigen presentation [1]. TNF-alpha increases the expression of adhesion molecules on endothelial cells, thus further increasing the recruitment of more inflammatory cells into the joints [9]. Activated macrophages also secrete pro-inflammatory chemokines – such as monocyte chemotactic protein-1 (MCP-1) that amplify the inflammatory response [10]. IL-1 and TNF-alpha stimulate FLSs to produce and secrete other pro-inflammatory cytokines such as IL-6 and IL-8, growth factors and matrix metalloproteinases (MMPs), which amplify inflammation and contribute to the damage of joint cartilage and bone. In particular, TNF-alpha directly promotes osteoclast differentiation and activation, whereas IL-1 directly induces cartilage destruction by inducing MMP production in chondrocytes [9,10].

In RA, the synovial membrane is rich in T cells, and autoreactive cells specific for citrullinated self-proteins have been identified, suggesting an ongoing local T cell-mediated B cell activation with specificity for self-proteins [11,12]. Despite RA has long been considered to be mediated by type 1 T helper cells, also type 17 T helper cells (Th17), have a role in the pathogenesis of RA. Th17 are a subset of T cells that produce IL-17 and TNF-alpha, which synergize in promoting the activation of FLSs and chondrocytes [13]. Finally, also an impaired function of T regulatory cells has been postulated, leading to an uncontrolled inflammatory process [14].

The production of RF and ACPA speaks in favour of an involvement of B cells in the pathogenesis of RA. Nevertheless, the role of B cells goes further than the mere autoantibody production, and

likely includes autoantigen presentation and cytokine production of IL-6 and TNF-alpha, as demonstrated by the efficacy of rituximab, a monoclonal antibody directed against CD20, which is not expressed by plasmablasts [15].

1.3 Synovial immunological processes and inflammation

The synovial membrane, also named "synovium" is the thin layer of cells lining the internal environment of diarthrodial joints, tendon sheaths and bursae. Its name contains the root of the word "egg" in Latin, which is likely due to the similar aspect shared by the synovium and the inner membrane of eggs [16,17].

It is important to know the normal structure of the synovial membrane, including the wide range of normal microscopic presentations, the different cellular populations dwelling in it, the normally present cytokines, enzymes and other biologically active proteins in order to comprehend the alterations in its microarchitecture and immunopathology typical of disease states. On the one hand, the structure of the normal synovial membrane is not as homogeneous as initially thought and reported; on the other hand, the normal synovial membranes share common characteristics that allow differentiating them from tissues of chronically inflamed joints.

The structure of the synovial membrane consists in a continuous thin layer of cells (intima) and the underlying tissue (sub-intima). The synovial intima consists of a layer of one or two cells, the synoviocytes, which are two types of cells interdigitated together: the macrophage-like synoviocytes, also known as synoviocytes A, and the FLSs, also called synoviocytes B [18]. The former originate from the peripheral blood, the latter likely derive from local mesenchymal precursors. The cells lay on a synovial sub-lining layer, the sub-intima, which contains few cells and only scattered blood and lymphatic vessels, resident fibroblasts, adipocytes, and few lymphocytes or macrophages, placed in an extracellular matrix rich in collagen [16]. A small amount of fluid is present between the two faces of the joint cavity, providing nourishment for chondrocytes

and a viscous medium rich in hyaluronan (hyaluronic acid). Unlike serosae, the synovial membrane derives from ectoderm and is not provided with a basal lamina.

There may be a relevant variation in the appearance of the normal synovial membrane, which may lack intimal cells or, in many locations, be completely absent, particularly where the underlying tissue is a fat pad or of fibrous tissue. [16,19].

The term "synovitis" denotes the inflammation of the diarthrodial joints. Synovitis, the hallmark of RA, implies a propensity to destruction of articular cartilage and subchondral bone, which are, together with the synovial membrane, the structural components of the diarthrodial joints.

Usually, inflammatory arthritides, such as RA, cause a marked thickening if the intimal layer that may be composed by up to 10-15 cell layers, due both to the proliferation of resident cells and to the influx of immune cells from the vascular compartment, mainly CD68+ macrophages into the joint. Even the sub-intima, which is usually acellular, undergoes inflammatory modifications becoming hypertrophic and oedematous and lymphoid aggregates may develop [17]. Changes involve the sub-intimal cell population, containing T and B cells, plasmacells and macrophages, the production of pro-inflammatory cytokines and chemokines, and the sub-intimal vascular and lymphatic web, which activates neoangiogenesis that is responsible for the increased vascularization that further amplify the inflammatory process. The so-called "*pannus*" tissue of RA invades the articular cartilage, whose destruction is directly driven by FLSs, also via the increased production of MMPs. As a result, subchondral bone erosions develop.

The synovial tissue from the joints of patients with RA has been extensively studied, leading to dramatic advances in the understanding of the pathogenesis of RA and, consequently, to the rational development of different effective therapies.

It is not clear why inflammatory arthritides such as RA typically involve the joints. One possible answer to this question is that the synovial membrane plays an essential role in the regulation of inflammation [20].

A more thorough comprehension of the different steps and chronological phases of the changes that occur in RA synovial membrane may help identify novel therapeutic targets, which may be useful at various stages of the evolution of the disease. Similarly, in the past, the discovery of TNF-alpha and IL-1-beta as the main pro-inflammatory cytokines in RA, led to their definition as possible therapeutic targets, to the design of biological agents directed against these cytokines and to the successful application of these drugs in clinical practice [17].

It is nowadays clear that the synovial membrane is not a mere "victim" of the inflammatory cells in the course of synovitis. In fact, the synovial membrane is not only the supervisor of the behaviour of circulating potentially harmful cells, but also the responsible of the potential damage of its own environment [21]. In normal conditions, the production of pro-inflammatory cytokines in the synovial membrane [17] consists of small amounts of IL-1, IL-6 and TNF-alpha, which are far lower than those seen in RA synovitis. On the contrary, the amount of anti-inflammatory cytokines such as IL-1 receptor antagonist (the natural blocker of IL-1), is considerably higher than that of pro-inflammatory cytokines, thus resulting in a normal suppression of any inflammatory processes. Also the expression of the ligand of receptor activator of NF-κB (RANKL), which is responsible for the development and activation of osteoclasts, is very low in normal synovium and far lower than that of its natural inhibitor, osteoprotegerin (OPG) [17]. The net result of this condition normally suppresses the differentiation of osteoclasts in the bone adjoining the synovium, thus preventing pathological bone erosions.

1.4 Fibroblast-like synoviocytes

In healthy conditions, FLSs, or type B synoviocytes, provide the joint and the surrounding tissues with nutritive proteins and lubrication [17]. The characteristics of RA-FLSs include surface markers VCAM-1, CD55, CD90 (Thy-1), cadherin-11 expression,c and the absence of CD14 or CD68 [22]. Only CD68- FLSs of the intimal layer own a high activity of the enzyme uridine-diphosphate (UDP)

glucose dehydrogenase (UDPGD), which is responsible for the transformation of UDP-glucose into UDP-glucuronate, one of the substrate necessary for assembling the hyaluronan polymer by the hyaluronan synthase [17]. Even *in vitro*, FLSs are able to produce proteoglycans, cytokines, chemokines, growth factors and MMPs during the first few weeks of culture; then, FLSs become quiescent after the third passage [2].

FLSs in RA have an activated phenotype and produce their own array of mediators, especially pro-inflammatory cytokines, prostanoids, and MMPs [23]. FLSs activation establishes a paracrine/autocrine loop that perpetuates the inflammation of the rheumatoid joint and leads to the formation of the rheumatoid *pannus*, which displays characteristics similar to a locally invasive tumour, due to its capability to expand, to damage cartilage and to erode bones [23]. RA-FLSs exhibit aggressive and invasive characteristics, which are similar to transformed cells [24]. Differently from FLSs from normal subjects and patients with osteoarthritis (OA), RA-FLSs can experimentally invade and destroy human cartilage [25]. Furthermore, RA-FLSs appear more resistant to apoptosis than OA-FLSs, although they have similar proliferation rates [1,23].

In the early 1980s, Fassbender described RA-FLSs as tumour-like cells [26]. However, it was not clear whether RA-FLSs were transformed aggressors because of inherent aggressive features or were passive responders to cytokine exposure in the rheumatoid milieu, *in vivo* [18]. Nowadays, it is common knowledge that the permanently altered phenotype of RA-FLSs is likely multifactorial. On the one hand, several observations demonstrated that the aggressive phenotype of RA-FLSs is not a transient consequence of the exposure to rheumatoid pro-inflammatory environment, and that they are markedly different from OA or normal FLSs, leading to the notion that FLSs are permanently altered [17,23]. On the other hand, exposure to pro-inflammatory cytokines can restore the secretory activity of quiescent RA-FLSs *in vitro* [2] and regulate the expression of key regulatory genes for migration and invasion [27], whereas over-expression of IL-10 and other anti-inflammatory stimuli are able to suppress RA-FLS invasive features [28]. Furthermore, RA-FLSs

are not truly transformed cells, as suggested by the oligoclonality of the cells present in the rheumatoid *pannus*. Finally, the imprinting due to the chronic exposure to pro-inflammatory cytokines may contribute to subsequently acquired mutations in key genes that may add to an already altered background [23].

The origin of the expanding FLSs is not completely clear. Both the migration and differentiation of stem cells from the bone marrow and the proliferation and differentiation of a stem cell pool dwelling in the synovial lining are plausible hypotheses. However, it is more likely that FLS expansion is due to an imbalance between proliferation and apoptosis of the cells already present in the synovial membrane [17,23].

Cell suspensions obtained by RA synovitis and cultured in tissue medium own fibroblast-like features and an elongated appearance [17,29]. RA-FLSs change their state from "resting" into "activated" in a similar manner as fibroblast behaviour, even before the inflammatory cells contribute to or maintain this activation, although the mechanisms underlying this process are still unknown [30]. RA-FLSs actively invade cartilage by secreting MMPs and expressing adhesion molecules, but also by producing pro-angiogenic factors and disease-relevant pro-inflammatory cytokines such as IL-1-beta, TNF-alpha and IL-6, and chemokines. Pro-inflammatory cytokines are also secreted by neutrophils, B and T cells recruited to the inflamed joints, thus further amplifying inflammation [30]. In return, RA-FLSs produce B cell activating factor, type-I IFN and chemokines that enhance cell survival and further recruit leukocytes into the affected area [31]. The activation of FLSs results in the conversion from "innocent" mesenchymal cells into destructive aggressors that show a tumour-like phenotype [2]. Although initially structural damage was thought to result, non-specifically, from chronic inflammation, currently, synovitis is reckoned to be responsible for the damage of both the articular cartilage and the contiguous subchondral bone in RA [21]. Actually, RA-FLSs are direct responsible of cartilage destruction but also indirect responsible for bone destruction by favouring osteoclast differentiation and activation [30]. RA-FLSs are characterised

by a number of unique and remarkable features such as the expression of adhesion molecules and an imbalanced production of MMPs, but also the reduced susceptibility to apoptosis [23,30]. The organization of FLSs into a synovial tissue occurs via the cell-to-cell interaction by means of cadherin-11. Cadherin-11 is necessary for the migration of FLSs to the articular cartilage, as demonstrated by the observation that mice deficient in cadherin-11 do not develop an experimental form of inflammatory arthritis [32]. Similarly, RANKL is primarily expressed and secreted by RA-FLSs at sites of bone invasion after toll-like receptor (TLR) signalling activation or pro-inflammatory stimuli [30].

The chronic inflammatory process in RA is driven by the local overexpression of pro-inflammatory cytokines and the production of autoantibodies in the joints [33]. These molecules are secreted by inflammatory cells, such as lymphocytes, macrophages, and mast cells that are dramatically increased in RA synovitis, both in the intima and the sub-intima. FLS phenotype has been analysed by electron microscopy and shows abundant rough endoplasmic reticulum, which is consistent with a relevant secretory activity. FLSs further contribute to the inflammatory process by producing themselves pro-inflammatory cytokines and other inflammatory mediators, but the correspondent contributions of the resident synovial cells and of the inflammatory cells recruited from the circulation still need to be clarified [21]. Indeed, the synovial hyperplasia of RA is caused by the imbalance between the proliferation of RA-FLS and their resistance to apoptosis [34].

1.5 Synovial fluid to reproduce joint microenvironment "in vitro"

Recent data from our group demonstrate that adding SF to the culture medium of growing FLSs collected from RA patients could be an innovative and useful method to recreate *in vitro* the peculiar physiopathological conditions that are observed in RA joints *in vivo* [35]. Under light microscopy FLSs cultured in complete tissue medium appear elongated or polygonal with few branched cytoplasmic processes [23]. After exposure to SF, the cells experience a significant growth advantage and DNA synthesis is enhanced more and for a longer time than in the presence of TNF-alpha, which has long been considered the reference standard as a proliferative stimulus. Furthermore, RA-FLSs cultured in the presence of SF, display more pro-inflammatory characteristics and a phenotype more similar to *in vivo* cells, with more branches. These morphological features are consistent with the functional changes that RA-FLSs undergo when cultured in SF, such as the observed activation of cytoskeleton, which reflects an enhanced ability of migration and invasiveness [35]. The composition of SF is complex and may also influence the expression of key genes. Indeed, not only disease-specific differences have been described, but also a great variation among donors, even though from a single disease group. This could be due, aside from interpersonal variability, to the use of different therapies that could influence the expression of pro-inflammatory or anti-apoptotic factors such as the pro-inflammatory cytokines in SF [36]. Nevertheless, previous data demonstrate that the use of a pool of SF collected by different patients reduces variability and that there are no differences among experiments performed with different pools [35], thus suggesting that the use of a pool of SF collected by different RA patients can be a reproducible experimental condition.

1.6 Therapeutic approaches for rheumatoid arthritis: state of the art

The prevention of joint damage and hence of disability is the main goal for RA treatment, which is obtained by means of an optimal disease remission. Biological therapies aimed at inhibiting cytokine-driven inflammation and joint damage have increased the likelihood of remission, thus changing RA prognosis in the last decade [37]. Currently available biologics for RA target cytokines produced from monocytes, such as TNF-alpha, IL-1-beta or IL-6, but also anti-co-stimulatory proteins and anti-CD20 expressed on B cells. Biological therapies are effective in most patients. However, approximately 30% of patients may experience an inadequate response to TNF-alpha antagonists, with a subsequent risk of unrestrained cartilage and joint damage [38]. Patients who have inadequate responses to TNF antagonists often benefit from a "shift" towards another TNF blocker or a "swap" with another biological agent. Similarly, the discontinuation rates for other biologics such as abatacept and tocilizumab range from 20% to 45% [39]. TNF-alpha, IL-6 and IL-1 are pro-inflammatory cytokines that amplify inflammation and enhance joint damage in RA through the activation of endothelial cells, FLSs, chondrocytes and osteoclasts. Anti-cytokine therapies can modulate the local and systemic inflammatory state, although their effect does not last once the drug is stopped, thus indicating that this therapeutic approach is able to suppress disease, but not to cure it [10]. RA synovial macrophages also produce other pro-inflammatory cytokines, such as IL-15. The absence of an effect on the other pro-inflammatory cytokines may explain the lack of response in a group of patients treated with the currently available anti-cytokine therapy [10].

FLSs have been demonstrated to play a pivotal role in RA by driving the destruction of articular cartilage and triggering osteoclast formation with subsequent bone damage. FLSs may not be directly affected by the currently available anti-cytokine therapies. A targeted therapy directed towards FLSs may provide interesting opportunities to directly interfere with the key cells of the pathological process of RA, thus going beyond the partial and temporary inhibition of inflammation.

1.7 Inhibitor of Apoptosis Proteins (IAPs)

The inhibitor of apoptosis proteins (IAPs) are a group of proteins that have been implicated in the regulation of apoptosis, mitosis and inflammation and were originally found in viruses and metazoans [40].

The anti-apoptotic activity of IAPs depends on the presence of baculoviral IAP repeat (BIR) domains (figure 2), which are shared by all the members of the IAP family. BIR domains are one to three in IAPs and are able to bind different caspases, with the result of inhibiting their function [41]. In the early '90s, IAPs were identified in baculoviral genomes as proteins with the capability of suppressing cell death in response to the host-cell interaction during viral infections [40].The human IAP family is composed by eight proteins: NAIP, which is the first identified human IAP protein, X-chromosome-linked IAP (XIAP), cellular IAP1 (cIAP1) and 2, survivin, Apollon/Bruce, livin and IAP-like protein 2. Among these, XIAP, cIAP1 and cIAP2 are especially involved in cell death regulation [42] and particularly in the engagement of TNF receptor (TNFR).

Apoptosis is a programmed cell death whose mechanisms are fine-tuned, leading to caspase activation. Pope et al. and Niedermeier et al. extensively reviewed the mechanisms underlying apoptosis and the possibility to target them in RA-FLSs [10,34,40]. Caspases are cysteine-dependent aspartic acid proteases that become active enzymes after cleavage by other proteases such as other caspases or dimerization. The apoptotic pathways involve both initiator (caspase-2, -8 and -9) and effector caspases (caspase-3, -6 and -7). Apoptosis or programmed cell death can be activated either via an extrinsic pathway, triggered by genotoxic stress or extrinsic stimuli, such as cytokines, which interact with death receptors (DRs) expressed by cells on their membrane surface, or via an intrinsic mitochondrial pathway as shown in Figure 3 [10,34].

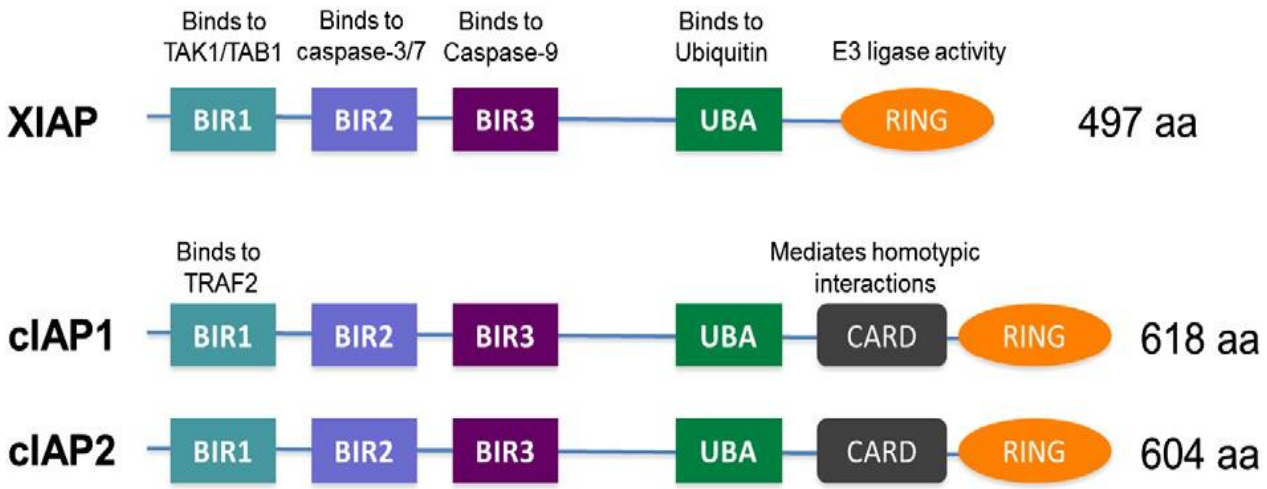


Figure 2. Schematic representation of domain structure in IAPs. BIR: baculoviral IAP repeat domain; UBA: ubiquitin-associated domain; CARD: caspase recruitment domain; RING: really interesting new gene finger domain [From Bai L. et al. Small-molecule SMAC mimetics as new cancer therapeutics, Pharmacology & Therapeutics 2014; 144:82–95].

The extrinsic pathway engages DRs, which are part of the TNFR superfamily, a group of transmembrane proteins with a common extracellular cysteine-rich domain. The members of the TNFR superfamily with the function of DRs have intracellular death domains (DD) and include TNFR1, Fas, DR3, -4 and -5. After binding to TNF-alpha, TNFR1 trimerizes, thus leading to the assembly of the receptor complex and initiation of signalling: the DD present in TNFR1 recruits TNFR-associated DD protein (TRADD), that allows binding of TRAF2, cIAP1 and cIAP2 to the receptor complex. In the absence of cIAPs, receptor interacting protein kinase 1 (RIP1) cannot be ubiquitinated. In these conditions, free RIP1 is able to form a complex with Fas—associated DD (FADD) and caspase-8 in the cytosol, eventually leading to apoptosis [43].

The intrinsic pathway is triggered by cellular stress, DNA damage, chemotherapeutics, radiations or other stress-related stimuli. Bcl-2-homology 3 only (BH3-only) proteins recognize these signals resulting in changes in mitochondria membrane potential and permeability with the subsequent release of cytochrome c (cyt c) and second mitochondria-derived activator of caspase (SMAC)/direct IAP binding protein with low pl (DIABLO). When released in the cytosol, Cyt c will bind apoptosis protease activating factor 1 (Apaf1), thus forming the "apoptosome", which is responsible for the activation at first of caspase-9, and secondly of caspase-3 and -7. On the one hand, XIAP inhibits caspase-3, -7 and -9 by direct binding, on the other hand, SMAC binds XIAP through its N-terminal IAP-binding motif (IBM), thus directly preventing the binding of XIAP to caspase-3, -7 and -9 and removing the apoptosis blockade [44].

