Fibroblasts as therapeutic targets in rheumatoid arthritis and cancer

Maria Juarez, Andrew Filer, Christopher D. Buckley

Rheumatology Research Group, IBR School of Immunity and Infection, University of Birmingham, United Kingdom

Summary

Rheumatoid arthritis (RA) is a common chronic inflammatory disease that causes progressive synovial inflammation resulting in irreversible joint destruction, chronic disability and premature mortality [1, 2]. Although it is recognised that in RA, inflammation and its persistence result from complex interactions between haematopoietic and stromal cells [3–5], research into the pathogenesis of the disease has traditionally concentrated on cells and cytokines of the immune system, neglecting the role of stromal cells. As a consequence, new biologic treatments have been developed, which have led to a step-change in the management of the disease [6]. Nevertheless, these treatments do not reverse tissue damage or lead to disease cure and are not effective for all patients. Furthermore, at best they induce a significant clinical response (ACR70) in less than 60% of patients, most of whom will relapse on treatment withdrawal, suggesting that additional therapeutic targets, responsible for complete resolution of inflammation, remain to be discovered [7]. An increasing body of evidence implicates RA synovial fibroblasts in driving the persistent, destructive characteristics of the disease. In this paper, we discuss the evidence implicating synovial fibroblasts in the pathogenesis of RA and explore their role as therapeutic targets.

Key words: stromal cells; fibroblasts; epigenetics; rheumatoid arthritis

Pathogenesis of RA

RA is an autoimmune disease, characterised by the development of innate and adaptive immune responses and, in a high percentage of patients, the presence of autoantibodies (rheumatoid factor and anti-cyclic citrullinated peptide antibodies) which may be detected in blood many years before disease onset and whose pathogenic role remains unclear [6]. Additionally, genetic factors such as most notably the presence of the shared epitope (a specific group of HLA-DR4 alleles) and single nucleotide polymorphisms in the PTPN22 gene are known to play a part in disease susceptibility and severity [8]. A number of environmental factors have also been described. The association of smoking with disease development in individuals who

carry the shared epitope and worse disease outcome is well established [9]. Local factors such as hypoxia, which is thought to be important in immune signalling and angiogenesis, are also relevant. However, a unified theory on how these factors interact together is still elusive (for a review on this topic see reference [10]).

At a local joint level, RA is characterised by a radical change of the two compartments of the synovium. The lining layer of the tissue situated adjacent to the synovial fluid compartment undergoes dramatic hyperplasia, sometimes reaching 10–15 cells in depth with expansion of both macrophage and fibroblast populations. At the articular borders the thickened synovial lining layer may become a mass of "pannus" tissue (rich in fibroblasts and osteoclasts) that invades the adjacent articular cartilage and subchondral bone. The sublining layer also undergoes expansion, with infiltrates of inflammatory cells including macrophages, mast cells, T cells, B cells and plasma and dendritic cells. T and B lineage cells may remain in diffuse infiltrates, or may coalesce into aggregates of cells varying from simple perivascular "cuffs" a few cells in diameter to structures resembling B-cell follicles in up to 20% of samples [11]. This increased activity is supported by further extracellular matrix (ECM) production and neoangiogenesis, although the inflamed synovium remains in a state of relative hypoxia [12].

The interactions between these different cell types are complex and mediated by numerous cytokine networks. The
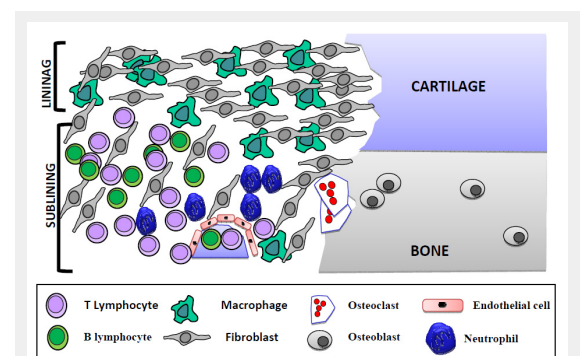

Figure 1

Representation of the different cell types in the rheumatoid synovium.

description of the relative contribution of each of these cell types to disease pathogenesis is beyond the remit of this paper but a schematic representation of the synovium's cellularity in RA has been depicted in figure 1.