Because resistance to apoptosis displayed by RA-FLSs has been identified as a key characteristic of these cells, the specific mechanisms by which RA-FLSs are protected from receptor-induced cell death are currently a matter of research (figure 4) [10]. Despite the expression of Fas, RA-FLSs are resistant to apoptosis induced by FasL. Furthermore, the expression of soluble Fas (sFas), which binds FasL and blocks FasL-induced apoptosis, is increased in the joints of RA patients, thus further contributing to the resistance to apoptosis displayed by RA-FLSs. However, also the overexpression

of specific anti-apoptotic molecules such as Fas-associated death domain-like IL-1-beta-converting enzyme (FLICE)-like inhibitory protein (FLIP) and the small ubiquitin-like modifier 1 (SUMO-1) have been implicated. In particular, FLIP inhibits caspase-8, whereas SUMO-1 is a small molecule that acts by modifying target proteins, thus altering their activation, interaction and degradation. This process is called SUMOylation and is similar to ubiquitination. The overexpression of SUMO-1 in RA-FLSs results in an altered recruitment of the pro-apoptotic adaptor molecule DAXX and a subsequently reduced apoptosis. Also several members of the Bcl family, important inhibitors of the mitochondrial pathway of apoptosis, are up-regulated in RA-FLS [34]. The anti-apoptotic molecule myeloid-cell leukaemia sequence 1 (Mcl-1) is a member of the Bcl family that counterbalances the effects of the pro-apoptotic intracellular factors Bax, Bak and Bim. Mcl-1 is overexpressed in RA-FLSs as a result of the induction by TNF-alpha and IL-1-beta [34]. The alteration of other apoptotic pathways (figure 4) may further contribute to the resistance to apoptosis in RA-FLSs.

Indeed, RA-FLSs are resistant also to TNF-induced apoptosis (figure 4) [10,34]. In fact, in RA-FLSs, TNF-alpha induces proliferation instead of cell death. The overexpression of XIAP and other IAPs may induce resistance to apoptosis.

Both cIAP1 and cIAP2, which are E3 ubiquitin ligases, can regulate receptor-mediated signalling through the recruitment in the TNFR1-associated complex, by binding TRAF2, normally blocking the activation of caspase-8. cIAP1 and cIAP2 are required for TNF-alpha-induced NF-κB activation and for TNF-alpha-induced RIP1 ubiquitination. Indeed, the loss of both cIAP1 and cIAP2, such as in genetic loss, dramatically reduces TNF-alpha-induced NF-κB activation; enhances sensitivity of cells to TNF-alpha-induced cell death; abrogates TNF-alpha-induced RIP1 ubiquitination; and causes TNF-alpha, TNF-related apoptosis-inducing ligand (TRAIL), and IL-1-beta to become pro-apoptotic stimuli to the majority of cancer cells [45-49].

Since their discovery, IAPs have been identified as regulators of programmed cell death. Nowadays, there is growing evidence that they also play an important role in the regulation of inflammatory intracellular signalling and production of pro-inflammatory cytokines [40]. The role of IAPs in shaping TNF-dependent inflammatory signalling has been a matter of research. In fact, independent of their inhibitory role in TNF-induced apoptosis, IAPs have a regulatory role in inflammation and IAP antagonists attenuate TNF-induced production of multiple pro-inflammatory cytokines and chemokines [50]. Both cIAP2 and RIP1 are required for optimal TNF-alpha-induced cytokine and chemokine production. Consequently, cIAP antagonism produces complex alterations to the pattern of spontaneous as well as TNF-alpha-induced production of pro-inflammatory cytokines and chemokines [50].

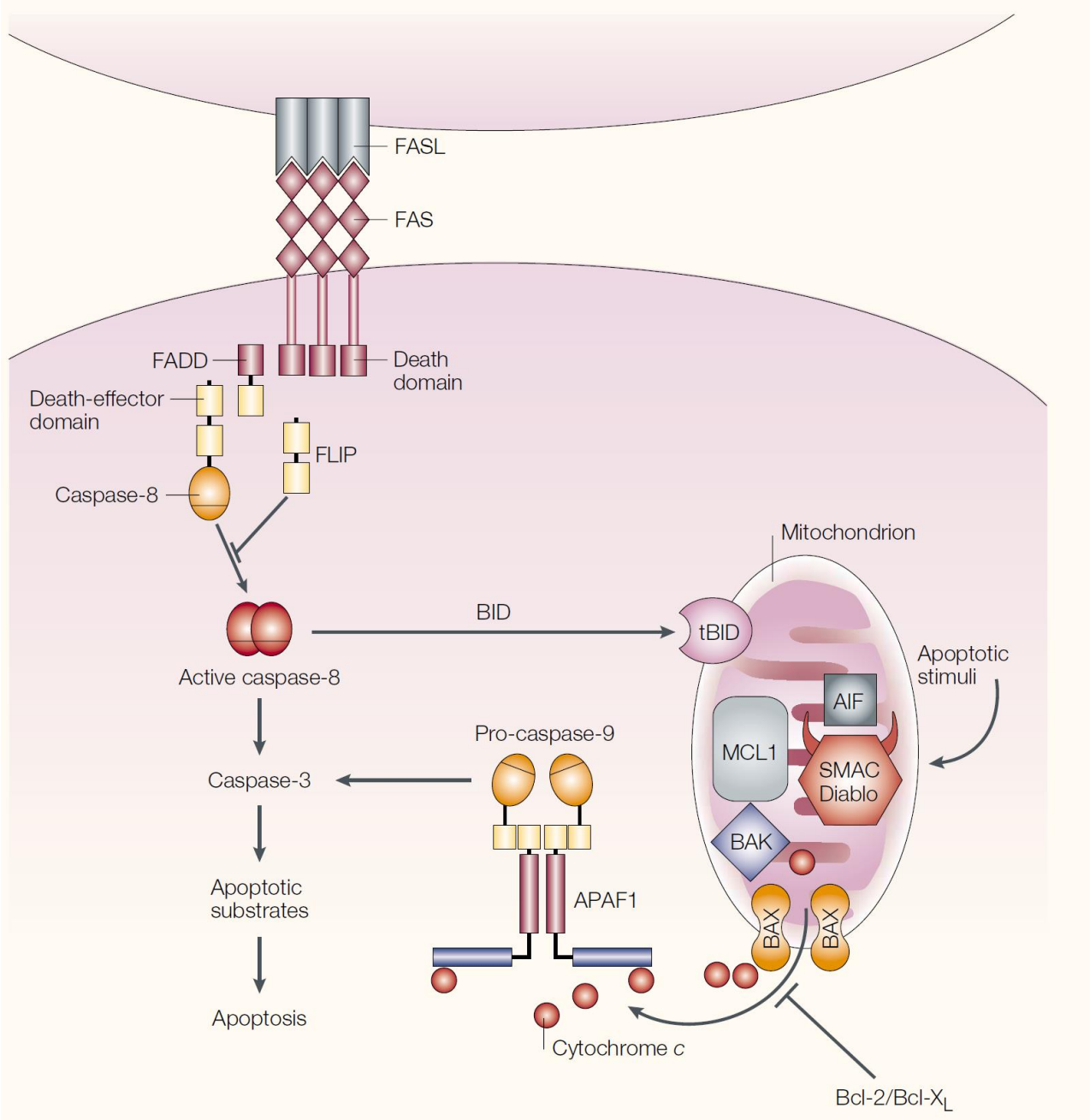


Figure 3. Apoptotic pathways. Apoptosis can be induced by death receptor (DR) ligation such as Fas-FasL or after loss of mitochondrial integrity as occurs in the case of DNA damage. FLIP suppresses DR-induced caspase activation by interfering with the activation of caspase-8. The activation of the DR pathway results in the cleavage of caspase-8, which can lead directly to the activation of caspase-3, without requiring mitochondrial damage, or it may proceed through BID, resulting in the loss of mitochondrial transmembrane potential and the release of cytochrome c (cyt c) into the cytosol. Cyt c, in the presence of Apaf1 and ATP activates caspase-9, which finally leads to the activation of caspase-3. The anti-apoptotic molecules Bcl-2 and Bcl-XL protect against the loss of mitochondrial transmembrane potential that is induced by pro-apoptotic molecules such as BAX and BAK. AIF, apoptosis-inducing factor; Apaf1, apoptotic protease-activating factor 1; BAK, Bcl-2-antagonist/killer; BAX, Bcl-2-associated X protein; BID, BH3-interacting death-domain agonist; FADD, FAS-associated death-domain protein; FASL, FAS ligand; FLIP, FADD-like IL-1-beta-converting enzyme-inhibitory protein; MCL-1, myeloid-cell leukaemia sequence 1; SMAC, second mitochondria-derived activator of caspase.
[From Pope RM, Apoptosis as a therapeutic tool in rheumatoid arthritis, Nature reviews, 2002, 2:1-9].

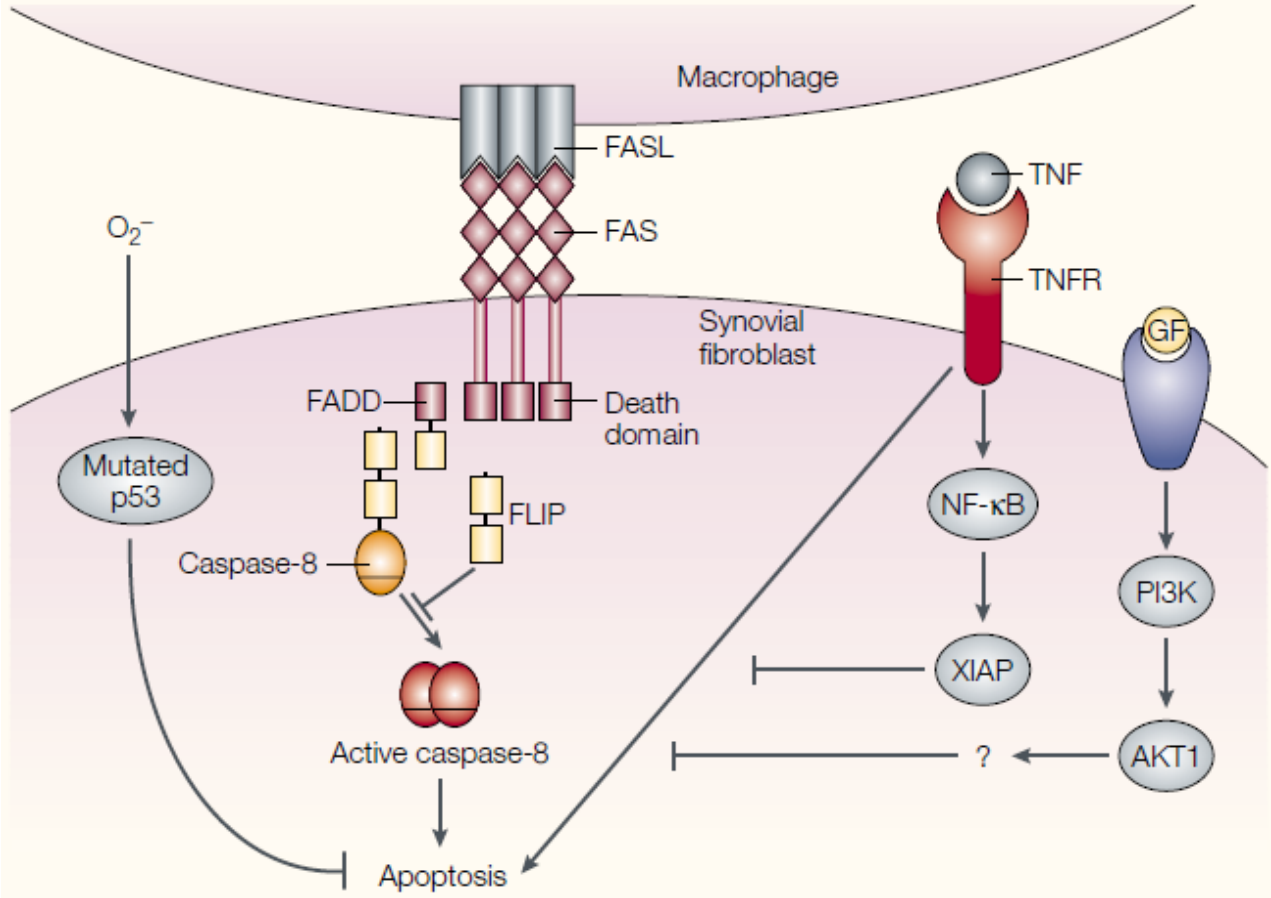


Figure 4. Apoptotic pathways in fibroblast-like synoviocytes (FLSs). Several mechanisms have been defined that might promote the survival of FLSs in the rheumatoid arthritis (RA) joint. FLSs express FLIP, and are resistant to FAS-mediated apoptosis in the RA joint. Activation of both the NF-κB and the PI3K–AKT1 pathways has been shown to protect against tumour necrosis factor (TNF)-induced apoptosis. The data indicate that XIAP might be involved in the NF-κB pathway, although the mechanism(s) that regulate the protection that is conferred through the PI3K–AKT1 pathway remain to be clarified. Finally, p53 is up-regulated in RA-FLSs. Inactivating mutations have been identified in the expressed p53 that are capable of suppressing the induction of apoptosis. FADD: FAS-associated death domain; FASL: Fas ligand; FLIP: FADD-like IL-1β-converting enzyme-inhibitory protein; GF: growth factor; PI3K: phosphatidylinositol 3-kinase; TNF: tumour necrosis factor; TNFR: TNF receptor; XIAP: X-linked inhibitor of apoptosis protein [From Pope RM, Apoptosis as a therapeutic tool in rheumatoid arthritis, Nature reviews, 2002, 2:1-9].

1.8 SMAC-mimetic compounds

The naturally occurring antagonist of IAPs is SMAC/DIABLO. The role of SMAC has been thoroughly studied in several oncologic diseases [40,51]. In fact, one of the typical features of immortal cancer cells is resistance to apoptosis. In several malignant cell lines, the overexpression of XIAP is one of the responsible for resistance to apoptosis. In certain cancer cells, IAPs may not be efficiently inhibited by the physiological amounts of SMAC/DIABLO released from the mitochondria.

With this as background, SMAC-mimetic compounds (SMCs) have been specifically designed to promote apoptosis by removing caspase inhibition in cancer cells overproducing XIAP (figure 5) [52]. Different formulations of SMCs (peptides, monovalent or bivalent molecules) have been demonstrated effective on many cancer cells both *in vitro* and *in vivo* in many pre-clinical studies [53-69]. For example, peptides are effective in MCF-7 breast cancer cells, in human acute leukaemia Jurkat T cells, in cervical cancer HeLa cells, L428 and L540 Hodgkin lymphoma cell lines, Caki-1 renal cancer cells, multiple pancreatic cancer cell lines, non-small cell lung cancer H460 cells, U87MG malignant glioma cells, SHEP and SH-SY5Y neuroblastoma cells, and multiple melanoma cell lines. Polynucleotides are effective in inducing apoptosis in leukaemia cell lines K562 and CEM, renal carcinoma NC65 and Caki-1 cells, and human leukaemia Jurkat T cells. Small molecules are effective in human glioblastoma T98G cells, cervical cancer HeLa cells, several non-small-cell lung cancer cell lines, in different ovarian cancer cell lines, in several breast cancer cell lines, many colon cancer cell lines, human promyelocytic leukemia, acute myelogenous and acute lymphoblastic leukemia, B cell chronic leukemia, and multiple myeloma cells [53-69].

Many clinical trials have been designed with the aim to study the effects of SMCs on different cancer cell types, both as monotherapy and in combination with other anti-cancer drugs, and are currently ongoing.

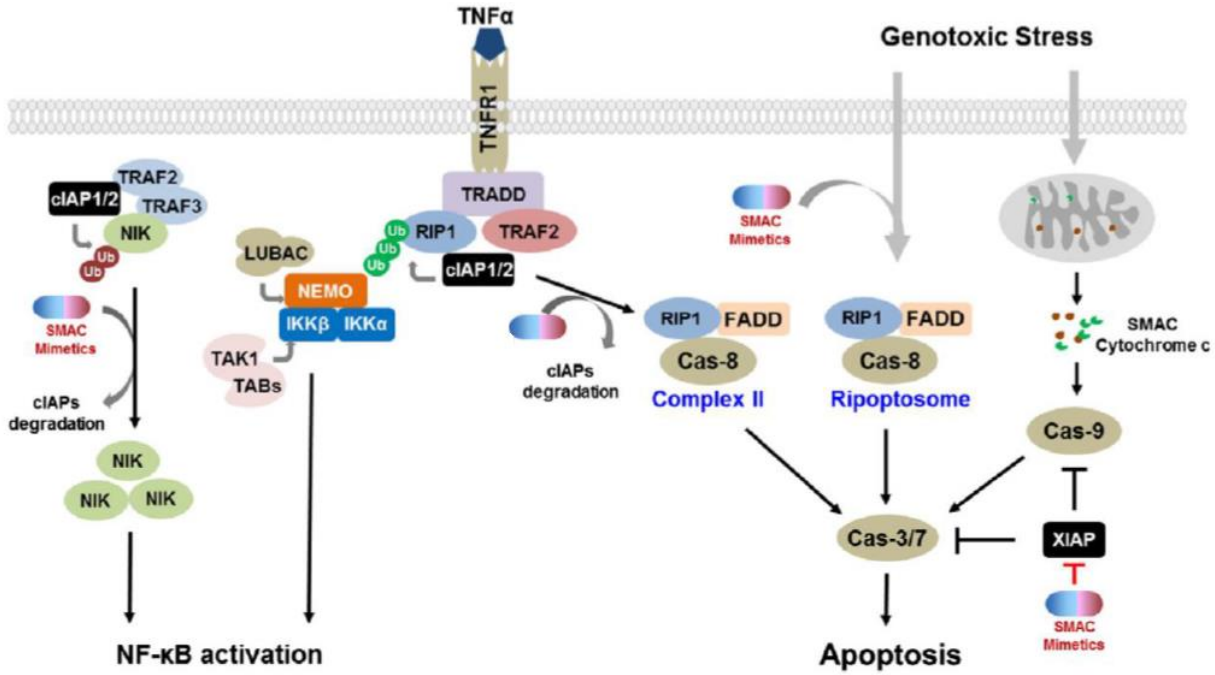


Figure 5. The roles of IAPs in regulation of NF-κB signalling and cell death pathway in cancer cells. Under resting conditions, NIK levels are low because the E3 complex TRAF3:TRAF2:cIAP1/2 constitutively targets NIK for degradation. TRAF3 and TRAF2 work as adaptor proteins in the complex by bringing NIK into position for cIAP-mediated ubiquitination. SMCs are responsible for cIAP quick autoubiquitination and proteasomal degradation. NIK is then stabilised, activated and free to phosphorylate IKK-alpha, which in turn phosphorylates p100. Phosphorylated p100 undergoes limited proteasome-mediated proteolysis of p100 to p52. RelB:p52 heterodimers then translocate to the nucleus and activate the expression of NF-κB target genes. NF-κB-mediated induction of TNF results in activation of TNFR1. In the absence of cIAPs. RIP1 recruits FADD and caspase-8 to form complex-IIB. Cas: caspase; FADD: Fas—associated death domain, IAP: inhibitor apoptosis proteins; IKK: inhibitor of κB kinase; LUBAC: linear ubiquitin (Ub) chain assembly complex; NEMO: NF-κB essential modulator; NF-κB: nuclear factor kappa-B; NIK: nuclear factor κB-inducing kinase; SMC: SMAC-mimetic compounds; RIP: receptor interacting protein kinase; TAK1: TGF-beta-activated kinase 1; TAB: TAK1-binding proteins; TRAF: TNF receptor associated protein [Modified from Bai L. et al. Small-molecule SMAC mimetics as new cancer therapeutics, Pharmacology & Therapeutics 2014; 144:82–95].