Synovial fibroblasts in the pathogenesis of RA

Over the last decade, researchers have demonstrated that in addition to their well-known structural role in "landscaping" the microenvironment, synovial fibroblasts are implicated in the pathogenesis of RA at two fundamental levels.

Fibroblasts as mediators of persistent inflammation

Firstly, activated RA synovial fibroblasts play crucial roles in determining the site at which inflammation occurs, and in the subsequent maintenance of persistent inflammation in the joint microenvironment, as reflected in hyperplasia of resident stromal cells and the organisation of T and B lymphocyte infiltration discussed above [11]. Much work has been devoted to the description of this microenvironment and of the complex haematopoietic-stromal cell interactions that determine its stability [3–5]. We have proposed that the maintenance of the persistent leukocyte infiltrate results from a distorted homeostatic balance between leukocyte recruitment, proliferation, emigration and death [3], and that synovial fibroblasts are key determinants of this imbalance. In the rheumatoid synovium, different subpopulations of stromal cells exist. Stromal cells of the RA synovium differ from those of the normal synovium. Additionally synovial fibroblasts from the lining layer differ in biological and morphological characteristics to those of the sublining layer. The so-called activated synovial fibroblasts found in RA tissue (RASFs) are the key stabilisers of the abnormal synovial microenvironment. In a series of experiments, our group and others have demonstrated that activated RASFs produce inflammatory chemokines (e.g.,
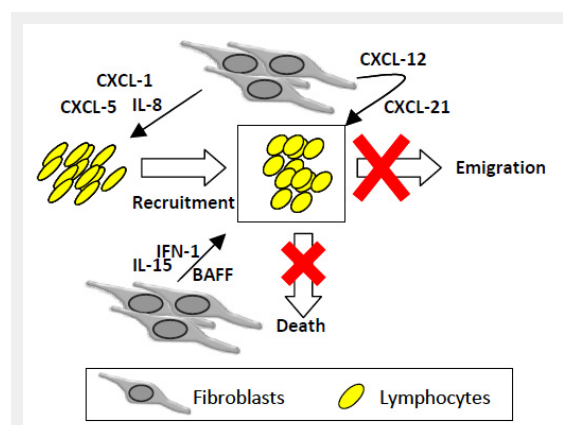
IL-8, CCL5, CXCL1) [13, 14] that actively recruit leukocytes such as neutrophils and B and T lymphocytes to the diseased synovial tissue. By contrast RASFs also inappropriately secrete so-called constitutive chemokines (e.g., CXCL12, CXCL13, CCL21) that are normally restricted to lymphoid tissues in order to coordinate the recirculation of lymphocytes [3, 15–18]. In this way, infiltrating leukocytes are prevented from leaving the synovial tissue. RASFs also produce survival factors (e.g., type I interferon, IL-15, BAFF) that prevent leukocyte subpopulations from dying by apoptosis as would normally occur during the resolution phase of an inflammatory response [19–21]. Thus fibroblast activation results in the accumulation, survival and retention of leukocytes at sites of disease, mimicking the microenvironment seen in lymphoid tissues, and thereby preventing the resolution of chronic inflammation (fig. 2).

Fibroblasts as mediators of joint destruction

Secondly, RASFs are key mediators of cartilage and bone destruction. They act directly on cartilage via secretion of matrix metalloproteinases (MMPs), cathepsins and inflammatory cytokines [22, 23], and indirectly on bone via regulation of monocyte to osteoclast differentiation [24]. In the early RA joint, activated synovial fibroblasts attach to and overgrow the cartilage surface, then invade and destroy cartilage and induce bone resorption (fig. 3). Active degradation of cartilage *in vivo* has been elegantly demonstrated in the SCID mouse model of arthritis, in which *in vitro* cultured RASFs (but not normal or osteoarthritis synovial fibroblasts) attach to and invade co-implanted healthy
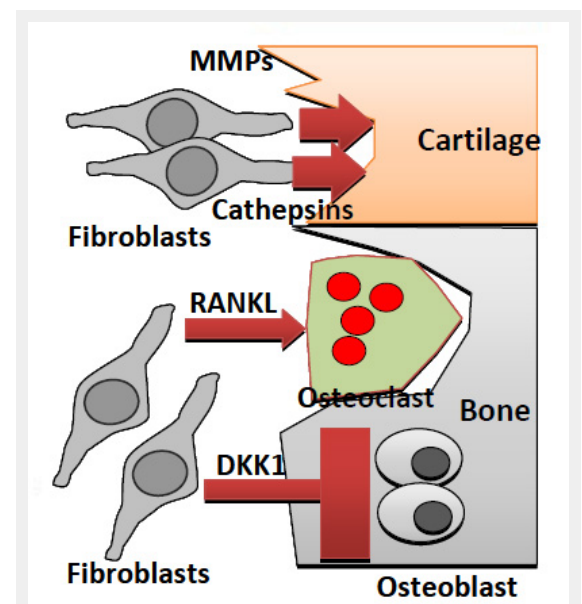