Table 1. SMCs currently under clinical development. 5-AZA-C 5-azacytidine; ALL acute lymphoblastic leukemia; AML acute myelogenous leukemia; CBDCA carboplatin; CT chemotherapy; CTX cyclophosphamide; CY cytarabine; DAU daunorubicine; GCB gemcitabine; MM multiple myeloma; PTX paclitaxel; RT radiotherapy

Drug	Developer	Phase	Study type	Disease condition	Status
GDC-0152	Genentech	1	Dose escalation	Solid cancer	Completed
GDC-0917	Genentech	1	Dose escalation	Solid tumors Lymphoma	Ongoing
(CIJDC427)	Curis	1	Dose esclaltion	Solid tumors Lymphoma	Completed
SM-406 (AT-406, Debio1143)	University of Michigan Ascenta Therapeutics	1	Dose escalation	Advanced solid tumors Lymphomas	Recruiting
	Debiopharm	1 1 1	+ DAU and CY + CT-RT + CBDCA and PTX	Acute myelogenous leukemia Squamous cell carcinoma head and neck Solid tumors	Terminated Recruiting Recruiting
LCL161	Novartis	1 1 1 1 2 2 2	Dose escalation Single agent + PTX + GCB and nab-PTX + PTX + CTX Single agent	Advanced solid cancers Advanced solid cancers Advanced solid cancers Metastatic pancreatic cancers Triple negative breast cancer MM Leukemia	Completed Recruiting Recruiting Recrutiing Recruiting Recruiting Not recruiting yet
Birinapant (TL32711)	Tetralogic Pharmaceutics	1 1 1/2 1/2 1/2 1 2	Dose escalation + GCB hydrochloride + 5-AZA-C + CT Single agent + conatumumab Single agent	Solid tumors or lymphoma Advanced solid tumors Myelodysplastic syndrome Advanced solid tumors AML, Myelodysplastic syndrome, ALL Ovarian cancer Advanced ovarian cancer	Completed Terminated Recruiting Ongoing Recruiting Recruiting Ongoing
AEG40826 (HGS1029)	Aegera Human Genome Sciences	1 1	Dose escalation Dose escalation	Advanced solid cancer Lymphoid malignancies	Completed Terminated

Furthermore, several SMCs have been tested in patients with advanced solid tumours and haematological malignancies (see Table 1): the monovalent GDC-0152, GDC-0917, SM-406, LCL-161; the bivalent Birinapant (TL32711) and AEG40826 (HGS1029) have been evaluated for their pharmacokinetics, pharmacodynamics, safety, maximum tolerated dose and initial efficacy [42].

Indeed, the effectiveness of SMC therapy is different, based on the cancer cell line. In particular, not all cancers respond to SMCs successfully. Likely, this is due to the presence or absence of aberrant apoptotic pathways.

More than ten years ago, Sun et al designed SMAC 001, a molecule able to bind the BIR3 domain on XIAP with sub-micromolar potency, by resembling the SMAC N-terminal AVPI sequence [70]. SMAC 001 and the following compounds derived from it, also show the capability of binding BIR3 domain on cIAP1 as well, thus promoting its ubiquitination and subsequent degradation [71]. Starting from the structure of SMAC 001, Seneci and its team designed many more SMCs with high affinity for BIR3 domains [72]. Among these, SMAC 066 is effective in inducing apoptosis also in human RA-FLSs, as recently demonstrated by our work team [73]. Figure 6 shows the chemical structure of SMAC 127, a monomeric compound that was developed in order to ameliorate bioavailability (Seneci et al., unpublished data). In particular, SMAC 127 was synthesised to enhance lipophilicity, thus promoting intracellular uptake. SMAC 127 has never been tested in RA or other autoimmune diseases.

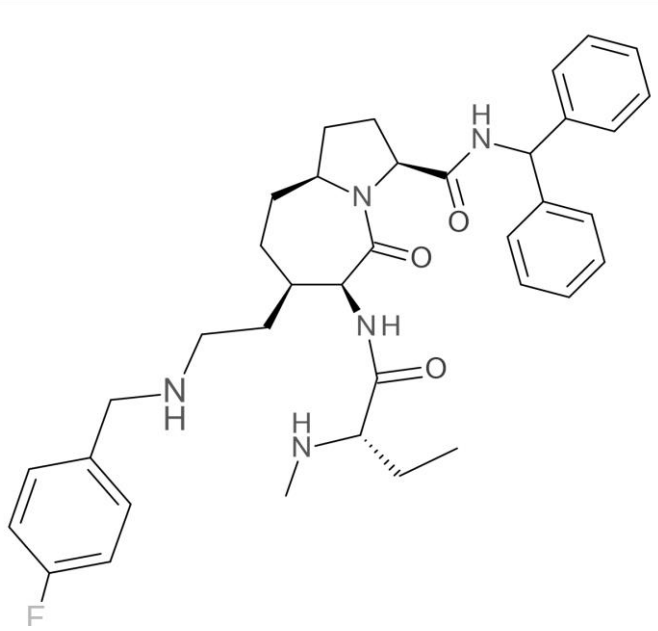


Figure 6. The chemical structure of SMAC 127. The figure shows the structure of the monomeric compound SMAC 127 [Courtesy of Seneci et al., unpublished data].

1.9 Mechanism of action of SMAC-mimetic compounds

SMCs were designed to restore apoptosis in cancer cells, based on their antagonistic activity on XIAP, cIAP1 and cIAP2. When used in cancer cells, SMCs induce cell death in a caspase-8 dependent manner, although their expected effect should depend on caspase-9. In fact, SMCs interact with the BIR domain of IAPs. By directly binding XIAP through the N-terminal IBM, SMCs prevent XIAP binding to caspase-9, -3, and -7 [74]. Nevertheless, it has been demonstrated that SMCs also induce autoubiquitination and proteasome degradation of cIAP1 and cIAP2 [44]. In cancer cells, this process occurs short after the exposure to SMCs and likely leads to the activation of NF-κB and to the secretion of TNF-alpha in an autocrine/paracrine manner thus inducing the activation of caspase-8 and finally to apoptosis [74].

How SMCs stimulate cIAP1 autoubiquitination is still to be clarified. *In vivo,* natural SMAC acts after dimerization and binding to the BIR2-BIR3 domain of cIAPs. Nevertheless, the dimerization is not necessarily required to obtain ubiquitination and degradation of cIAPs, considered that the monovalent SMCs are equally effective. It is possible that SMCs are able to induce an alteration in the conformation of cIAPs that allows an easier access of Lys residues on SMCs to the E2s present in the RING domain [74].

Hence, in cancer cells, treatment with SMCs has two main consequences: the first is to induce the autoubiquitination and the consequent proteasomal degradation of cIAPs, leading to NIK stabilization and RIP1 recruitment, finally resulting in the activation of the non-canonical and, indirectly, of the canonical NF-κB pathways, which both lead to TNF-alpha production [74]. The second is to restore sensitivity to TNF-alpha-induced cell death, by favouring the formation of a RIP1-dependent caspase-8-activating complex in the absence of cIAPs. In support of these observations, the pro-apoptotic effect of SMCs is blocked after inhibition of NF-κB-driven TNF-alpha production with a super-repressor form of inhibitor of κB (IκB) protein or by inhibiting TNF-alpha with TNF-alpha-blocking antibodies [75]. Furthermore, evidence for a role of TNF-alpha as

a DR ligand, in cancer cell apoptosis induced by SMCs, is provided by three independent studies [47,75,76]. In these studies, the blockade of TNFR1 by means of siRNAs or the specific knockdown of TNF-alpha prevented cancer cells from SMC-induced apoptosis, whereas the knockdown of Fas, TRAIL-receptor 1 (TRAIL-R1), and TRAIL-R2 or of FasL or TRAIL did not interfere with SMC effects [47,76]. Activation of the NF-κB signalling pathway leads to the induction of many genes among which, in the case of cancer cells, TNF-alpha is an important effector for SMC-induced apoptosis [47,75]. Nevertheless, a recent study demonstrates that also other pathways may be involved in SMC-induced cell death, independent of TNF-alpha production. There is evidence that sensitive malignant cells produce TNF-alpha constitutively [76]. It is unclear which one of the canonical and non-canonical NF-κB pathways are necessary for SMC-induced TNF-alpha expression. Unlike other DR ligands, the main function of TNF-alpha is not to specifically induce cell death, on the contrary it is able to activate NF-κB and MAP kinases (MAPK), leading to cell survival and cell activation [74]. A secondary TRADD-RIP1-FADD-caspase-8 complex likely drives TNF-alpha-induced cell death, also independent of TNFR1 [43]. In fact, RIP1 is necessary for SMC-induced cell death, both in the case of sensitive malignant cells and in the case of cells resistant to SMCs alone, but responsive to co-stimulation with TNF-alpha [76]. RIP1 is involved both in TNF-alpha-induced NF-κB activation in normal conditions thus contributing to cell survival [77], and in induction of apoptosis when it is overexpressed [78].

It is reckoned that there are three different kinds of cancer cells, based on their response to SMC therapy. The first type of cells is sensitive to SMCs used alone, because of induction or perhaps enhancement of the autocrine TNF-alpha production, thus responsible of induction of apoptosis after the assembly of RIP1-dependent caspase-8- activating complex. The second kind of malignant cells are those that are sensitive to SMCs, only when exogenous TNF-alpha is provided, thus it is likely that, although TNF-alpha secretion is not induced, cells become sensitive to apoptosis induced by several other DRs (figure 7) or other signalling pathways different from NF-κB could be activated,

such as c-Jun N-terminal kinase (JNK) [79]. There is a third sort of cancer cells: those that do not respond to SMCs neither alone nor after co-stimulation with TNF-alpha. In these cells, the RIP1-dependent caspase-8-activating complex is not assembled even after the addition of TNF-alpha. However, SMCs are able to induce the degradation of cIAPs in all cell kinds, thus it is possible that sensitivity to SMCs depends on other mechanisms [47].

Apart from the capability to induce apoptosis in malignant cells, SMCs could also have a role in the therapy of autoimmune diseases, due to the important roles played by IAPs in the regulation of inflammation. A reduction of TNF-alpha-induced cytokine production has been reported after SMC treatment [50]. This is not a mere consequence of cell death, but a direct consequence of IAP antagonism, because when Z-VAD-fmk, an inhibitor of TNF/SMC initiated cell death was added, cytokine production was still inhibited. Furthermore, cytokine production was suppressed also in cells not responsive to TNF-induced apoptosis [50]. The local inhibition of NF-κB and maybe of other signalling pathways, in combination with TNF-alpha produced locally, may enhance the therapeutic potential of SMCs, restoring sensitivity to apoptosis. Finally, SMC treatment is able to reduce leukocyte infiltration into tissues during inflammation in mouse models, via the down-regulation of the intercellular adhesion molecule-1 (ICAM-1) on endothelial cells, therefore reducing the interaction between these cells and circulating leukocytes [80]. SMC treatment seems to suppress TNF-alpha mediated induction of pro-inflammatory cytokines and to block transforming growth factor beta activated kinase-1 (TAK1)-dependent activation of MAPK and JNK pathways [80,81]. Similarly, SMC therapy can inhibit the pro-inflammatory stimuli induced by TLR activation in macrophages without affecting the responses to anti-inflammatory cytokines and IFN [82]. In conclusion, IAPs also contribute to the modulation of inflammatory processes that do not directly involve their function of inhibitors of caspase activity. In particular, cIAP1, cIAP2 and XIAP seem to be involved in the regulation of different aspects of the innate and adaptive immune system. Although it is not clear how the antagonism of IAPs can lead to the different regulation of

signalling pathways such as NF-κB, MAPK, JNK, or the inflammasome pathways, it seems reasonable to study the effects of SMCs also in autoimmune inflammatory diseases such as RA.

Based on these findings, confirmed by the demonstration that IAPs are up-regulated in RA-FLSs [10,73], our group recently demonstrated that the monomeric compound SMAC 066 is able to induce apoptosis and to down-regulate the pro-inflammatory cytokine IL-15 [73]. It was the first study to demonstrate that SMCs are effective not only in cancer cells, but also in cells from patients with RA.

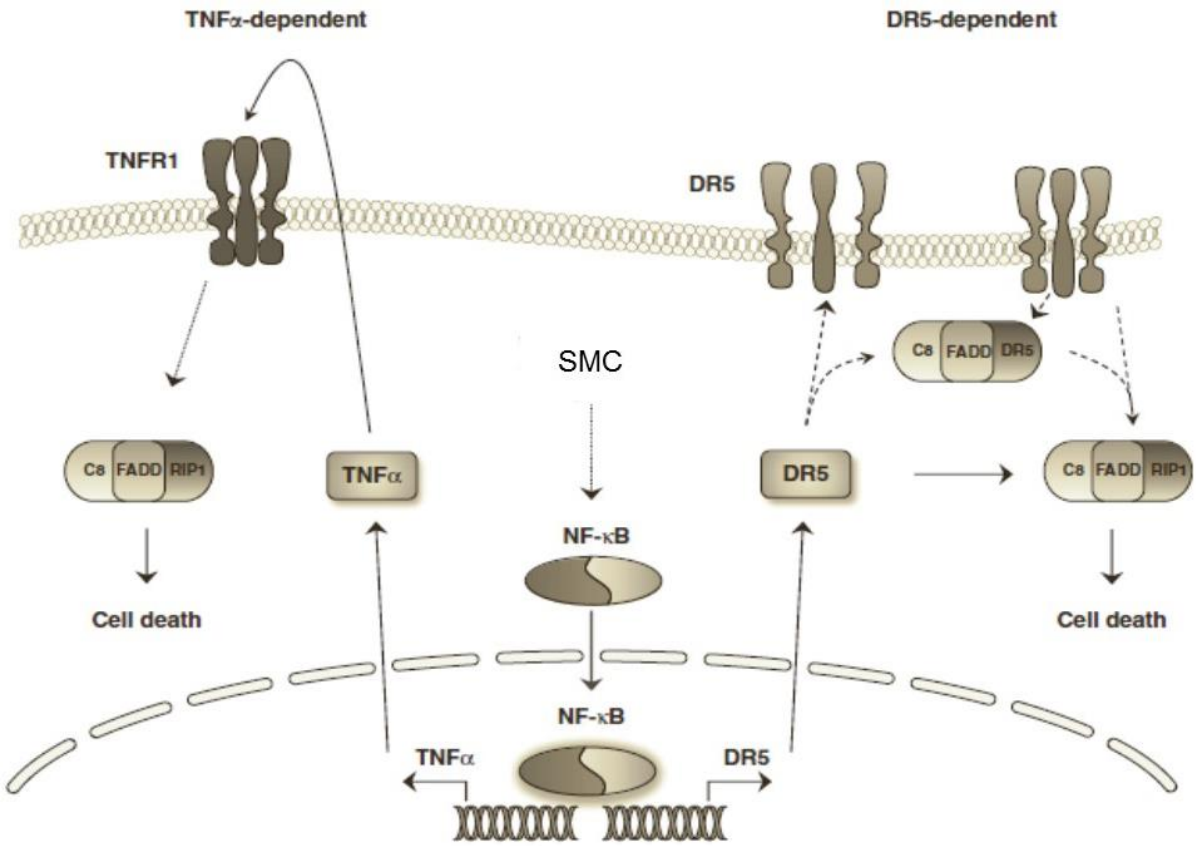


Figure 7. A simplified scheme of SMC-induced signalling pathways. C8: caspase-8; DR-5: death receptor 5; FADD: Fas-associated death domain; NF-κB: nuclear factor kappaB; RIP: receptor interacting protein kinase; SMC: SMAC-mimetic compound; TNF: tumour necrosis factor [Modified from Eckhardt I, Roesler S and Fulda S. Identification of DR5 as a critical, NF-kappaB-regulated mediator of Smac-induced apoptosis. Cell Death Dis 2013;4:e936].

2. RATIONALE OF THE STUDY

The synovial membrane harbours a special cell population named FLSs. These cells are responsible for the initiation and progression of RA [83]. RA-FLSs are different from normal FLSs and display local tumour-like, destructive and invasive characteristics. Subsequently, they accumulate in the synovial membrane, which becomes hyperplastic and for this reason is called "rheumatoid *pannus*". Once activated, RA-FLSs secrete numerous pro-inflammatory cytokines, chemokines, and MMPs, all engaged in the progressive destruction of cartilage and bone and responsible for the recruitment of inflammatory cells that further amplify inflammation and help preserving the aggressive state of RA-FLSs and osteoclasts [24,26].

We chose to study the effects of SMAC 127, a newly synthesised monomeric compound, on FLSs because of their pivotal role in RA pathogenesis. For this reason, FLSs represent an interesting target for novel therapeutic approaches in RA. Furthermore, FLSs can be easily isolated from the synovial membranes and cultured in tissue medium even for a prolonged time [23]. These *in vitro* models provide further insights for the pathogenesis of RA and for the evaluation of new targets for RA treatment. The enhanced survival of RA-FLSs, the escape from the mechanisms of immune surveillance and the unresponsiveness to cytotoxic therapies are likely due to a reduced susceptibility of these cells to apoptosis [84]. Indeed, a deficient apoptosis results from up-regulated IAPs [85]. Among the members of the IAP family directly involved in apoptosis regulation, are cIAP1, cIAP2 and XIAP. The natural antagonist of IAPs is SMAC/DIABLO, which is released from the mitochondria after triggering by pro-apoptotic stimuli, thus blocking IAP anti-apoptotic activity [86,87]. SMCs are synthetic monomeric and dimeric molecules designed to be similar to the N-terminal amino acid residues of SMAC. These mimetics can bind several members of the IAP family - among which XIAP, cIAP1, and cIAP2 - and to induce apoptosis through the down-regulation of IAP expression [42].

The activity of SMCs has been extensively investigated on cancer cells with satisfying results, and recently also in autoimmune diseases such as RA [73]. Apart from their role in conferring resistance to apoptosis of RA-FLSs when overexpressed, the growing evidence that IAPs also have a role in the regulation of the immune system and in the inflammatory signalling pathway [73], makes them a suitable target for RA. With this as background, we believe that these compounds could represent a new valid therapeutic approach for RA patients resistant to both traditional and biological DMARDs.

3. AIM OF THE STUDY

The aim of this study is to investigate the pro-apoptotic and anti-inflammatory activity of the newly synthesized monomer, SMAC 127, on RA-FLSs. We decided to compare data of SMAC 127 with previous data from the same Laboratory, regarding SMAC 060 and SMAC 066 [73]. In order to optimize our *in vitro* model, we decided to test the effects of SMAC 127 on RA-FLSs in different conditions of culture: complete tissue medium alone, in the presence of TNF-alpha or in the presence of SF [accepted, Mediators of Inflammation, SMAC 127 has pro-apoptotic and anti-inflammatory effects on rheumatoid arthritis fibroblast-like synoviocytes, D.Lattuada, **R.Gualtierotti**, K. Crotta et al.].

4. DESIGN OF THE STUDY

After enzymatic digestion of the synovial tissue, a single cell lining will be allowed to adhere to the flasks. When the tissue medium will be changed, detached cells as well as macrophage-like (type A) cells, which have a short life span *in vitro*, will be removed in order to obtain, after three or four passages, FLS-type as the dominant cell type. The result will be a homogeneous population with a doubling time of around one week [23,29]. We will test the effectiveness of SMAC 127 in different culture conditions: complete tissue medium alone and after the addition of a pool of synovial fluid

(SF), which is the actual milieu of the cells *in vivo*. We will compare our results with data regarding other SMCs from a previous study of our group. We will evaluate the proliferation of cells and the number of apoptotic cells in cultures of RA-FLSs after exposure to SMAC 127 in different culture conditions. Then, we will investigate the expression of IAPs in RA-FLSs in different culture conditions in order to verify whether SMAC 127 is able to down-regulate IAPs and we will test the effects of IAP inhibition on the regulation of the inflammatory process by evaluating the levels of pro-inflammatory and anti-inflammatory cytokines also after the addition of TNF-alpha to the medium. To verify the specificity of the effects of SMAC 127 on RA we will also perform experiments on FLSs from OA patients in an *in vitro* model.

5. MATERIALS AND METHODS

5.1 Study population

Adult patients affected by RA and OA, independent of disease duration and localization of the disease, undergoing surgery at Ist. Ort. G. Pini in Milan, First Division of Orthopaedic Surgery were asked to provide informed consent. Inclusion criteria were fulfilment of RA classification criteria [88]. The collection and analysis of synovial membranes and synovial fluids for research purposes was approved by the Ethics Local Committee of the Gaetano Pini Hospital of Milan on March 27, 2012.

5.2 SMAC 127

We will test SMAC 127 and compare its effects with data regarding other SMCs from previous studies of our Laboratory. SMAC 127 synthesis was performed as described by Seneci et al. [52,89]. SMAC 127 will be reconstituted in water.

5.3 Synovial fluid samples

SF was collected in heparinized tubes by arthroscopic procedure and was centrifuged to separate cells from the fluid part for 10 minutes at 1000xg. The acellular portion was collected and stored at -80°C before use. We will mix SF collected from 10 RA patients to create a pool that will be used 1:8 dilution in culture medium.

5.4 Fibroblast-like synoviocytes

We collected samples (synovial tissue and synovial fluid) from patients with RA (n = 22) (female 75%, mean age 47.43 ± 13.42) or OA (n=5) in the course of synovectomies after providing informed written consent. No significant correlations were found with age, sex, disease duration and therapy and the results of our experiments.