Figure 2

Fibroblasts orchestrate chronic inflammation in RA. The maintenance of persistent leukocyte infiltrates results from a distorted homeostatic balance between leukocyte recruitment, proliferation, emigration and death. Activated RASFs produce inflammatory chemokines (IL-8, CXCL5, CXCL1) implicated in leukocyte recruitment to diseased synovium, survival factors (type I interferon, IL-15, BAFF) and constitutive chemokines (e.g. CXCL12, CCL21). The net result is the chronic accumulation, survival and retention of leukocytes at sites of disease.

Figure 3

Fibroblast contribute to joint damage. Pannus tissue consists of activated RASFs that attach to and damage cartilage through production of MMPs and cathepsins. In addition, fibroblasts secrete RANKL, which promotes osteoclast differentiation and activation, leading to bone erosions. Furthermore production of DKK-1 inhibits Wnt signaling pathways that normally promote anabolic osteoblast activity, preventing repair of bone erosions. Abbreviations: MMPs: matrix metalloproteinases; RANKL: receptor activator of nuclear factor k B ligand; DKK-1: dickkopf-1.

human cartilage in the absence of cells of the immune system, indicating that this invasive phenotype is both stable and disease specific [25]. Furthermore, RASFs maintain this destructive phenotype even after multiple *in vitro* passages, and their *in vitro* invasiveness correlates with clinical rates of bone erosion in individual patients [26].

Initial and persistent activation of RASFs

It is therefore clear that activated RASFs are not passive players in immune responses but key effectors in the pathogenesis of RA. One of the questions arising from these observations is how these cells become activated and how this activated phenotype persists.

It has been proposed that activation arises from molecular cross-talk between RASFs and other cells of the synovial microenvironment early in disease. Pro-inflammatory cytokines of the initial rheumatoid microenvironment such as TNFα and IL-1 play a key role in the activation of RASFs [23]. Once activated, RASFs produce TNFα, IL-1 and IL-6 that are involved in sustaining regulatory feedback loops and induce production of MMPs, cathepsins, aggrecanases and their inhibitors [6]. However, independently of the fact that pro-inflammatory cytokines induce fibroblast activation, the fact that not all fibroblasts in a joint become activated suggests that there must be other mechanisms in play (intrinsic to these cells) that make them "permissive" to such activation. Indeed, it has conclusively been shown that the persistence of the activated RASFs phenotype is independent of the pro-inflammatory cytokines that initiated the process [25] This may also explain the clinical observation that cessation of anti cytokine therapy (e.g., anti TNF therapy) in the context of clinical RA leads to disease flare [10].

Mechanisms of activation

So, what could these intrinsic mechanisms be? Cellular DNA and the epigenetic machinery that regulates its expression are the most plausible candidates to explain unique cellular characteristics.