Human synovial tissues will be minced and digested with collagenase in Dulbecco's modified Eagle Medium (DMEM) (Euroclone, Pero; Italy) for 2 h at 37°C to isolate cells. The synoviocytes will be centrifuged at 1000xg and suspended in DMEM supplemented with 20% fetal calf serum (FCS) (Fetalclone1 Hyclone Logan, UT, USA) at first, then 10% from the first passage, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (Euroclone, Pero, Italy), then plated in 75 cm^2 flasks. Cultures will be kept in a humidified incubator at 37°C under 5% CO_2. The purity of the cells has been tested before the beginning of the experiments by flow-cytometry analysis using phycoerythrin-conjugated anti-CD14 (Pharmingen, San Diego, CA, USA) and fluorescein isothiocyanate phycoerythrin-conjugated anti-CD3, anti-CD19, anti-CD14, or anti-Thy-1 (CD90) monoclonal antibodies (R&D Systems Minneapolis, MN). A FACS Calibur flow cytometer (488Ex/620Em) (Becton Dickinson, San Jose', CA, USA) was used for the analysis.

At confluence, the cells will be trypsinized and passaged, and used for the experiments between passage 3 and 8. We will change tissue medium after 2-3 days.

In this study, we will refer to "complete tissue medium" as DMEM containing 2mM L glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. Based on previous data demonstrating that the use of a pool of SF collected by different patients reduces variability and that there are no differences among experiments performed with different pools [35], we will use a pool of SF collected by ten different RA patients. We will refer to cells cultured in the presence of SF when complete tissue medium is added with the SF pool with a 1:8 dilution. We will refer to cells grown in TNF-alpha when complete tissue medium is added with recombinant TNF-alpha 25 ng/ml for 96 h; for experiments performed in the presence of anti-TNF-alpha we will use monoclonal anti-TNF-alpha antibody (Perprotech #500-M26) added in the cultures in the quantity of 5 μM for 24 h.

Cells that will not be used immediately will be frozen with the following procedure: each flask that is to be pooled (freeze pool) will be examined microscopically for contamination and any unusual growth pattern. One flask will be maintained as a "backup" flask until the viability of the freeze can be checked. We will aspirate the growth medium from each flask, add trypsin/EDTA 1x in PBS in each flask without dislodging cells and incubate at 37°C for 5 minutes. Then we will check each flask microscopically to make sure the cells begin to round. We will centrifuge the freeze pool at 1000xg for 8 minutes at 4°C. Then we will remove the supernatant and suspend the cells in complete tissue medium +50% FCS, 1flask=400 microliter +5% dimethyl sulfoxide (DMSO) in cryovials. We will freeze the cryovials at a rate of 1°C per minute. Frozen cell stocks will be stored in the liquid nitrogen tanks.

Thawing will be performed by removing one cryovial from frozen storage and placing it immediately in a 37°C water bath. We will remove the content of cryovials using a sterile transfer pipette and place them in a tissue culture flask containing 5 ml of complete tissue medium+20% FCS that will be placed in the incubator.

5.5 Proliferation assay

RA-FLSs will be cultured in the presence of SMAC 127 (15 μM, 24 h) or complete tissue medium alone. Furthermore, we will investigate the proliferation in the presence of SF (1:8 dilution) for five days or after stimulation with TNF-alpha 25 ng/ml for 96 h. DNA synthesis will be evaluated by adding 10 μM 5-ethynyl-2 –deoxyuridine (EdU) labelled with Alexa Fluor 488 dye (Click-iT® EdU Alexa Fluor® 488 HCS Assay, Invitrogen Carlsbad, CA). After a 24 h-incubation, cells will be fixed and permeabilised, and EdU incorporated into newly synthesized DNA will be detected using the fluorescent Alexa Fluor 488 label. Hoechst staining will be used to count total nuclei. Analysis will be performed using a confocal microscopy at an external Centre (see acknowledgements).

5.6 Apoptosis assay

RA-FLSs will be treated with 15 μM of SMAC 127 for 24 h. Apoptotic cells will be detected with the Annexin V-FITC apoptosis detection kit (Abcam, Cambridge, UK), according to the manufacturer's instructions. All samples will be analysed with a FACSCalibur flow cytometer equipped with an argon laser at 488 nm (Becton Dickinson, San Jose', CA, USA). FITC-conjugated Annexin V emission will be collected in the FLH-1 channel whereas propidium iodide (PI; for detecting necrotic or dead cells) emission will be collected in the FLH-3 channel. Data will be analysed with Cell Quest software. Both early ($Annexin^{+}PI^{-}$) and late apoptotic cells ($Annexin^{+}PI^{+}$) will be considered in the analysis.

5.7 Western Blots

Whole cell extracts will be prepared by direct lysis of cells in lysis buffer, and cell lysates will be processed so that the protein concentration can be measured by the BCA method (Thermo Scientific, USA) according to the manufacturer's instructions. The cell lysates will be separated on NuPAGE

4-12% Bis-Tris pre-cast gels for IAPs detection (Life Technologies, Carlsbad, CA) with constant 130 mV voltage and transferred onto nitrocellulose membranes (Life Technologies, Carlsbad, CA) with constant 50 mA electrical current. Membranes will be blocked for 3 h with 5% non-fat dry milk (Lab Scientific) in 0.1% PBS-Tween 20 (SIGMA-Aldrich, St Louis, MO) and probed with primary antibody overnight at 4°C. Secondary antibodies will be conjugated to horseradish peroxidase (Thermo Scientific, USA), and the gels developed using Western Lightning Plus ECL (PerkinElmer OH, USA). Densitometry will be performed using Image J Software (NIH, Bethesda, USA). The following primary antibodies will be used: anti-cIAP1 (R&D Systems), anti-cIAP2 (BD Pharmigen, MA, USA), anti-XIAP (Cell Signalling Technology, Europe) whereas α-actin (SIGMA-Aldrich, St Louis, MO) will be used as the loading control.

5.8 Caspase detection

RA-FLSs will be cultured in complete tissue medium in a humidified incubator at 37°C under 5% CO_2. SF will be added to the tissue medium for five days when necessary. SMAC 127 15 μM will be added for 6 h in complete tissue medium and for 18 h in the presence of SF. Apoptosis will be induced by addition of staurosporine 20 μM as a positive control. Then RA-FLSs will be collected for cell lysates and western blot analysis will be performed as described above, using NuPAGE 10% (Life Technologies, Carlsbad, CA) and anti-caspase-3 rabbit mAb (#31A1067 Enzo).

5.9 Cytokine dosage

RA-FLSs will be cultured in complete tissue medium, in the presence of TNF-alpha as described above, or in the presence of SF. When the cells will be sub-confluent, for all cytokines except IL-15, we will add 2μL/well of BMS00-4980-03 4 x 100 mL Protein Transport Inhibitor Cocktail (500X)(PRODOTTI GIANNI) to block the secretion of the proteins 18 h before the end of

incubation. The fixing will be performed with the following procedure: the cells will be incubated at room temperature for 30 minutes in FCS for saturation of specific bounds, then centrifuged for 7 minutes at 1000xg and the pellet will be suspended in PFA 3% in PBS (100 µL/sample), then incubated at room temperature for 15 minutes. The cells will be washed with 1 mL of PBS+0.5% FCS at 4°C, then centrifuged as above. The pellet will be suspended and the cells permeabilised with TRITON 0.3% in PBS 100 µL/sample. The cells will be incubated at room temperature for 5 minutes without shaking and then washed as above and centrifuged for 10 minutes at 1200xg. The anti-cytokine antibodies will be added 1:50 µL/sample (TNF-alpha/FITC #398040 Ancell monoclonal anti-human, IL-15 RD system PE 10 µL/sample, IL-8 and IL-6 BIOLEGEND 5 µL/sample) and tubulin 1:250 will be used as a control of permeabilization. Cells will be incubated at room temperature for 30 minutes, washed as above and centrifuged for 10 minutes at 1200xg. The samples with tubulin will be incubated with anti-mouse IgG PE 5 µL/pellet wet for 30 minutes at room temperature, then washed and suspended in 500 µL of PBS. The anti-cytokine antibody is conjugated with fluorescein (FITC) and the laser ray elicits a response. We will use Cytofluorimeter Laser Argon 488 nm. FACS Calibur Becton Dickinson. The instrument will be calibrated based on the auto-fluorescence emitted by the control cells.

For Odyssey cytokine dosage, RA-FLSs will be cultured in 96 wells plates, in complete tissue medium alone or in the presence of SF for five days, and then treated with SMAC 127 15 µM for 24 h. Cells will be fixed and permeabilised. The samples will be saturated with LI-COR Odyssey Blocking Buffer to avoid non-specific binding with slow shaking, for 2 h. Then, plates will be washed four times with washing solution (PBS added with 0.1% Tween 20). RA-FLSs will be incubated with 50 µl of several primary mouse monoclonal antibodies (anti human IL-15 (15 µg/ml), IL-6 (15 µg/ml), IL-10 (15 µg/ml), (Perprotech, Rocky Hill, NJ USA)) and mouse monoclonal antibodies (anti -MMP1 (1:40), RANKL (1:70) and OPG (1:15) (Novus, Italy)). After 2.5 h, plates will be washed and incubated with goat anti-mouse IRDye™ 800CW (1:800 dilution;

LI-COR) and Cell Tag 700CW (1:500; LI-COR) for 1 h. After the final wash, the plate will be scanned simultaneously at 700 nm and 800 nm using the Odyssey CLx Infrared Imaging System.

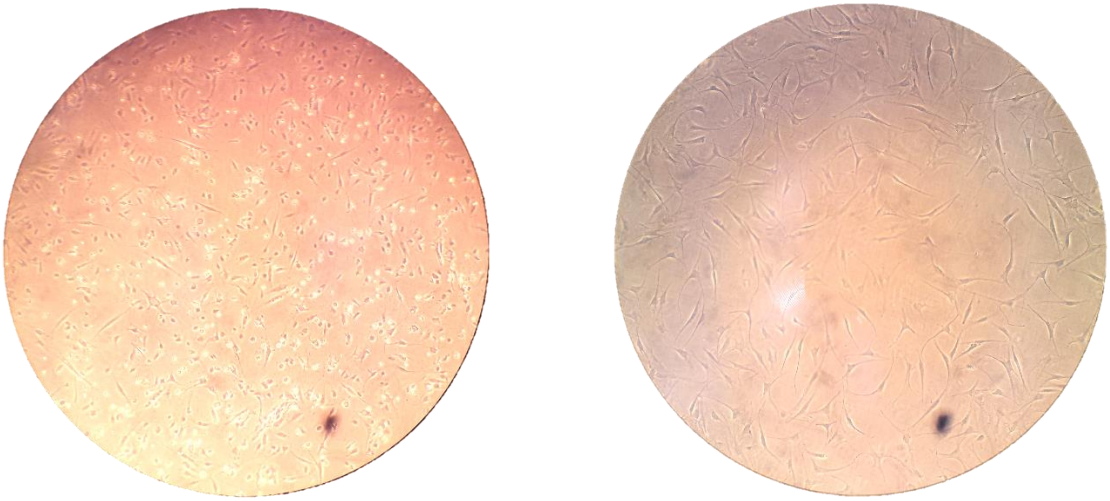
5.10 Statistical analysis

Mean, standard deviation and standard error will be calculated when appropriate. Student's t-test will be used to find correlations between the different groups. SPSS 21 (IBM) program will be used.

6. RESULTS

6.1 Fibroblast-like synoviocyte cultures in different culture conditions

As expected, FLSs displayed a different phenotype when cultured in complete tissue medium or SF. *Ex-vivo* RA-FLSs appeared round under light microscopy (figure 1A), then, after several passages, they acquired a ramified aspect (figure 1B). When cultured in the presence of SF, RA-FLSs displayed a phenotype more similar to *in vivo* cells, with much more branches and larger dimensions (figure 2).



A. **B.**

Figure 1. **Cultures of fibroblast-like synoviocytes.** A. Ex-vivo culture of fibroblast-like synoviocytes (FLSs) from a sample of synovial membrane collected from a patient with rheumatoid arthritis (RA). Cells are round-shaped and are only partially adherent to the flask bottom. B. RA-FLS culture after several passages. The cells display evident ramifying processes (10x magnification).

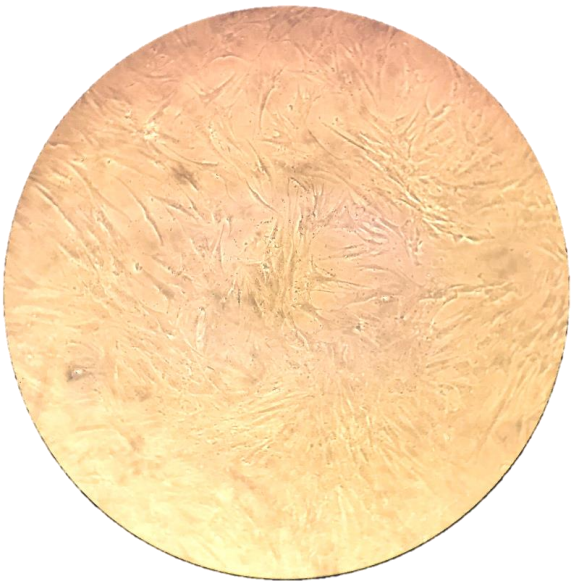
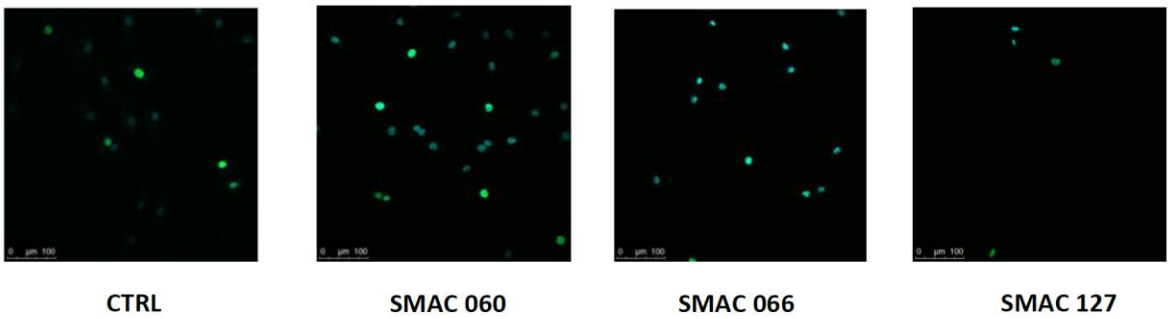


Figure 2. Fibroblast-like synoviocytes cultured in synovial fluid. RA-FLSs cultured in the presence of synovial fluid - as described in the 5.4 section of Materials and methods - after multiple passages display numerous branches as compared to those cultured in complete tissue medium alone. The cells also seem to be larger (hyperplasia) than the cells cultured in complete tissue medium alone (10x magnification).

6.2 Fibroblast-like synoviocyte proliferation assays

At first we evaluated the rate of proliferation of RA-FLSs cultured in the presence of the newly synthetized molecule SMAC 127, which has never been tested in RA [accepted, Mediators of Inflammation, SMAC 127 has pro-apoptotic and anti-inflammatory effects on rheumatoid arthritis fibroblast-like synoviocytes, D.Lattuada, **R.Gualtierotti**, K. Crotta et al.]. We used SMAC 060 and SMAC 066 for comparison because our group recently demonstrated the efficacy of SMAC 066 in inducing apoptosis in RA-FLSs , whereas SMAC 060 seems to have no effect on them. We used the dosage of 15 μM for 24 h. Figure 3A [73], shows a representative image of the analysis of newly synthetised DNA after 24 h incubation with 5-ethynyl-2'-deoxyuridine (EdU). The graph in Figure 3B shows the results of the three independent experiments, which clearly shows that SMAC 066 exerts a significant inhibition of the proliferation of RA-FLSs whereas SMAC 060 has effects comparable to controls [73]. It is evident that SMAC 127 is the most effective among these monomeric compounds.

A.



B.

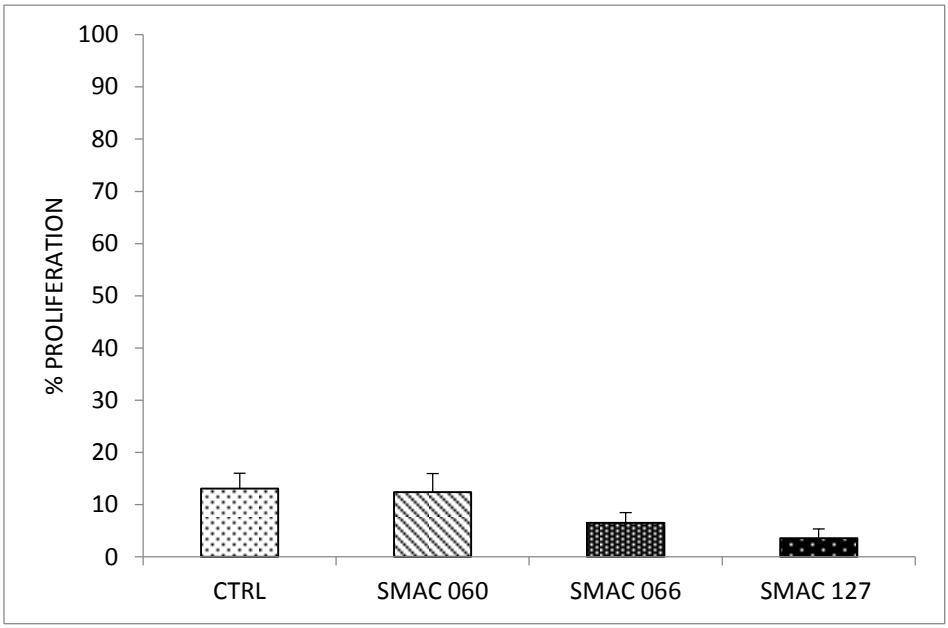


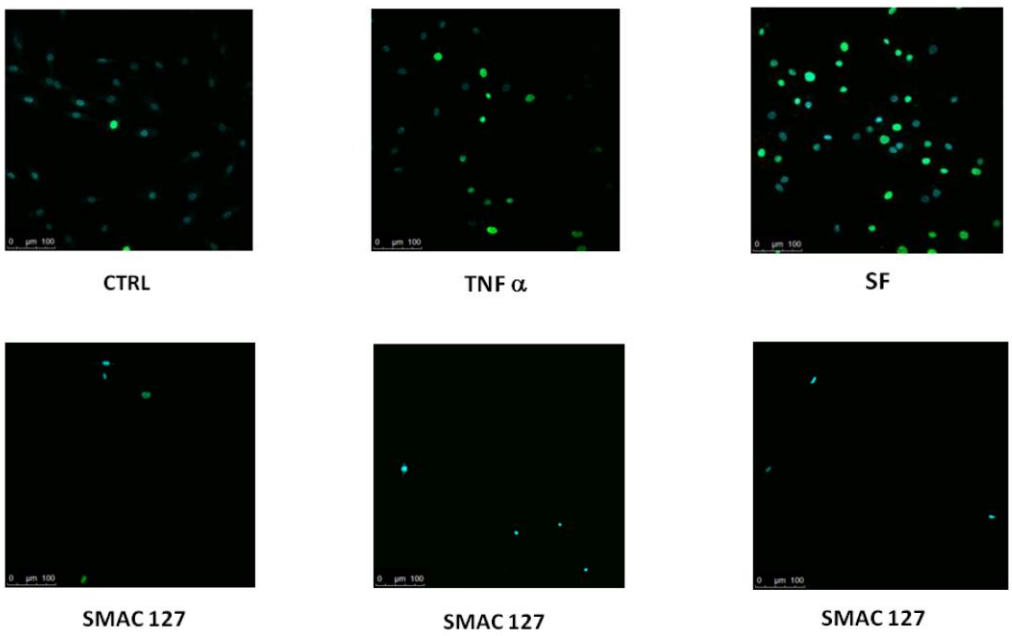
Figure 3. Proliferation assay of RA-FLSs in the presence of different SMCs. A. Confocal microscopy images of RA-FLS proliferation in the presence of SMAC 127 15 μM for 24 h or complete tissue medium alone (CTRL) as compared to SMAC 060 and 066. The newly synthesised DNA was analysed after 24 h incubation with 10 μM 5-ethynyl-2'-deoxyuridine (EdU) and the signal was detected using the fluorescent Alexa Fluor 488 azide. B. Results are shown as % of proliferation: %=(nuclei stained with Edu/total nuclei) x100 and represent the mean ± standard error of three independent experiments.
The images and data regarding SMAC 060 and 066 have been published in Lattuada et al. [73] and serve as a comparison with data regarding SMAC 127.

CTRL= complete tissue medium; *green*—newly synthesised DNA, *blue*—Hoechst staining of total DNA.

6.3 SMAC 127 inhibits proliferation in different culture conditions

In order to better evaluate the effects of SMAC 127 after stimulation of the cells, we evaluated RA-FLS proliferation in the presence of complete tissue medium alone, after addition of recombinant TNF-alpha (25 ng/ml) or in the presence of SF. In fact, TNF-alpha has long been considered the reference standard for stimulation of FLSs of RA patients, whereas culture in SF has been recently demonstrated to be a reliable way to recreate RA joint microenvironment [35]. DNA synthesis was evaluated after incubation with EdU (figure 4A). We found the highest rate of proliferation in the group of cells grown in SF, followed by those grown in TNF-alpha, and the lowest rate in the group grown in complete tissue medium alone (figure 4B). After treatment with SMAC 127, the percentage of proliferating cells in complete tissue medium was 3.6% (vs. 13.9% of CTRL), in the presence of TNF-alpha was 7.7% (vs. 25.8% of TNF-alpha alone), whereas in the presence of SF, the percentage of proliferating cells was 25% (vs. 61.8% of SF alone). Although SF reduced significantly its activity, nevertheless, SMAC 127 was effective also in this culture condition.

A.



B

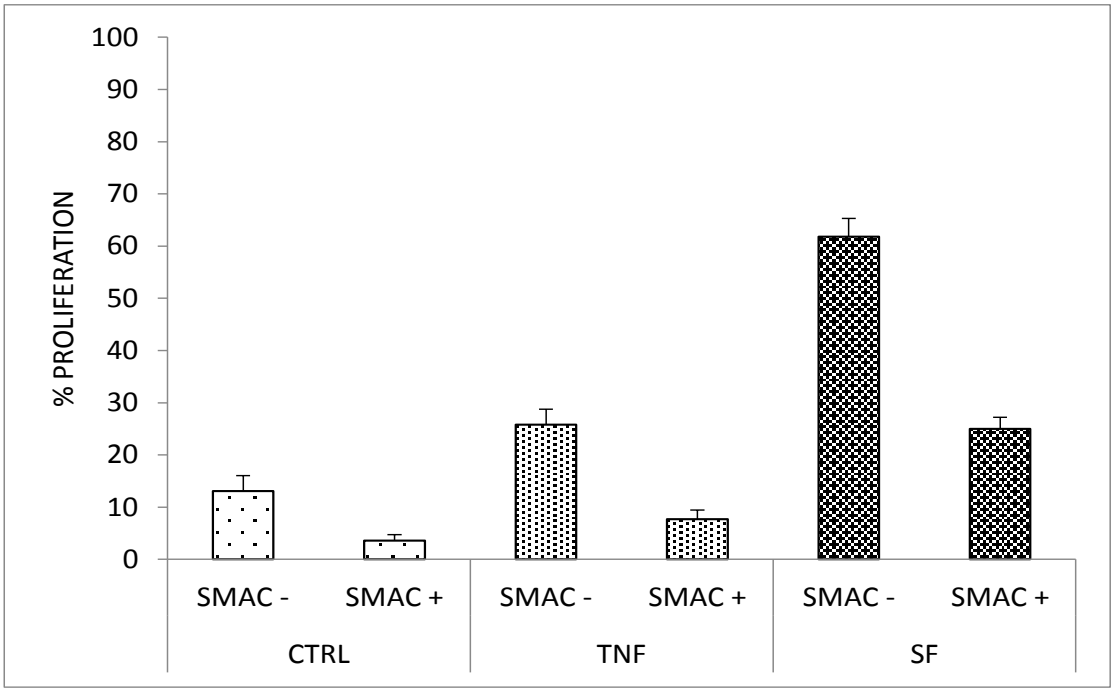


Figure 4. Proliferation assay of RA-FLSs in different culture conditions. A. RA-FLS proliferation in the presence of complete tissue medium alone (CTRL), synovial fluid (SF) 1:8 dilution in culture medium, or TNF-alpha (25 ng/ml) with or without the addition of SMAC 127 15 μM for 24 h. The figure is representative of three independent experiments. B. RA-FLS proliferation in the presence of SF, TNF-alpha or DMEM alone (CTRL) in the presence or absence of SMAC 127. Results are shown as % of proliferation: %=(nuclei stained with Edu/total nuclei) x 100 and represent the mean ± standard error of three independent experiments [the figure has been submitted in a poster at the 2013 Congress of the Biometra Department].
CTRL= controls, cells cultured in complete tissue medium only, SF= pool of SF from RA patients of the study; *green*—newly synthesised DNA, *blue*—Hoechst staining of total DNA

6.4 SMAC 127 induces significant apoptosis in RA-FLSs

Apoptosis was probed by Annexin V test with flow cytometer analysis. Both Annexin V^+PI^- FLS (i.e., early apoptotic) and Annexin V^+PI^+ FLS (i.e., late apoptotic) signals were calculated. Figure 5 shows the results of one experiment on RA-FLSs which is representative of all the experiments.

Finally, as SF is the actual milieu for RA-FLSs and in RA containes anti-apoptotic factors that may reduce the effectiveness of SMCs *in vivo* [35], we also performed several experiments on RA-FLSs cultured in the presence of SF for five days before adding SMAC 127 for 24 h (figure 6). As expected, the response of RA-FLSs to SMAC 127 was reduced when cells were cultured in the presence of SF, although it was still significant when compared to controls.

Then, as in cancer cells one of the hypothesized mechanism for SMC-induced apoptosis is the activation of an autocrine/paracrine loop of TNF-alpha secretion, we sought to understand whether TNF-alpha blockade could interfere with this mechanism in RA-FLSs. Figure 7 shows the results of FACS Annexin V test after exposure to SMAC 127 in the presence of anti-TNF-alpha monoclonal antibody, demonstrating a degree of apoptosis comparable to complete tissue medium alone. Figure 8 represents the mean of all the apoptosis assay experiments performed and a comparison between the different culture conditions. SMAC 127 is significantly effective compared to controls ($p<0.003$, $p<0.00001$ and $p<0.005$ respectively).

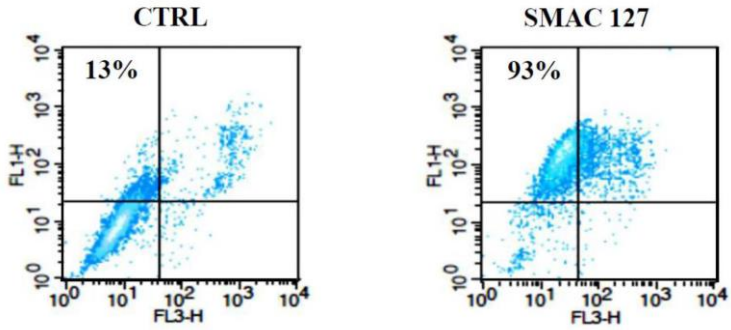


Figure 5. Apoptosis assays in RA-FLSs. FACS analysis shows the degree of apoptosis detected by Annexin V test in RA-FLSs grown in complete tissue medium alone (CTRL) and after the addition of SMAC 127 15 μM for 24 h. Both Annexin V^+PI^- FLS (i.e., early apoptotic) and Annexin V^+PI^+ FLS (i.e., late apoptotic) signals were measured. The total percentage of apoptotic cells is reported in the upper left corner. The image is representative of five individual experiments.
CTRL= complete tissue medium.

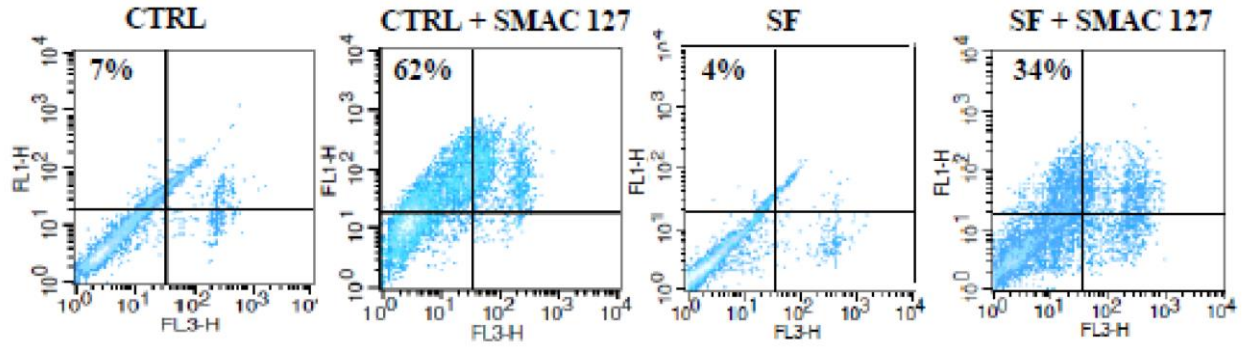


Figure 6. Apoptosis assays in RA-FLSs cultured in the presence of SF. FACS analysis shows the degree of apoptosis detected by Annexin V test in RA-FLSs grown in complete tissue medium alone (CTRL) or after exposure to SF for five days after the addition of SMAC 127 15 μM for 24 h. Both Annexin V^+PI^- FLSs (i.e., early apoptotic) and Annexin V^+PI^+ FLSs (i.e., late apoptotic) signals were calculated. The total percentage of apoptotic cells is reported in the upper left corner. The image is representative of five individual experiments.

CTRL= complete tissue medium, SF= synovial fluid.

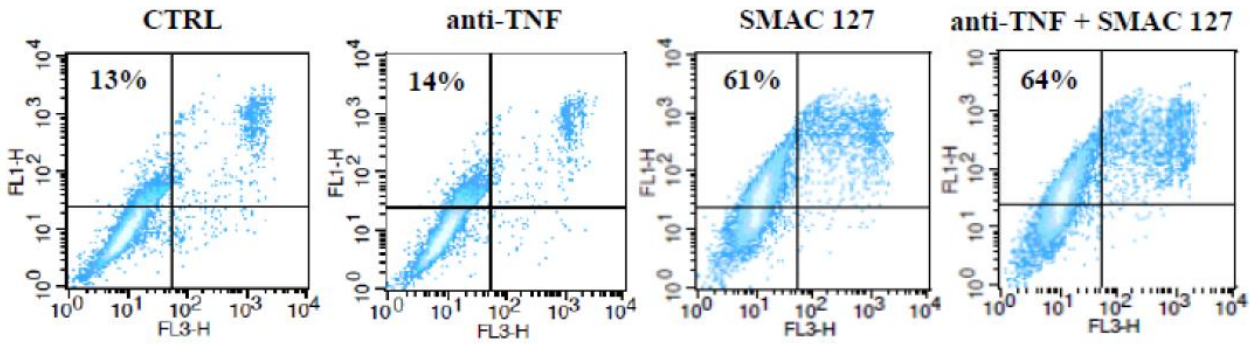


Figure 7. Apoptosis assays in RA-FLSs in the presence of antiTNF-alpha. FACS analysis shows the degree of apoptosis detected by Annexin V test in RA-FLSs grown in complete tissue medium alone (CTRL) and after the addition of SMAC 127 15 μM for 24 h, after the addition of monoclonal anti-TNF-alpha antibody and after the addition of SMAC 127 and anti-TNF-alpha together. Both Annexin V^+PI^- FLS (i.e., early apoptotic) and Annexin V^+PI^+ FLS (i.e., late apoptotic) signals were calculated. The total percentage of apoptotic cells is reported in the upper left corner. The image is representative of two individual experiments.

CTRL= complete tissue medium.

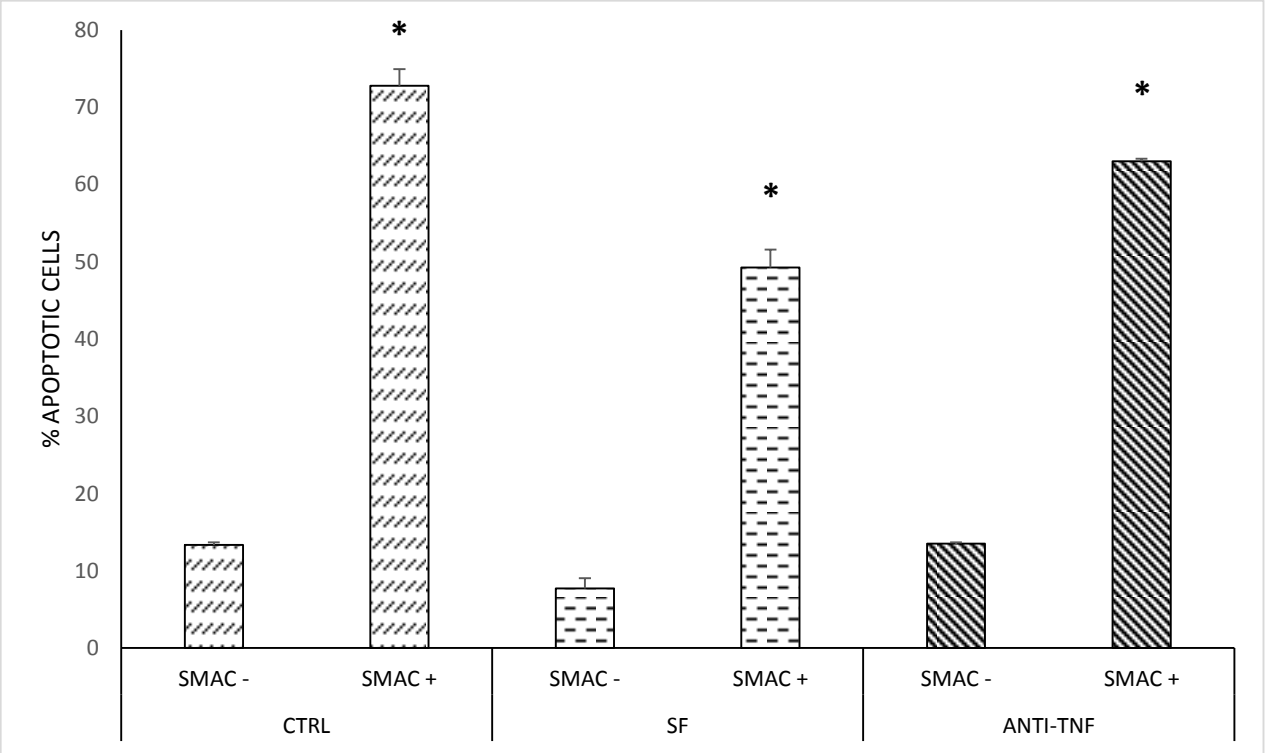


Figure 8. Apoptosis assays in RA-FLSs in different culture conditions. The graph represents the percentage of apoptotic cells in RA-FLSs cultured in complete tissue medium alone (CTRL), in the presence of synovial fluid (SF) for five days or after treatment with anti-TNF-alpha (ANTI-TNF). SMAC 127 15 μM was added 24 h before the esperiments. Apoptosis was detected by Annexin V test. Results are expressed as the mean percentage ± standard error from five individual experiments for CTRL and SF [accepted, Mediators of Inflammation, SMAC 127 has pro-apoptotic and antinflammatory effects on rheumatoid arthritis fibroblast-like synoviocytes, D.Lattuada, **R.Gualtierotti**, K. Crotta et al.], whereas data regarding SMAC 127 plus anti-TNF-alpha are the mean of two experiments.

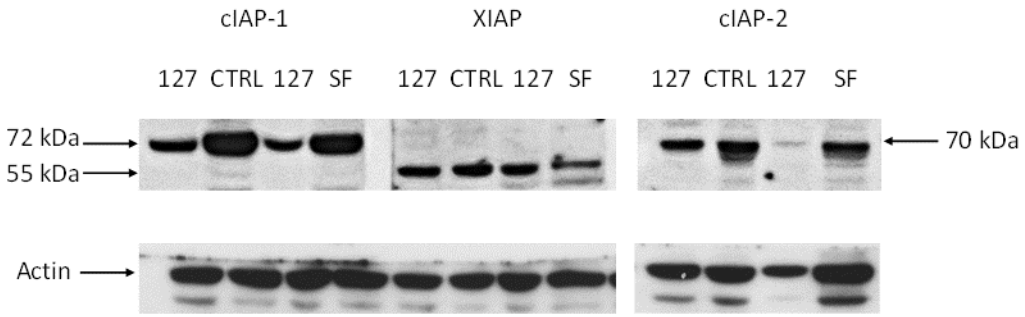
CTRL= complete tissue medium, SF= synovial fluid
* $p < 0.05$ vs CTRL, SF or anti-TNF.

6.5 Effects of SMAC 127 on IAPs in RA-FLSs

Because one of the antagonising effect of SMCs on IAPs is their down-regulation, we investigated the levels of IAPs expressed in RA-FLSs after exposure to SMAC 127. Considered the pro-inflammatory and anti-apoptotic milieu created in RA joints in the presence of SF, we decided to perform the western blots on cell lysates from RA-FLS cultured both in complete tissue medium and in the presence of SF.

Figure 9A represents a set of the experiments performed on the cells from the same patients, with actin used as a loading control. Figure 9B represents the densitometric analysis of four independent experiments [accepted, Mediators of Inflammation, SMAC 127 has pro-apoptotic and antinflammatory effects on rheumatoid arthritis fibroblast-like synoviocytes, D.Lattuada, **R.Gualtierotti**, K. Crotta et al.]. SMAC 127 induced a significant down-regulation of cIAP1, cIAP2 and XIAP in RA-FLSs, both when cultured in complete tissue medium and in the presence of SF. As shown in figure 9B, cIAP1 and XIAP were up-regulated when cells were cultured in SF, as compared to complete tissue medium alone. Differently from the other IAPs, cIAP2 was not up-regulated in SF, although the effects of SMAC 127 on its levels are evident in both conditions (figure 9B).

A.



B.

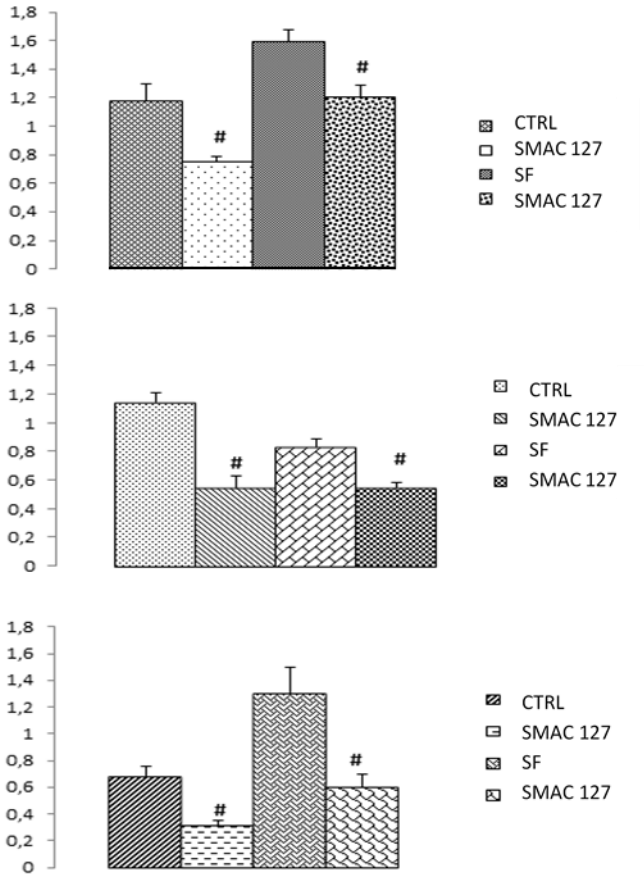


Figure 9. SMAC 127 down-regulates IAPs in RA-FLSs in different culture conditions. SMAC 127 15 μM was left for 48 h. The western blots (A) show the detection of cIAP1 (72 kDa), cIAP2 (70 kDa), and XIAP (55 kDa). As a loading control, we used actin (42 kDa). Densitometric analysis of the western blots (B) show mean ± standard error of the ratio of IAPs/actin protein from four independent experiments (cIAP1, cIAP2 and XIAP respectively from top to bottom) [figure B is accepted in Mediators of Inflammation, SMAC 127 has pro-apoptotic and antinflammatory effects on rheumatoid arthritis fibroblast-like synoviocytes, D.Lattuada, **R.Gualtierotti**, K. Crotta et al.].

CTRL= complete tissue medium, SF= synovial fluid.
# $p<0.005$

6.6 Cleavage of caspase-3 in RA-FLSs by SMAC 127

In order to investigate further the apoptotic activity of SMAC 127, we analysed whether the activation of caspase 3 occurred after the addition of SMAC 127 in RA-FLSs cultured in complete tissue medium or in the presence of SF. As shown in Figure 10A, caspase-3 was effectively cleaved not only in complete tissue medium, but also in the presence of SF (figure 10B) [accepted, Mediators of Inflammation, SMAC 127 has pro-apoptotic and anti-inflammatory effects on rheumatoid arthritis fibroblast-like synoviocytes, D.Lattuada, **R.Gualtierotti**, K. Crotta et al.]. As a positive control, we used cells incubated with the apoptosis inducer staurosporine.