Several lines of evidence indicate that responses to pro-inflammatory stimuli result from persistent changes in the transcription levels of disease relevant genes. RASFs show upregulated expression of proto-oncogenes (c-myc) [27],

Figure 4

Schematic representation of epigenetics. Epigenetic changes mark the genome at specific sites acting as switches that determine whether a particular gene is transcribed (switch "on") or not (switch "off"). In the figure an activating mark results in normal transcription of a tumour suppressor gene and maintenance of normal cell phenotype whilst a repressing mark in the same gene results in lack of transcription and malignant transformation of that cell line. Abbreviations: P: promoter; E: exon.

adhesion molecules (VLA-3, VLA-4, VLA-5) [28] and MMPs (MMP13, MT-MMP) [29, 30] whilst displaying low expression of tumor suppressor genes such as PTEN and p53 [31]. These persistent changes in gene transcription are ultimately responsible for the aggressive behaviour of RASFs. Differential gene expression patterns are not only apparent in disease but also in health and indeed fibroblasts from different anatomical regions have been shown to display characteristic transcriptional profiles that are maintained *in vitro* [32]. The HOX genes encode the important transcription factors that confer such region-specific identity to tissues [32–34]. But how is this differential gene expression pattern obtained and regulated to provide persistent changes in phenotype? The field of epigenetics explains such regulation, and is increasingly implicated as the determinant of the RASFs phenotype.

Epigenetic changes are hereditable changes in gene expression that do not result from alteration of the underlying DNA sequence [35]. Figure 4 provides a schematic representation of epigenetic mechanisms. The most studied epigenetic modifications to date include non-coding RNA species, DNA methylation and histone modifications. These modifications can be thought of as chemical "switches" that regulate gene expression determining whether a particular gene is expressed ("on") or not ("off"). In healthy fibroblasts, expression of a non-coding RNA (termed HOTAIR) residing in the HOXC locus results in silencing of genes in the HOXD locus, explaining epigenetic control of HOX gene expression in health [36]. In RA, RASFs display global genomic DNA hypomethylation (an epigenetic change associated with up-regulation of gene expression) that causes up-regulation of disease relevant genes (growth factors, adhesion molecules and MMPs) [37]. Most notably, treatment of normal synovial fibroblasts with the DNA methylation inhibitor 5-aza-2′deoxycytidine (decitabidine) resulted in their transformation into cells resembling activated RASFs [37]. Although this evidence lends support to epigenetic changes as determinants of the RASFs persistent phenotype, further research in this area is needed to unequivocally demonstrate a significant role in the pathogenesis of disease.

Perhaps more noteworthy is that the epigenetic hypothesis may not only provide a mechanistic explanation for this phenotype but a tool to modify it. Most epigenetic modifications are reversible processes catalysed by specific enzymes and cofactors that allow cells to change their gene expression patterns in response to various stimuli. The enzymes and molecular complexes involved in these processes represent very attractive targets for future therapies. As a result, much interest is currently being devoted to this area.

Cancer associated fibroblasts

The importance of the stromal microenvironment in disease has been recognised in the cancer field for years. It is now widely accepted that cancer develops as a result of genetic and epigenetic alterations in clonal cells, but that the growth, survival and metastasis of these cells are regulated by stromal-cancer cell interactions [38]. Studies in human lung, breast, colon and prostate cancer have unequivocally demonstrated a key role for stromal cells (so-called cancer
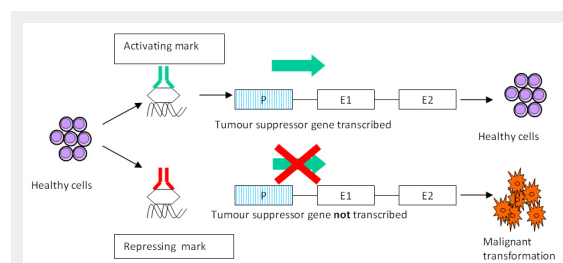
associated fibroblasts (CAFs)) in initiation and progression of disease [38]. The term "cancer stroma" has been coined to refer to macroscopic and functional differences between this and the corresponding normal tissue stroma. Cancer stroma is characterized by a modified ECM composition, increased microvessel density, inflammatory cells and activated fibroblasts [39]. Autocrine and paracrine mechanisms resulting in secretion of cytokines, chemokines and growth factors by the stromal microenvironment have been implicated in activation and transformation of fibroblasts into CAFs [40]. Platelet derived growth factor (PDGF) [41] and transforming growth factor beta (TFGβ) are the most important factors in this process. They regulate disease relevant gene expression (fibronectin, PAI-1 and cyclin dependent kinase inhibitors) [42, 43] through stimulation of specific signaling cascades like serine/threonine kinase receptors and the STAT (Signal Transduction and Activation of Transcription) family of signaling pathway regulators. Confirmation of the importance of CAFs comes from in *vivo* studies: co-implantation of pre-malignant prostate epithelial cells with prostate tumour CAFs, but not normal prostate fibroblasts, leads to malignant transformation and proliferation of epithelial cells [44]. In breast cancer, overexpression of TGFβ in mouse fibroblasts induces initiation of breast cancer within normal human epithelium [45].

Targeting CAFs
This compelling evidence demonstrates that tumour-host cross talk at the level of the resident fibroblast cell determines the behaviour of malignant cells. As a result, therapies targeting the tumour stroma are being developed and a number of strategies have been proposed:
1. Targeting signals responsible for activation of fibroblasts: the key role of PDGF and TGFβ signaling pathways has resulted in the development of inhibitors against these molecules. Imatinib (Glivec) inhibits the PDGF-receptor tyrosine kinase and is used for the treatment of chronic myeloid leukaemia and other types of cancer [46]. TGFβ inhibitors are currently in development (e.g., lerdelimumab, metelimumab) [47].
2. Targeting fibroblast specific molecules that initiate and promote tumour growth: an example of this approach is the use a humanised monoclonal antibody (sibrotuzumab) against the fibroblast activation protein (FAP) [48]. FAP is a membrane bound glycoprotein with serine protease activity. It is highly expressed in the tumour stroma and has been shown to enhance tumour growth *in vivo* [49]. Efficacy has been shown in phase I studies in colorectal cancer. [50]
3. Eliminating activated CAF subpopulations: efforts are being made to determine the differential transcriptional profile of normal fibroblasts compared to CAFs in a variety of malignancies in order to identify unique transcriptional signatures. This will then be further investigated in order to develop new therapies aimed towards elimination of CAFs.
4. Epigenetic therapies. Although still in its early days, research has shown that epigenetic modifications determine the phenotype of cancer and tumour stroma cells promoting their invasiveness and survival. CAFs in human gastric carcinomas exhibit global DNA

hypomethylation [51]. More broadly, cancerous epithelial cells are characterized on the one hand by global DNA hypomethylation that promotes chromosomal instability and activates proto-oncogenes, and on the other by regional promoter hypermethylation of specific genes that results in down-regulation of tumour suppressor genes [52]. The understanding of the molecular basis of these epigenetic modifications has led to the development of epigenetic therapies. The DNA methylation inhibitor 5-aza-2′deoxycytidine is currently used for the treatment of myelodysplastic syndromes (MDS), acting by reversing pathological hypermethylation (and hence increasing expression) of the tumour suppressor gene CDKN2B in affected cells [52].

As the understanding of global epigenetic mechanisms in cancer advances, a combinatorial approach using different epigenetic approaches along with standard chemotherapy holds significant promise for effective future treatment. For example, it has been shown that treatment of MDS with 5-aza-2′deoxycytidine is limited by a short duration of response that is improved upon use of combination chemotherapy [53].

Towards targeting synovial fibroblasts in the treatment of rheumatoid arthritis
Obvious parallels can be drawn between CAFs and RASFs, and indeed current RA research is focusing increasingly upon targeting stromal cells therapeutically.

Non- Fibroblast specific mechanisms
1. Cytokine blockade. Existing strategies in RA have indirectly targeted stromal cells either by targeting cytokines which impact upon fibroblasts such as anti-TNF therapies (infliximab, etanercept, adalimumab) or by blocking their significant products (anti-IL-6 receptor, tocilizumab) therapies respectively. These therapies share the shortcomings of existing biologics, inhibiting key pleiotropic proinflammatory cytokines with widespread generic roles, leading on the one hand to significant adverse events despite submaximal efficacy, while on the other hand failing to address persistence of disease.
2. Cell signalling pathways. Cellular MAPKinase and NF-κB signalling pathways in particular are heavily implicated in the pro-inflammatory and cartilage damaging activities of synovial fibroblasts. However, the consequences of inhibiting such generic pathways in terms of off-target effects has been likened to opening Pandora's box [54]. As described in the field of cancer medicine, the approach of targeting more specific upstream activators of pathologically altered fibroblasts via inhibitors of tyrosine kinase family members may lead to fewer off-target effects; imatinib inhibits a narrow family of tyrosine kinases including the PDGF receptor, c-fms and c-kit. It shows promise as a therapy in RA that has been shown to modulate synovial fibroblast proliferation rates [55, 56].