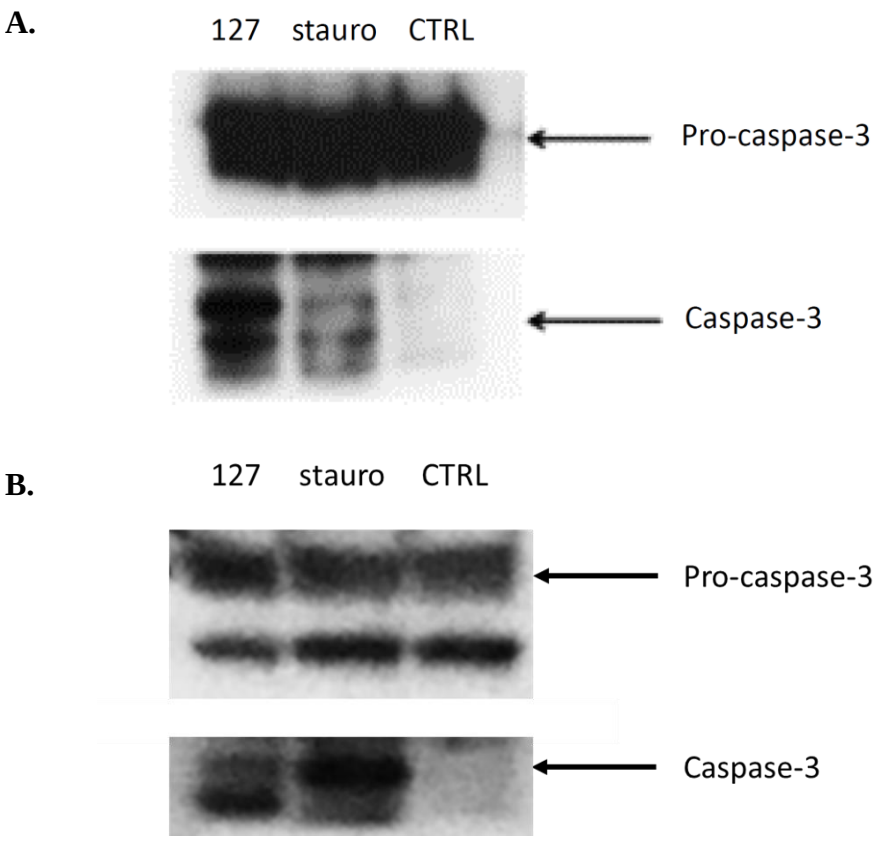


Figure 10. SMAC 127 effectively cleaves procaspase-3 to caspase-3. A: RA-FLSs cultured in complete tissue medium alone (CTRL) or with the addition of SMAC 127 15 μM for 6 h (127). B: RA-FLSs cultured in the presence of SF (SF) or with the addition of SMAC 127 for 18 h (127). Staurosporine worked as a positive control (Stauro). The figure is representative of three independent experiments [accepted, Mediators of Inflammation, SMAC 127 has pro-apoptotic and anti-inflammatory effects on rheumatoid arthritis fibroblast-like synoviocytes, D.Lattuada, **R.Gualtierotti**, K. Crotta et al.].

127= SMAC 127, CTRL= complete tissue medium, stauro= staurosporine, i.e. positive controls, SF= synovial fluid.

6.7 SMAC 127 activity is specific for RA-FLSs

IAPs are not up-regulated in OA-FLSs as demonstrated in a previous work from our Laboratory [73]. Likely, this is the reason why SMCs are not effective in OA-FLSs. Although it is known that RA- and OA-FLSs have a different functional phenotype and different responses to pro-inflammatory cytokines or stimuli such as TNF-alpha [18,23], in order to thouroughly test our hypothesis that SMCs are specific on RA-FLSs, we added TNF-alpha to OA-FLS cultures at baseline (figure 11). Then, we compared the degree of apoptosis in complete tissue medium alone or after the addition of TNF-alpha to the cultures, following the time and dosage as previously reported in the literature [35] after exposure of SMAC 127 for 24 h.

No significant differences in the percentage of apoptotic cells were found between the two groups, with Annexin V test.

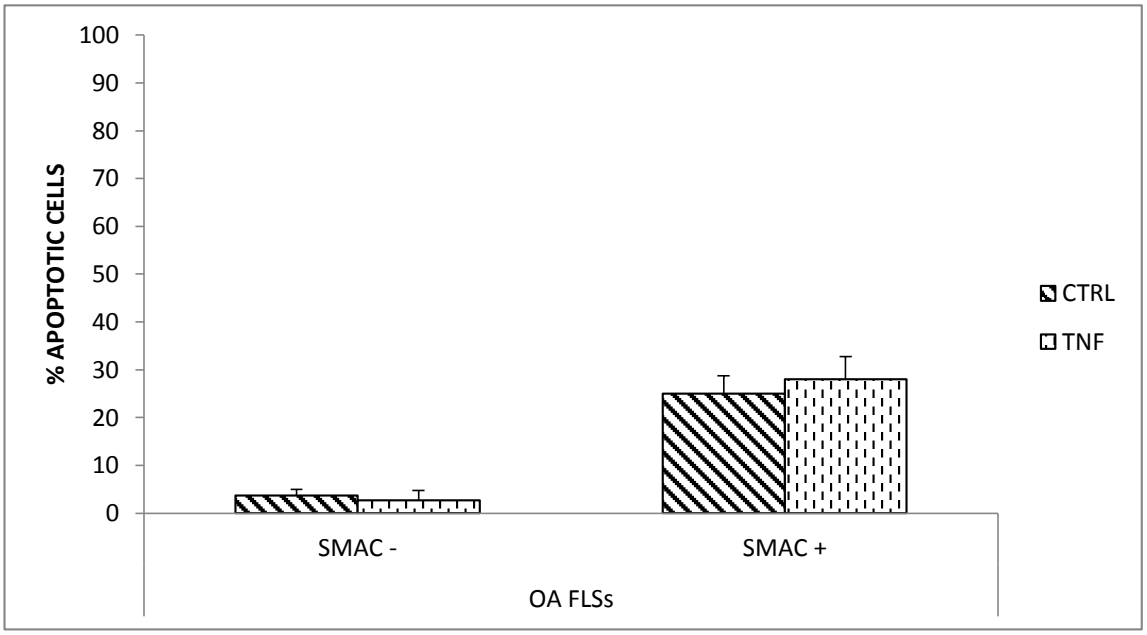


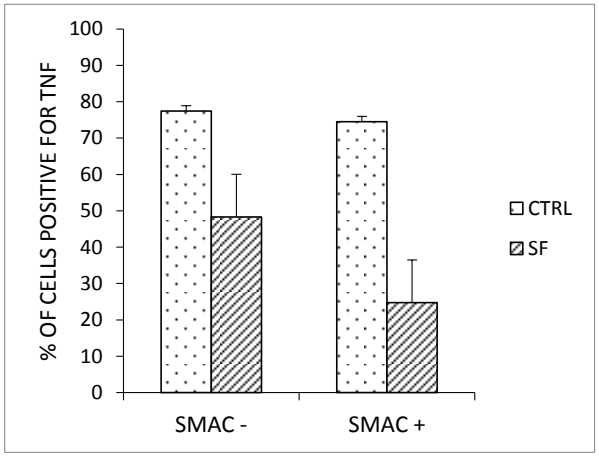
Figure 11. SMAC 127 is ineffective on OA-FLSs even after TNF-alpha stimulation. The graph shows the results of Annexin V test for apoptotic cells after the addition of SMAC 127 15 μM to OA-FLS cultures for 24 h. We compared cells cultured in complete tissue medium alone and cells exposed to TNF-alpha 25 ng/mL added at baseline when OA cells were sub-confluent and leaved in culture for 96 h. We observed no significant difference in the percentage of apoptotic cells between the two groups. The graph represents the mean ± standard error of five independent experiments.

CTRL= complete tissue medium.

6.8 Effects of SMAC 127 on pro-inflammatory cytokines, IL-10, MMP-1, RANKL and OPG

We evaluated the levels of the pro-inflammatory cytokines TNF-alpha, IL-15 and IL-6 and of the anti-inflammatory cytokine IL-10 after addition of SMAC 127 for 24 h. At first we measured TNF-alpha levels in RA-FLSs at baseline (figure 12A), when cultured in complete tissue medium alone (CTRL) or in the presence of SF (SF), and then after the addition of SMAC 127 for 24h. We also measured IL-8 levels in complete tissue medium after TNF-alpha stimulation for 96h and after addition of SMAC 127 15 μM for 24 h (figure 12B), but we found a weak effect of SMAC 127 on IL-8 levels and decided not to further investigate this cytokine. Then cells were grown in different culture conditions (figure 12C): complete tissue medium alone (CTRL), SF exposure for five days (SF) or TNF-alpha exposure for 96 h (TNF-ALPHA). Figure 12C shows that the levels of IL-6 and IL-15 were significantly down-regulated when cells were treated with SMAC 127, whereas IL-10 was significantly up-regulated.

A.



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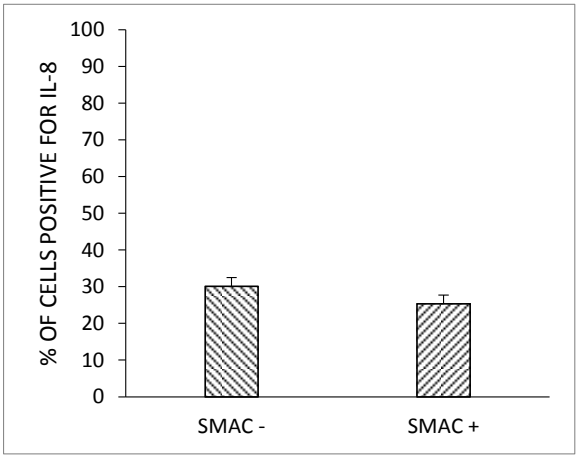

C.

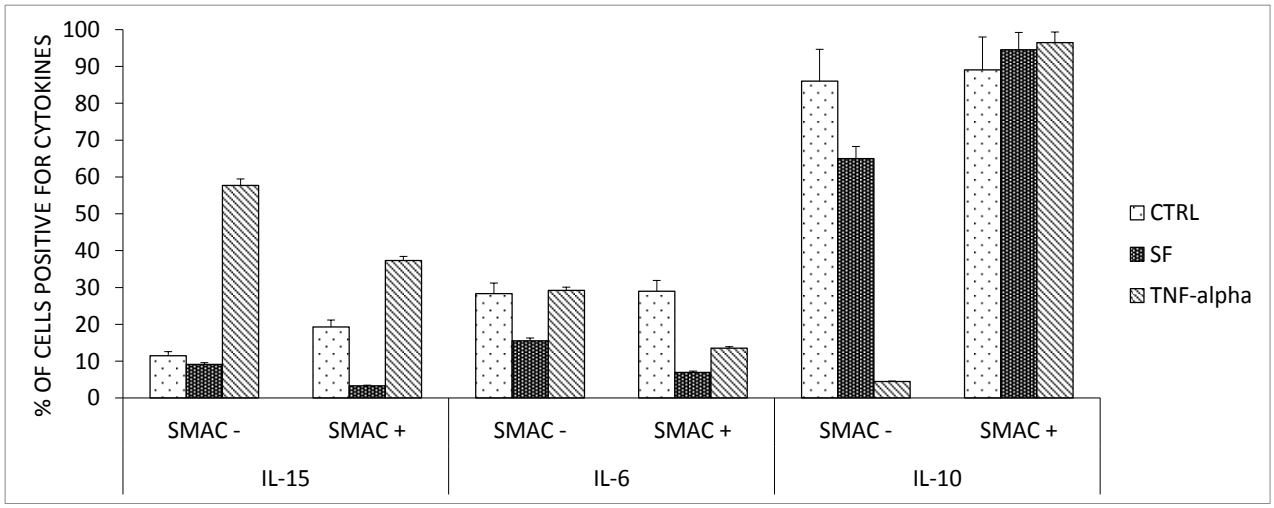

Figure 12. FACS analysis of cytokine regulation in RA-FLSs after exposure to SMAC 127. In A, TNF-alpha levels at baseline (CTRL) and after addition of SMAC 127 15 μM for 24 h were measured in complete tissue medium (CTRL) or SF for 5 days (SF). B: IL-8 levels in complete tissue medium after TNF-alpha stimulation for 96h and after the addition of SMAC 127 15 μM for 24 h. C: the levels of pro-inflammatory cytokines IL-15 and IL-6 and of the anti-inflammatory cytokine IL-10 in different culture conditions - complete tissue medium (CTRL), after stimulus with SF for five days (SF) or TNF-alpha for 96 h (TNF-alpha) - at baseline and after addition of SMAC 127 15 μM for 24 h. Values are expressed as the mean ± standard error of ten independent experiments. Cytokines were analysed by FACS analysis.

CTRL= complete tissue medium, SF= synovial fluid, SMAC= SMAC 127

Data have been confirmed also with the Odyssey platform, which allows quick reading with multiple array of different cytokines of the same sample in the presence of a lower number of cells. We performed experiments both in complete tissue medium and in the presence of SF, which we decided to be our reference standard instead of TNF-alpha exposure [35]. We measured IL-15, IL-6, IL-10, whereas we did not further analyse IL-8, on which SMAC 127 has a weak activity based on FACS cytokine measurement. MMP-1 is one of the main endopeptidases responsible for cartilage destruction the are up-regulated in RA-FLSs, after triggering by inflammatory cytokines [90]. Then we studied the RANK/RANKL/OPG axis. In the presence of pro-inflammatory cytokines RANKL is up-regulated, whereas OPG is down-regulated [91]. Furthermore, because of previous observations in cancer cells reporting that bone resorption could be a side effect of SMC treatment, we decided to investigate the effects of SMC treatment on the RANK/RANKL/OPG axis [92].

As shown in figure 13 (A-C), after the addition of SMAC 127 to the cultures, the pro-inflammatory cytokines IL-15 and IL-6 were down-regulated, anti-inflammatory IL-10 was up-regulated (C); MMP-1 was down-regulated (D), whereas RANKL and OPG levels were not significantly affected, apart from the levels of RANKL, which were higher in SF as compared to complete tissue medium, as expected [accepted, Mediators of Inflammation, SMAC 127 has pro-apoptotic and antinflammatory effects on rheumatoid arthritis fibroblast-like synoviocytes, D.Lattuada, **R.Gualtierotti**, K. Crotta et al.].

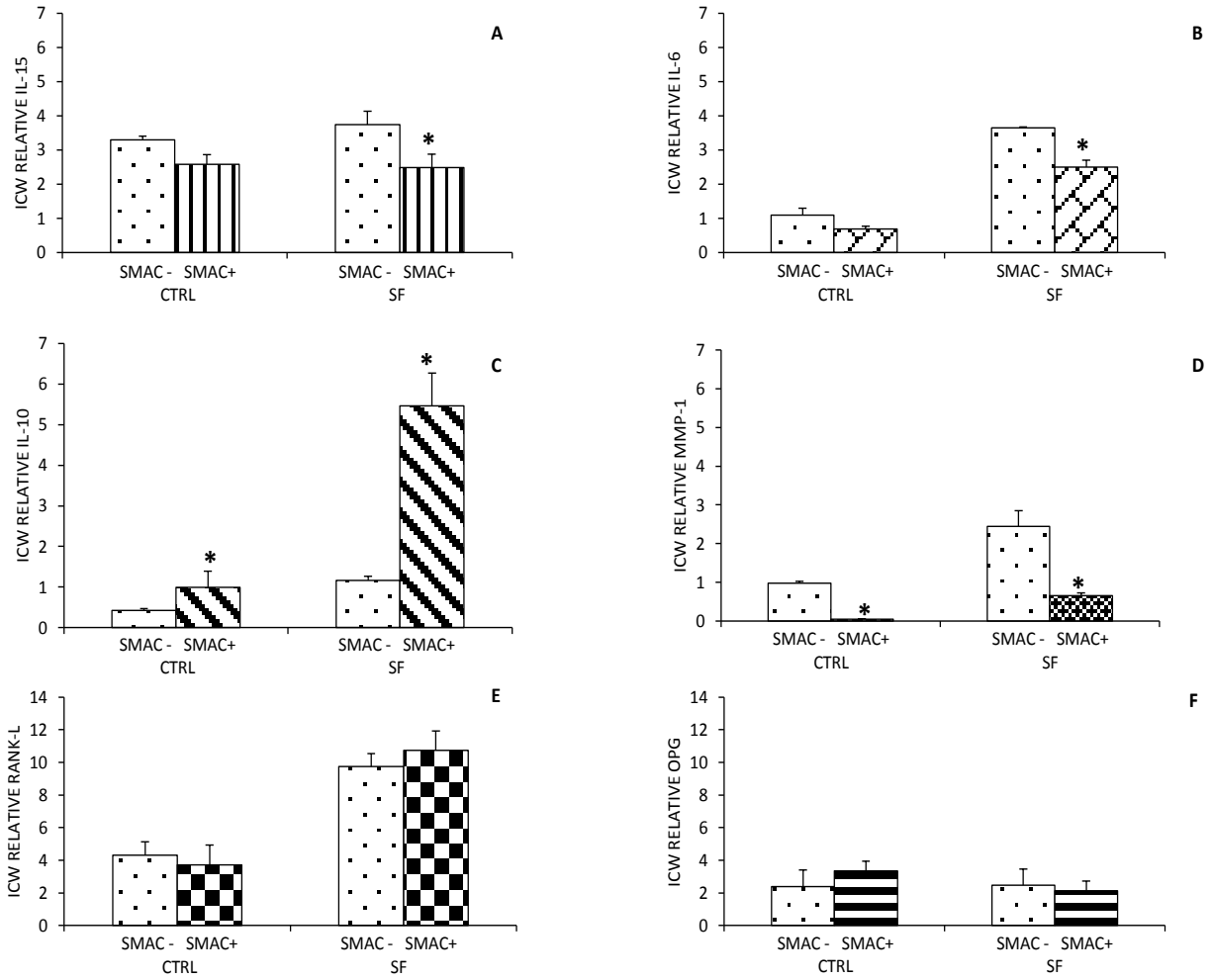

Figure 13. In-cell Western analysis of pro-inflammatory cytokines, IL-10, MMP-1, RANKL and OPG in RA-FLSs after SMAC 127. RA-FLSs grown in complete tissue medium alone (CTRL) or in the presence of synovial fluid for five days (SF) were exposed to SMAC 127 15 μM for 24 h. In-cell western analyses were performed with 800 channel Odyssey Li-COR infrared platform. Data are reported as In-cell western relative unite measure (ICW relative). We analysed the expression of IL-15 (A), IL-6 (B), IL-10 (C), MMP-1 (D) RANKL (E) and OPG (F), which are reported as mean ± standard error of ten independent experiments [data have been submitted to Mediators of Inflammation, SMAC 127 has pro-apoptotic and antinflammatory effects on rheumatoid arthritis fibroblast-like synoviocytes, D.Lattuada, **R.Gualtierotti**, K. Crotta et al.].

CTRL= complete tissue medium, ICW= in-cell Western, SF= synovial fluid, SMAC= SMAC 127
* $p < 0.05$ vs untreated cells.

7. DISCUSSION

Biological therapy represented a breakthrough in RA treatment in the last decades. Currently, the biological drugs approved for the treatment of RA include anti-TNF-alpha monoclonal antibodies, anti-CD20 receptor; tocilizumab, a humanized monoclonal anti IL-6 receptor antibody; abatacept, a CTLA-4 mimic that blocks the co-stimulation of T-cells, and anakinra, an antagonist of the IL-1 receptor [93,94]. Anti-cytokine therapies, but also anti-co-stimulatory and anti-CD20 therapy were designed for the first time as a targeted therapy for RA, in contrast to small molecules which were designed for different purposes and demonstrated to have pleiotropic effects also on the immune system. Nevertheless, many patients with RA do not respond in a high percentage of cases [10]. Furthermore, none of the currently available therapies for RA has aetiological effects. Almost all of the available therapies for RA have anti-inflammatory or immunosuppressive effects.

Among the many factors involved in the pathogenesis of RA, RA-FLSs play a pivotal role both in the initiation and in the progression of RA. RA-FLSs drive the destruction of joints in RA, especially of articular cartilage and subchondral bone [23]. Moreover, FLSs are the main cells responsible for RA synovial hyperplasia due to their resistance to apoptosis. Resistance to apoptosis in these cells depends, among other factors, also on IAPs up-regulation [10]. The anti-apoptotic activity of IAPs, in normal conditions, is blocked by the naturally occurring antagonist, the mitochondrial protein SMAC/DIABLO, which is set free into the cytosol in response to a pro-apoptotic stimulus [86,95], and is able to activate the so-called intrinsic apoptotic pathway. Several SMCs have been synthesised, both monomers and dimers that imitate the structure of SMAC. SMCs were initially designed as new pro-apoptotic anti-neoplastic drugs for cancer cells with up-reglated levels of IAPs and have been used with satisfying results, both in combination with traditional chemotherapeutics and radiation or, most surprisingly, alone [42].

Recently, the role of IAPs in regulating the inflammatory pathway and the immune system has emerged [81]. Furthermore, a previous study from our Laboratory recently demonstrated that SMCs are effective in inducing apoptosis also in RA-FLSs and can down-regulate the pro-inflammatory cytokine IL-15 [73]. These findings and the evidence from the literature encouraged us to perform this study, which is the first to show that the monomer SMAC 127 is effective in inducing apoptosis and to induce a down-regulation of pro-inflammatory cytokines with up-regulation of IL-10 in FLSs from patients with RA.

To do so, samples of synovial membranes were collected from 22 patients affected by RA, which were later processed and analysed. As control, we used synovial membranes from five patients with OA. No significant correlations were found between the results of our experiments and age, sex, disease, duration and therapy. This may be due to the fact that the cells isolated by patients were used after multiple passages. These passages likely allow the complete metabolisation of DMARDs. First of all, we confirmed the previous observations regarding RA-FLSs cultured in the presence of synovial fluid, which gradually acquired a ramifying aspect (figure 2) as compared with RA-FLSs cultured in complete tissue medium alone (figure 1).

Then, we observed that SMAC 127 is a monomeric compound with a stronger pro-apoptotic activity on RA-FLSs as compared to the previously studied SMAC 066 [73]. In fact, the proliferation assay demonstrated a lower proliferation rate in the presence of SMAC 127 as compared with previous data regarding SMAC 066 and 060 (figure 3) [73]. This is not surprising, due to the fact that SMAC 127 molecule was synthesised in order to enhance lipophilicity and promote intracellular uptake, as compared to previous compounds. We also tested SMAC 127 effects on proliferation in different culture conditions (figure 4). In particular, we cultured RA-FLSs in the presence of TNF-alpha, which has long been considered the reference standard stimulus for RA-FLS proliferation, and in the presence of SF from patients with RA. In order to reduce variability we chose to use a pool of SF from ten RA patients, added to culture medium with a 1:8 dilution. We found the highest rate of

proliferation in the group of cells grown in SF, followed by those grown in TNF-alpha, and the lowest rate in the cells grown in complete tissue medium alone. We hypothesised that the presence in the cultures of SF from patients with RA could favour the resistance to apoptosis and may blunt the effects of SMCs on RA-FLSs. In fact, SF from RA patients characterizes and modulates the microenvironment of joints, by means of its complex composition. In fact, SF collected from patients with RA contains a great amount of inflammatory cytokines, including IL-1β, IL-17 and TNF-alpha, growth factors and anti-apoptotic factors, many of which are still unknown [35]. Therefore, we tested the effectiveness of SMAC 127 even in the presence of stimulatory factors such as SF or of TNF-alpha. Despite these adverse culture conditions, we found that SMAC 127 was effective in reducing proliferation of RA-FLSs in all the experimental conditions (figure 4B). As expected, SF dampened the effects of SMCs on RA-FLSs proliferation, as compared to complete tissue medium alone. On the one hand, we confirmed our hypothesis that SF from patients with RA has an inhibitory effect on SMC activity. On the other hand, the results of our experiments confirmed that SMAC 127 is effective also in conditions that reproduce the actual milieu of RA-FLSs.

In order to test the pro-apoptotic effects of SMAC 127 on RA-FLSs, we used the Annexin V test. Its rationale is based on the flow cytometric analysis of the Annexin V, which binds the phosphatidylserine residues exposed on the outer cell surface during apoptosis (figure 5). Both Annexin V^+PI^-FLSs (i.e., early apoptotic) and Annexin V^+PI^+FLSs (i.e., late apoptotic) signals were considered in the analysis. In order to obtain the most reliable results, we tested SMAC 127 activity in different culture conditions (figures 5-8). The mean percentage of apoptotic cells after treatment with SMAC 127 was higher for cells cultured in complete tissue medium alone, as compared with cells cultured in the presence of SF. These results further confirm that SF is a more reliable medium to reproduce RA-FLS *in vivo* conditions [accepted, Mediators of Inflammation, SMAC 127 has pro-apoptotic and antinflammatory effects on rheumatoid arthritis fibroblast-like synoviocytes, D.Lattuada, **R.Gualtierotti**, K. Crotta et al.]. Then, we sought to verify whether the mechanism of

action of SMCs on RA-FLSs was based on an enhancement in TNF-alpha production, as hypothesised for cancer cells. Therefore, we added anti-TNF-alpha to the culture medium and found that the degree of apoptosis was higher when cells were exposed to SMAC 127 as compared to controls, although the number of apoptotic cells was lower than with SMAC 127 alone (figures 7 and 8). Our results are consistent with the recent demonstration that the induction of TNF-alpha could be dispensable for SMC-induced apoptosis, which may also involve the ligand-independent activity of DR5 [97]. In conclusion, SMAC 127 results effective in inhibiting the proliferation of RA-FLSs in all culture conditions.

Then, as SMCs exert their functions by means of IAP antagonism, we evaluated the levels of IAPs in RA-FLSs cultured both in complete tissue medium and in SF. We focused on cIAP1, cIAP2 and XIAP for different reasons. First, cIAP1, cIAP2 and XIAP play a pivotal role in apoptosis regulation [42]. Secondly, a previous study from our laboratory demonstrated that cIAP1, cIAP2 and XIAP are up-regulated in RA-FLSs, thus explaining the resistance to apoptosis and providing the rationale for the use of SMCs in RA [60]. Finally, there is growing evidence for a key role of cIAP1, cIAP2 and XIAP in the regulation of innate immunity and inflammatory responses [96]. As expect, SMAC 127 was able to significantly down-regulate cIAP1, cIAP2, and XIAP, both in the presence of complete tissue medium and in the presence of SF, as demonstrated by western blotting (figure 9) [accepted, Mediators of Inflammation, SMAC 127 has pro-apoptotic and antiinflammatory effects on rheumatoid arthritis fibroblast-like synoviocytes, D.Lattuada, **R.Gualtierotti**, K. Crotta et al.]. SMAC 127 treatment leads to the down-regulation of IAPs via the induction of both auto-ubiquitination and proteasomal degradation [44]. Consistent with our hypothesis that SF contributes to the resistance of apoptosis, we also observed that cIAP1 and XIAP levels were up-regulated in cells grown in SF as compared to complete tissue medium alone, although cIAP2 did not follow this trend. As IAPs are redundant proteins, it is possible that the main responsible for the resistance to apoptosis in RA-FLSs, particularly after stimulation with

SF, could be due to an up-regulation of cIAP1 and XIAP with no or limited effect on cIAP2. Further studies are needed to verify this hypothesis. Nevertheless, all the IAPs we studied – also cIAP2 – were down-regulated, after exposure to SMAC 127 and we have already demonstrated a significantly higher degree of apoptosis in cells treated with SMAC 127. Therefore, we can conclude that SMAC 127 was able to restore a normal apoptotic machinery in RA-FLSs.

Our team has already demonstrated that the proapoptotic effects of SMCs are due to the inhibitory effects on IAPs and consequent activation of caspases in RA-FLSs [73]. Therefore, we decided to investigate if also SMAC 127 was able to promote the activation of the effector caspase in these cells. As expected, we found (figure 10) that also SMAC 127 is able to promote the proteolytic activation of pro-caspase-3 into the active caspase-3 [accepted, Mediators of Inflammation, SMAC 127 has pro-apoptotic and antiinflammatory effects on rheumatoid arthritis fibroblast-like synoviocytes, D.Lattuada, **R.Gualtierotti**, K. Crotta et al.]. In fact, SMCs directly interfere with XIAP and specifically block their ability to bind and inhibit caspase-3. Furthermore, SMCs can activate the E3 ligases of cIAP1 and cIAP2, which are engaged in their autoubiquitination and proteasomal degradation [40,97].

A previous work from our team demonstrated that, differently from RA-FLSs, the expression of IAPs in OA-FLSs is not up-regulated, and likely for this reason SMCs have no activity on OA-FLSs [73]. Nonetheless, as a further confirmation of our hypothesis, we decided to use SMAC 127 on OA-FLSs after the stimulation with TNF-alpha. We found no significant difference in the percentage of apoptotic cells after SMAC 127 treatment as compared to controls, even when TNF-alpha was added at baseline and leaved in culture of OA-FLSs for 96 h (figure 11). This is due to the different physiopathologic features and responses to pro-inflammatory cytokines displayed from RA-FLSs vs. OA-FLSs [18].

In RA, FLSs are important mediators of the inflammatory damage in joints, as they exaggeratedly produce and respond to pro-inflammatory cytokines [34]. In this study we focused on TNF-alpha,

IL-6, IL-8 and IL-15. We chose TNF-alpha that is involved in the inflammatory process underlying the development and progression of RA. TNF-alpha is a major pro-inflammatory cytokine that in normal conditions exerts an integral role in mounting the inflammatory response against invading pathogens. It is secreted by activated monocytes, macrophages and T-cells. It is involved in the inflammatory process underlying the development and progression of RA [98]. Anti-TNF-alpha treatment was the first targeted therapy in autoimmune diseases and proved effective in relieving symptoms and reducing the radiographic progression of patients treated with it [99]. The blockade of spontaneously produced TNF-alpha down-regulates many pro-inflammatory cytokines among which even IL-6 and IL-8 [100]. IL-6 is involved in both initiation and progression of RA, where FLSs are both its target and responsible for its production. The introduction of the anti-IL-6 receptor monoclonal antibody, tocilizumab, for RA therapy and its effectiveness in clinical practice, clearly confirm a major role of this cytokine in the pathogenesis of joint and systemic inflammation in RA [101,102]. IL-8 production contributes to the perpetuation of RA-associated synovitis in a similar manner as IL-6 and its production by FLSs is triggered by TNF-alpha [100]. IL-15, a pro-inflammatory cytokine that shares many functions with IL-2, is overexpressed in the synovial membrane of patients with RA, particularly in the SF of early RA patients. IL-15 was initially reckoned to be a T-cell growth factor, but it is also important for the differentiation of B cells [103]. IL-15 also drives osteoclastogenesis [104].

In order to investigate the role of SMAC 127 on the inflammatory response, we evaluated the levels of the pro-inflammatory cytokines TNF-alpha, IL-6 and IL-15 and of the anti-inflammatory cytokine IL-10 after addition of SMAC 127 after incubation of cells in different culture conditions: complete tissue medium alone, SF or TNF-alpha exposure. As demonstrated in Figure 12, SMAC 127 inhibited the secretion of the pro-inflammatory cytokines TNF-alpha, IL-15 and IL-6, whereas on IL-8 we observed a weak effect and decided not to perform further analyses on it. Furthermore, IL-10 levels were significantly up-regulated.

These results were confirmed also by Odissey analysis (figure 13), that we decided to perform in cells cultured in complete tissue medium and in SF. The Odyssey platform allows to perform In-cell Western (ICW) analyses, immunocytochemical assays, which can be conducted on microplates. In this analysis, target-specific primary antibodies are bound by secondary antibodies with infrared dye labels. The use of infrared fluorescence allows to reduce interferences, as compared to standard methods. The advantages of this procedure are the enhanced accuracy and the high sensitivity, because target proteins are detected in their cellular context [www.licor.com].

In addition, we measured MMP-1, RANKL and OPG with this platform. In RA, cartilage destruction is driven by RA-FLSs, responsible for the production of MMPs, an ample family of zinc-dependent endopeptidases whose activity is directed against extracellular matrix [90]. Among these, MMP-1 secretion is up-regulated in RA-FLSs after triggering by inflammatory cytokines [105]. By contrast, bone resorption is regulated by the RANK/RANKL/OPG axis: the interaction of RANKL with the correspondent receptor, RANK, expressed on the cell membrane of osteoclasts, induces their differentiation, multinucleation and activation, with subsequent bone resorption. The natural inhibitor of RANKL-RANK interaction is the decoy receptor OPG. RANKL is known to be up-regulated, whereas OPG is down-regulated in RA-FLSs in the presence of pro-inflammatory cytokines [91]. We sought to verify whether SMAC 127 could interfere with the RANK/RANKL/OPG axis consistent with previous observations in cancer cells reporting that bone resorption could be a side effect of SMC treatment [92]. Infact, SMCs induce NIK-mediated non-canonical NF-κB pathway activation that could lead to an up-regulation of RANKL and down-regulation of OPG. This asset may lead to osteoclast differentiation and activation, with a subsequent higher risk of bone metastases – such as in cancer cells, or of bone erosions - in the case of RA [42].

Data regarding the down-regulation of the pro-inflammatory cytokines IL-15 and IL-6, and of an up-regulation of anti-inflammatory cytokine IL-10 were confirmed also with the Odyssey platform.

Therefore, apart from restoring the sensitivity to apoptosis, SMCs can also induce the down-regulation of pro-inflammatory cytokines. The mechanisms by which SMCs can induce TNF down-regulation and up-regulation of IL-10 will be the subject of our next study. We found up-regulated levels of IL-10 in RA-FLSs. IL-10 is elevated in RA SF where it is likely responsible for the perpetuation of antibody production but also for the negative feedback on the synthesis of pro-inflammatory cytokines, such as TNF-alpha, IL-1 and GM-CSF [106,107]. Furthermore, MMP-1 was down-regulated by SMAC 127. As expected, RANKL levels were higher when cells were cultured with SF than with complete tissue medium. After exposure to SMAC 127 we found that RANKL and OPG levels were not significantly affected [accepted, Mediators of Inflammation, SMAC 127 has pro-apoptotic and antinflammatory effects on rheumatoid arthritis fibroblast-like synoviocytes, D.Lattuada, **R.Gualtierotti**, K. Crotta et al.]. Our findings potentially exclude the risk of an enhanced bone resorption induced by SMCs, at least in RA patients.

In cancer cells, up-regulation of IAPs is one of the major responsible for resistance to TNF-alpha-induced apoptosis and SMC treatment can resptore the response to TNF-alpha induced apoptosis [108]. Similarly, our results confirmed the rationale of using SMAC 127 in RA, where up-regulated IAPs in RA-FLSs provide resistance to apoptosis and SMCs can remove it. Therefore, SMCs could be useful since the early stage of RA in order to reduce FLS hyperplasia and the subsequent inflammation-driven articular cartilage and bone destruction. We may hypothesize that SMAC 127 can restore TNF-alpha induced apoptosis, as already hypothesised for cancer cells . However, based on our findings, and consistent with previous studies, TNF-alpha blockade does not inhibit SMC-induced apoptosis, thus demonstrating that the autocrine/paracrine TNF-alpha/TNFR1 loop is dispensable for SMC-induced cell death [97]. Instead, DR5, which is a member of the TNFR superfamily and one of the genes regulated by NF-κB, was shown to be up-regulated after SMC treatment [97]. This could be an interesting explanation for the restored sensitivity to apoptosis also

in RA-FLSs. In fact, DR5 signalling may induce the activation of the apoptotic pathway in a ligand/TNF-alpha independent way. We will perform further experiments to test this hypothesis.

Furthermore, IAPs up-regulation may also be responsible for the perpetuation of severe inflammation in RA-FLSs. In fact, apart from their role in apoptosis regulation, IAPs are involved in the modulation of the inflammatory responses [108]. We demonstrated that SMCs can reduce the production of pro-inflammatory citokines and can up-regulate the production of IL-10. This is consistent with previous findings that IAP antagonism can suppress the induction of pro-inflammatory cytokines in response to TNF-alpha by acting on different signalling pathways such as the canonical pathway of NF-κB and reduce the activity of kinases such as MEK/ERK, p38MAPK and JNK, which are important mediators for the TNF-induced expression of pro-inflammatory cytokines and chemokines [50,81,108]. Additionally, although it does not prevent NF-κB activation, SMC treatment can differentially modulate the inflammatory responses mediated by TLR4, by selectively reducing the expression of pro-inflammatory citokines without interfering with the expression of IL-10 [82]. Another possible explanation is that SMC treatment may restore a normal homeostasis in RA-FLS through the regulation of TNFR1 and TNFR2, which are both expressed on the cell surface of RA-FLSs and drive different signal pathways [109]. On the one hand, TNFR1 mediates pro-inflammatory signals. On the other hand, the role of TNFR2 is highly context-dependent and may be polarized to anti-inflammatory signals [110] and even anti-apoptotic signals [111]. As mentioned above, IAPs are necessary for TNFR1 pro-inflammatory signalling. SMC treatment, via depletion of cIAP1 and cIAP2, allows the formation of RIP1/FADD/caspase-8 complex and a shift towards a pro-apoptotic signalling [43]. The specific inhibition of TNFR1 could leave TNFR2 normal functions unaltered.

Furthermore, evidence form animal models speaks in favour of a reduced recruitment of leukocytes into tissues by down-regulation of the endothelial ICAM-1 [80]. Although the mechanisms of action

of SMCs have not been completely elucidated, SMC treatment seems to be suitable for treatment in RA.

8. CONCLUSIONS

In conclusion, we demonstrated that treatment of RA-FLS with SMCs induces apoptosis by acting on IAPs, which are up-regulated in RA-FLSs, especially when cultured in SF. Aside from directly inhibiting XIAP, SMCs also induce ubiquitination of cIAPs finally leading to cleavage of caspase-3. Finally and importantly, we demonstrated that SMC treatment also down-regulates pro-inflammatory cytokines and up-regulates IL-10. We observed all these effects of SMC treatment in an *in vitro* ideal model, but we reckon that these effects may apply to an *in vivo* scenario as well. In fact, a reduced RA-FLS hyperplasia and a dampened inflammation in the joints may effectively interpose with the maintenance of the chronicity of RA. Based on our findings, SMC treatment may be suitable both in early and in late RA. Experiments *in vivo* will be necessary in order to evaluate the pharmacokinetics, the way of administration and the safety of SMCs.

REFERENCES

References

1. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. N Engl J Med 2011 December 8;365(23):2205-19.

2. Firestein GS. Invasive fibroblast-like synoviocytes in rheumatoid arthritis. Passive responders or transformed aggressors? Arthritis Rheum 1996 November;39(11):1781-90.

3. MacGregor AJ, Snieder H, Rigby AS, Koskenvuo M, Kaprio J, Aho K et al. Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins. Arthritis Rheum 2000 January;43(1):30-7.

4. Gregersen PK, Silver J, Winchester RJ. The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum 1987 November;30(11):1205-13.

5. Klareskog L, Ronnelid J, Lundberg K, Padyukov L, Alfredsson L. Immunity to citrullinated proteins in rheumatoid arthritis. Annu Rev Immunol 2008;26:651-75.

6. Symmons DP, Bankhead CR, Harrison BJ, Brennan P, Barrett EM, Scott DG et al. Blood transfusion, smoking, and obesity as risk factors for the development of rheumatoid arthritis: results from a primary care-based incident case-control study in Norfolk, England. Arthritis Rheum 1997 November;40(11):1955-61.

7. De RL, Peene I, Hoffman IE, Kruithof E, Union A, Meheus L et al. Rheumatoid factor and anticitrullinated protein antibodies in rheumatoid arthritis: diagnostic value, associations with radiological progression rate, and extra-articular manifestations. Ann Rheum Dis 2004 December;63(12):1587-93.

8. Capellino S, Cosentino M, Wolff C, Schmidt M, Grifka J, Straub RH. Catecholamine-producing cells in the synovial tissue during arthritis: modulation of sympathetic neurotransmitters as new therapeutic target. Ann Rheum Dis 2010 October;69(10):1853-60.

9. Feldmann M, Maini RN. Anti-TNF alpha therapy of rheumatoid arthritis: what have we learned? Annu Rev Immunol 2001;19:163-96.

10. Pope RM. Apoptosis as a therapeutic tool in rheumatoid arthritis. Nat Rev Immunol 2002 July;2(7):527-35.

11. Cantaert T, Brouard S, Thurlings RM, Pallier A, Salinas GF, Braud C et al. Alterations of the synovial T cell repertoire in anti-citrullinated protein antibody-positive rheumatoid arthritis. Arthritis Rheum 2009 July;60(7):1944-56.

12. Humby F, Bombardieri M, Manzo A, Kelly S, Blades MC, Kirkham B et al. Ectopic lymphoid structures support ongoing production of class-switched autoantibodies in rheumatoid synovium. PLoS Med 2009 January 13;6(1):e1.

13. Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. N Engl J Med 2009 August 27;361(9):888-98.

14. Behrens F, Himsel A, Rehart S, Stanczyk J, Beutel B, Zimmermann SY et al. Imbalance in distribution of functional autologous regulatory T cells in rheumatoid arthritis. Ann Rheum Dis 2007 September;66(9):1151-6.

15. Edwards JC, Szczepanski L, Szechinski J, Filipowicz-Sosnowska A, Emery P, Close DR et al. Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. N Engl J Med 2004 June 17;350(25):2572-81.

16. Smith MD, Walker JG. The synovium. In: Hochberg, Silman, Smolen, Weinblatt, Weisman, editors. Rheumatology. 5th ed. 2011. p. 51-6.

17. Smith MD. The normal synovium. Open Rheumatol J 2011;5:100-6.

18. Bottini N, Firestein GS. Duality of fibroblast-like synoviocytes in RA: passive responders and imprinted aggressors. Nat Rev Rheumatol 2013 January;9(1):24-33.

19. Smith MD, Barg E, Weedon H, Papengelis V, Smeets T, Tak PP et al. Microarchitecture and protective mechanisms in synovial tissue from clinically and arthroscopically normal knee joints. Ann Rheum Dis 2003 April;62(4):303-7.

20. Avouac J, Fransen J, Walker UA, Riccieri V, Smith V, Muller C et al. Preliminary criteria for the very early diagnosis of systemic sclerosis: results of a Delphi Consensus Study from EULAR Scleroderma Trials and Research Group. Ann Rheum Dis 2011 March;70(3):476-81.

21. Lipsky PE. Why does rheumatoid arthritis involve the joints? N Engl J Med 2007 June 7;356(23):2419-20.

22. Ahn JK, Kim H, Lee J, Bae EK, Cha HS, Koh EM. Phenotypic characterization and invasive properties of synovial fluid-derived adherent cells in rheumatoid arthritis. Inflammation 2008 December;31(6):365-71.

23. Bartok B, Firestein GS. Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. Immunol Rev 2010 January;233(1):233-55.

24. Lafyatis R, Remmers EF, Roberts AB, Yocum DE, Sporn MB, Wilder RL. Anchorage-independent growth of synoviocytes from arthritic and normal joints. Stimulation by exogenous platelet-derived growth factor and inhibition by transforming growth factor-beta and retinoids. J Clin Invest 1989 April;83(4):1267-76.

25. Lefevre S, Knedla A, Tennie C, Kampmann A, Wunrau C, Dinser R et al. Synovial fibroblasts spread rheumatoid arthritis to unaffected joints. Nat Med 2009 December;15(12):1414-20.

26. Fassbender HG. Histomorphological basis of articular cartilage destruction in rheumatoid arthritis. Coll Relat Res 1983 March;3(2):141-55.

27. You S, Yoo SA, Choi S, Kim JY, Park SJ, Ji JD et al. Identification of key regulators for the migration and invasion of rheumatoid synoviocytes through a systems approach. Proc Natl Acad Sci U S A 2014 January 7;111(1):550-5.

28. Muller-Ladner U, Evans CH, Franklin BN, Roberts CR, Gay RE, Robbins PD et al. Gene transfer of cytokine inhibitors into human synovial fibroblasts in the SCID mouse model. Arthritis Rheum 1999 March;42(3):490-7.

29. Rosengren S, Boyle DL, Firestein GS. Acquisition, culture, and phenotyping of synovial fibroblasts. Methods Mol Med 2007;135:365-75.

30. Lefevre S, Meier F, Neumann E, Muller-Ladner U. Role of synovial fibroblasts in rheumatoid arthritis. Curr Pharm Des 2014 August 25.

31. Ohata J, Zvaifler NJ, Nishio M, Boyle DL, Kalled SL, Carson DA et al. Fibroblast-like synoviocytes of mesenchymal origin express functional B cell-activating factor of the TNF family in response to proinflammatory cytokines. J Immunol 2005 January 15;174(2):864-70.

32. Lee DM, Kiener HP, Agarwal SK, Noss EH, Watts GF, Chisaka O et al. Cadherin-11 in synovial lining formation and pathology in arthritis. Science 2007 February 16;315(5814):1006-10.

33. Niu X, Chen G. Clinical biomarkers and pathogenic-related cytokines in rheumatoid arthritis. J Immunol Res 2014;2014:698192.

34. Niedermeier M, Pap T, Korb A. Therapeutic opportunities in fibroblasts in inflammatory arthritis. Best Pract Res Clin Rheumatol 2010 August;24(4):527-40.

35. Casnici C, Lattuada D, Tonna N, Crotta K, Storini C, Bianco F et al. Optimized "in vitro" culture conditions for human rheumatoid arthritis synovial fibroblasts. Mediators Inflamm 2014;2014:702057.

36. Leijs MJ, van Buul GM, Lubberts E, Bos PK, Verhaar JA, Hoogduijn MJ et al. Effect of Arthritic Synovial Fluids on the Expression of Immunomodulatory Factors by Mesenchymal Stem Cells: An Explorative in vitro Study. Front Immunol 2012;3:231.

37. Furst DE, Emery P. Rheumatoid arthritis pathophysiology: update on emerging cytokine and cytokine-associated cell targets. Rheumatology (Oxford) 2014 September;53(9):1560-9.

38. Hyrich KL, Lunt M, Watson KD, Symmons DP, Silman AJ. Outcomes after switching from one anti-tumor necrosis factor alpha agent to a second anti-tumor necrosis factor alpha agent in patients with rheumatoid arthritis: results from a large UK national cohort study. Arthritis Rheum 2007 January;56(1):13-20.

39. Leffers HC, Ostergaard M, Glintborg B, Krogh NS, Foged H, Tarp U et al. Efficacy of abatacept and tocilizumab in patients with rheumatoid arthritis treated in clinical practice: results from the nationwide Danish DANBIO registry. Ann Rheum Dis 2011 July;70(7):1216-22.

40. de Almagro MC, Vucic D. The inhibitor of apoptosis (IAP) proteins are critical regulators of signaling pathways and targets for anti-cancer therapy. Exp Oncol 2012 October;34(3):200-11.

41. Miller LK. An exegesis of IAPs: salvation and surprises from BIR motifs. Trends Cell Biol 1999 August;9(8):323-8.

42. Bai L, Smith DC, Wang S. Small-molecule SMAC mimetics as new cancer therapeutics. Pharmacol Ther 2014 May 16.

43. Micheau O, Tschopp J. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 2003 July 25;114(2):181-90.

44. Vaux DL, Silke J. Mammalian mitochondrial IAP binding proteins. Biochem Biophys Res Commun 2003 May 9;304(3):499-504.

45. Dougan M, Dougan S, Slisz J, Firestone B, Vanneman M, Draganov D et al. IAP inhibitors enhance co-stimulation to promote tumor immunity. J Exp Med 2010 September 27;207(10):2195-206.

46. Vandenabeele P, Bertrand MJ. The role of the IAP E3 ubiquitin ligases in regulating pattern-recognition receptor signalling. Nat Rev Immunol 2012 December;12(12):833-44.

47. Varfolomeev E, Blankenship JW, Wayson SM, Fedorova AV, Kayagaki N, Garg P et al. IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. Cell 2007 November 16;131(4):669-81.

48. Beug ST, Tang VA, LaCasse EC, Cheung HH, Beauregard CE, Brun J et al. Smac mimetics and innate immune stimuli synergize to promote tumor death. Nat Biotechnol 2014 February;32(2):182-90.

49. Varfolomeev E, Goncharov T, Fedorova AV, Dynek JN, Zobel K, Deshayes K et al. c-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor alpha (TNFalpha)-induced NF-kappaB activation. J Biol Chem 2008 September 5;283(36):24295-9.

50. Kearney CJ, Sheridan C, Cullen SP, Tynan GA, Logue SE, Afonina IS et al. Inhibitor of apoptosis proteins (IAPs) and their antagonists regulate spontaneous and tumor necrosis factor (TNF)-induced proinflammatory cytokine and chemokine production. J Biol Chem 2013 February 15;288(7):4878-90.

51. Anguiano-Hernandez YM, Chartier A, Huerta S. Smac/DIABLO and colon cancer. Anticancer Agents Med Chem 2007 July;7(4):467-73.

52. Seneci P, Bianchi A, Battaglia C, Belvisi L, Bolognesi M, Caprini A et al. Rational design, synthesis and characterization of potent, non-peptidic Smac mimics/XIAP inhibitors as proapoptotic agents for cancer therapy. Bioorg Med Chem 2009 August 15;17(16):5834-56.

53. Bockbrader KM, Tan M, Sun Y. A small molecule Smac-mimic compound induces apoptosis and sensitizes T. Oncogene 2005 November 10;24(49):7381-8.

54. Checinska A, Hoogeland BS, Rodriguez JA, Giaccone G, Kruyt FA. Role of XIAP in inhibiting cisplatin-induced caspase activation in non-small cell lung cancer cells: a small molecule Smac mimic sensitizes for chemotherapy-induced apoptosis by enhancing caspase-3 activation. Exp Cell Res 2007 April 1;313(6):1215-24.

55. Franklin MC, Kadkhodayan S, Ackerly H, Alexandru D, Distefano MD, Elliott LO et al. Structure and function analysis of peptide antagonists of melanoma inhibitor of apoptosis (ML-IAP). Biochemistry 2003 July 15;42(27):8223-31.

56. Arnt CR, Chiorean MV, Heldebrant MP, Gores GJ, Kaufmann SH. Synthetic Smac/DIABLO peptides enhance the effects of chemotherapeutic agents by binding XIAP and cIAP1 in situ. J Biol Chem 2002 November 15;277(46):44236-43.

57. Guo F, Nimmanapalli R, Paranawithana S, Wittman S, Griffin D, Bali P et al. Ectopic overexpression of second mitochondria-derived activator of caspases (Smac/DIABLO) or cotreatment with N-terminus of Smac/DIABLO peptide potentiates epothilone B derivative-(BMS 247550) and Apo-2L/TRAIL-induced apoptosis. Blood 2002 May 1;99(9):3419-26.

58. Yang QH, Du C. Smac/DIABLO selectively reduces the levels of c-IAP1 and c-IAP2 but not that of XIAP and livin in HeLa cells. J Biol Chem 2004 April 23;279(17):16963-70.

59. Yang L, Mashima T, Sato S, Mochizuki M, Sakamoto H, Yamori T et al. Predominant suppression of apoptosome by inhibitor of apoptosis protein in non-small cell lung cancer H460 cells: therapeutic effect of a novel polyarginine-conjugated Smac peptide. Cancer Res 2003 February 15;63(4):831-7.

60. Fulda S, Wick W, Weller M, Debatin KM. Smac agonists sensitize for Apo2L/T. Nat Med 2002 August;8(8):808-15.

61. Jia L, Patwari Y, Kelsey SM, Srinivasula SM, Agrawal SG, Alnemri ES et al. Role of Smac in human leukaemic cell apoptosis and proliferation. Oncogene 2003 March 20;22(11):1589-99.

62. Petrucci E, Pasquini L, Petronelli A, Saulle E, Mariani G, Riccioni R et al. A small molecule Smac mimic potentiates TRAIL-mediated cell death of ovarian cancer cells. Gynecol Oncol 2007 May;105(2):481-92.

63. Bank A, Wang P, Du C, Yu J, Zhang L. SMAC mimetics sensitize nonsteroidal anti-inflammatory drug-induced apoptosis by promoting caspase-3-mediated cytochrome c release. Cancer Res 2008 January 1;68(1):276-84.

64. Sun H, Nikolovska-Coleska Z, Lu J, Meagher JL, Yang CY, Qiu S et al. Design, synthesis, and characterization of a potent, nonpeptide, cell-permeable, bivalent Smac mimetic that concurrently targets both the BIR2 and BIR3 domains in XIAP. J Am Chem Soc 2007 December 12;129(49):15279-94.

65. Weisberg E, Kung AL, Wright RD, Moreno D, Catley L, Ray A et al. Potentiation of antileukemic therapies by Smac mimetic, LBW242: effects on mutant FLT3-expressing cells. Mol Cancer Ther 2007 July;6(7):1951-61.

66. Chauhan D, Neri P, Velankar M, Podar K, Hideshima T, Fulciniti M et al. Targeting mitochondrial factor Smac/DIABLO as therapy for multiple myeloma (MM). Blood 2007 February 1;109(3):1220-7.

67. Servida F, Lecis D, Scavullo C, Drago C, Seneci P, Carlo-Stella C et al. Novel second mitochondria-derived activator of caspases (Smac) mimetic compounds sensitize human leukemic cell lines to conventional chemotherapeutic drug-induced and death receptor-mediated apoptosis. Invest New Drugs 2011 December;29(6):1264-75.

68. Kashkar H, Seeger JM, Hombach A, Deggerich A, Yazdanpanah B, Utermohlen O et al. XIAP targeting sensitizes Hodgkin lymphoma cells for cytolytic T-cell attack. Blood 2006 November 15;108(10):3434-40.

69. Bilim V, Yuuki K, Itoi T, Muto A, Kato T, Nagaoka A et al. Double inhibition of XIAP and Bcl-2 axis is beneficial for retrieving sensitivity of renal cell cancer to apoptosis. Br J Cancer 2008 March 11;98(5):941-9.

70. Sun H, Nikolovska-Coleska Z, Yang CY, Xu L, Liu M, Tomita Y et al. Structure-based design of potent, conformationally constrained Smac mimetics. J Am Chem Soc 2004 December 29;126(51):16686-7.

71. Cossu F, Mastrangelo E, Milani M, Sorrentino G, Lecis D, Delia D et al. Designing Smac-mimetics as antagonists of XIAP, cIAP1, and cIAP2. Biochem Biophys Res Commun 2009 January 9;378(2):162-7.

72. Cossu F, Malvezzi F, Canevari G, Mastrangelo E, Lecis D, Delia D et al. Recognition of Smac-mimetic compounds by the BIR domain of cIAP1. Protein Sci 2010 December;19(12):2418-29.

73. Lattuada D, Casnici C, Crotta K, Seneci PF, Corradini C, Truzzi M et al. Proapoptotic activity of a monomeric smac mimetic on human fibroblast-like synoviocytes from patients with rheumatoid arthritis. Inflammation 2015 February;38(1):102-9.

74. Wu H, Tschopp J, Lin SC. Smac mimetics and TNFalpha: a dangerous liaison? Cell 2007 November 16;131(4):655-8.

75. Vince JE, Wong WW, Khan N, Feltham R, Chau D, Ahmed AU et al. IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell 2007 November 16;131(4):682-93.

76. Petersen SL, Wang L, Yalcin-Chin A, Li L, Peyton M, Minna J et al. Autocrine TNFalpha signaling renders human cancer cells susceptible to Smac-mimetic-induced apoptosis. Cancer Cell 2007 November;12(5):445-56.

77. Kelliher MA, Grimm S, Ishida Y, Kuo F, Stanger BZ, Leder P. The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. Immunity 1998 March;8(3):297-303.

78. Stanger BZ, Leder P, Lee TH, Kim E, Seed B. RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. Cell 1995 May 19;81(4):513-23.

79. Chang L, Kamata H, Solinas G, Luo JL, Maeda S, Venuprasad K et al. The E3 ubiquitin ligase itch couples JNK activation to TNFalpha-induced cell death by inducing c-FLIP(L) turnover. Cell 2006 February 10;124(3):601-13.

80. Mayer BA, Rehberg M, Erhardt A, Wolf A, Reichel CA, Kracht M et al. Inhibitor of apoptosis proteins as novel targets in inflammatory processes. Arterioscler Thromb Vasc Biol 2011 October;31(10):2240-50.

81. Beug ST, Cheung HH, LaCasse EC, Korneluk RG. Modulation of immune signalling by inhibitors of apoptosis. Trends Immunol 2012 November;33(11):535-45.

82. Tseng PH, Matsuzawa A, Zhang W, Mino T, Vignali DA, Karin M. Different modes of ubiquitination of the adaptor TRAF3 selectively activate the expression of type I interferons and proinflammatory cytokines. Nat Immunol 2010 January;11(1):70-5.

83. Huber LC, Distler O, Tarner I, Gay RE, Gay S, Pap T. Synovial fibroblasts: key players in rheumatoid arthritis. Rheumatology (Oxford) 2006 June;45(6):669-75.

84. Korb A, Pavenstadt H, Pap T. Cell death in rheumatoid arthritis. Apoptosis 2009 April;14(4):447-54.

85. Hunter AM, LaCasse EC, Korneluk RG. The inhibitors of apoptosis (IAPs) as cancer targets. Apoptosis 2007 September;12(9):1543-68.

86. Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 2000 July 7;102(1):33-42.

87. Takahashi R, Deveraux Q, Tamm I, Welsh K, Assa-Munt N, Salvesen GS et al. A single BIR domain of XIAP sufficient for inhibiting caspases. J Biol Chem 1998 April 3;273(14):7787-90.

88. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, III et al. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum 2010 September;62(9):2569-81.

89. Bianchi A, Ugazzi M, Ferrante L, Lecis D, Scavullo C, Mastrangelo E et al. Rational design, synthesis and characterization of potent, drug-like monomeric Smac mimetics as pro-apoptotic anticancer agents. Bioorg Med Chem Lett 2012 March 15;22(6):2204-8.

90. Burrage PS, Mix KS, Brinckerhoff CE. Matrix metalloproteinases: role in arthritis. Front Biosci 2006;11:529-43.

91. Takayanagi H, Iizuka H, Juji T, Nakagawa T, Yamamoto A, Miyazaki T et al. Involvement of receptor activator of nuclear factor kappaB ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis. Arthritis Rheum 2000 February;43(2):259-69.

92. Yang C, Davis JL, Zeng R, Vora P, Su X, Collins LI et al. Antagonism of inhibitor of apoptosis proteins increases bone metastasis via unexpected osteoclast activation. Cancer Discov 2013 February;3(2):212-23.

93. Choy EH, Kavanaugh AF, Jones SA. The problem of choice: current biologic agents and future prospects in RA. Nat Rev Rheumatol 2013 March;9(3):154-63.

94. Mocsai A, Kovacs L, Gergely P. What is the future of targeted therapy in rheumatology: biologics or small molecules? BMC Med 2014;12:43.

95. Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE et al. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 2000 July 7;102(1):43-53.

96. Estornes Y, Bertrand MJ. IAPs, regulators of innate immunity and inflammation. Semin Cell Dev Biol 2015 March;39:106-14.

97. Eckhardt I, Roesler S, Fulda S. Identification of DR5 as a critical, NF-kappaB-regulated mediator of Smac-induced apoptosis. Cell Death Dis 2013;4:e936.

98. Chu CQ, Field M, Feldmann M, Maini RN. Localization of tumor necrosis factor alpha in synovial tissues and at the cartilage-pannus junction in patients with rheumatoid arthritis. Arthritis Rheum 1991 September;34(9):1125-32.

99. Feldmann M. Development of anti-TNF therapy for rheumatoid arthritis. Nat Rev Immunol 2002 May;2(5):364-71.

100. Butler DM, Maini RN, Feldmann M, Brennan FM. Modulation of proinflammatory cytokine release in rheumatoid synovial membrane cell cultures. Comparison of monoclonal anti TNF-alpha antibody with the interleukin-1 receptor antagonist. Eur Cytokine Netw 1995 July;6(4):225-30.

101. Naugler WE, Karin M. The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer. Trends Mol Med 2008 March;14(3):109-19.

102. Smolen JS, Beaulieu A, Rubbert-Roth A, Ramos-Remus C, Rovensky J, Alecock E et al. Effect of interleukin-6 receptor inhibition with tocilizumab in patients with rheumatoid arthritis (OPTION study): a double-blind, placebo-controlled, randomised trial. Lancet 2008 March 22;371(9617):987-97.

103. Benito-Miguel M, Garcia-Carmona Y, Balsa A, Bautista-Caro MB, Arroyo-Villa I, Cobo-Ibanez T et al. IL-15 expression on RA synovial fibroblasts promotes B cell survival. PLoS One 2012;7(7):e40620.

104. Park MK, Her YM, Cho ML, Oh HJ, Park EM, Kwok SK et al. IL-15 promotes osteoclastogenesis via the PLD pathway in rheumatoid arthritis. Immunol Lett 2011 September 30;139(1-2):42-51.

105. Tolboom TC, Pieterman E, van der Laan WH, Toes RE, Huidekoper AL, Nelissen RG et al. Invasive properties of fibroblast-like synoviocytes: correlation with growth characteristics and expression of MMP-1, MMP-3, and MMP-10. Ann Rheum Dis 2002 November;61(11):975-80.

106. Schlaak JF, Pfers I, Meyer Zum Buschenfelde KH, Marker-Hermann E. Different cytokine profiles in the synovial fluid of patients with osteoarthritis, rheumatoid arthritis and seronegative spondylarthropathies. Clin Exp Rheumatol 1996 March;14(2):155-62.

107. Katsikis PD, Chu CQ, Brennan FM, Maini RN, Feldmann M. Immunoregulatory role of interleukin 10 in rheumatoid arthritis. J Exp Med 1994 May 1;179(5):1517-27.

108. Gyrd-Hansen M, Meier P. IAPs: from caspase inhibitors to modulators of NF-kappaB, inflammation and cancer. Nat Rev Cancer 2010 August;10(8):561-74.

109. Taylor DJ. Studies on the expression of the TNF alpha receptors (p55 and p75) and their relative contributions to prostanoid production and glycolytic rate by rheumatoid synovial fibroblasts in vitro. Rheumatol Int 1993;13(3):89-93.

110. Higuchi Y, McTiernan CF, Frye CB, McGowan BS, Chan TO, Feldman AM. Tumor necrosis factor receptors 1 and 2 differentially regulate survival, cardiac dysfunction, and remodeling in transgenic mice with tumor necrosis factor-alpha-induced cardiomyopathy. Circulation 2004 April 20;109(15):1892-7.

111. Youn J, Kim HY, Park JH, Hwang SH, Lee SY, Cho CS et al. Regulation of TNF-alpha-mediated hyperplasia through TNF receptors, TRAFs, and NF-kappaB in synoviocytes obtained from patients with rheumatoid arthritis. Immunol Lett 2002 September 2;83(2):85-93.