Fibroblast specific mechanisms

1. Novel fibroblast markers. Historically, the study of fibroblasts has been hampered by a lack of specific cell surface markers. As a result, they have traditionally been identified by their spindle-shaped morphology, elaboration of ECM and lack of markers associated with endothelial, epithelial or haematopoietic cells. This situation is now rapidly changing; an early discovery in this field revealed that the lining layer of synovial fibroblasts is associated with specific expression of cadherin-11, an adhesion molecule responsible for homotypic adhesion between lining layer stromal cells which self organise in the absence of a basement membrane [57]. Not only does this marker co-localise with cells of the lining layer, but in cadherin-11 knockout mice subjected to models of inflammatory arthritis, damage to the joint cartilage is ameliorated [58]. Although not a realistic therapeutic target in isolation, this provided proof of concept that deletion of a stromal sub-population marker could impact on progression of animal models of disease. Further synovial stromal markers have been identified in the rheumatoid synovium with strong overlap with CAF markers. These include FAP, and the sub-lining marker CD248 (endosialin), with restricted stromal expression, and important roles in cancer progression, linkage of hypoxia to angiogenesis, and stromal responses to inflammation [59–61]. Maia et al. have shown that full knockouts and cytoplasmic tail mutants of CD248 result in amelioration of murine arthritis induced by anti-collagen antibodies, offering potential as a therapeutic target [62]. Other specific markers include PBEF (Visfatin), an adipokine with expression closely linked to RA disease activity [63], and FAP, which is expressed in the lining layer of the pathological rheumatoid joint. Inhibitors exist which specifically inhibit both molecules, though exploring their use in arthritis is at an early stage [64, 65].

2. Epigenetic therapies. *In vitro* and *in vivo* studies of the use of epigenetic therapies in arthritis have started to emerge. Histone acetylation/decacetylation is an epigenetic modification that has a critical role in regulation of gene transcription by altering chromatin structure. Jüngel and colleagues [66] treated RASFs with the histone deacetylase inhibitor Trichostatin A (TSA). Treatment resulted in inhibition of their proliferation and sensitisation to tumour necrosis factor related apoptosis-inducing ligand (TRAIL) induced apoptosis. Subsequently, Nasu and colleagues [67] conducted an elegant *in vivo* study to analyse the effects of TSA treatment in the collagen antibody induced arthritis (CAIA) mouse model. Mice were given subcutaneous injections of TSA and histological analysis showed amelioration of clinical arthritis in a dose dependent manner. These studies provide proof of concept that epigenetic therapies may become a reality for the treatment of arthritis. Non-coding RNA species such as microRNAs (miRs) are also becoming rapidly recognised as relatively specific controllers of aberrant gene expression [68]. MiR expression is itself frequently under epigenetic control, highlighting a central mechanism in cancer pathology. One such example in arthritis is miR203. This molecule is under the direct control of DNA methylation, and drives expression of IL-6 and MMP production by synovial fibroblasts. Not only are levels of miR203 raised in longstanding RA compared to osteoarthritis and normal synovial fibroblasts, but expression in the earliest phases of RA when disease is not fully differentiated occurs at an intermediate level, suggesting that the epigenetic control of synovial fibroblast behaviour remains plastic in the earliest phases of disease [69].

At present, experimental approaches to epigenetic modifications have used broad specificity drugs such as TSA and 5-aza-2′deoxycytidine. However, as discussed above, pathological epigenetic changes in carcinoma cells involve complex combinations of gene activation and repression. These therapies do not therefore represent a final goal for rational drug design. The potential for targeted therapy is made possible by the complexes that effectively "read" and "write" the epigenetic code via DNA methylation and a multitude of histone modifications. These complexes, such as the acetyl-lysine reading bromodomains [70] demonstrate high specificity for certain modifications and groups of genes. Engineering of specific antagonists to active sites in these complexes can radically alter disease, as in the case of a malignancy inducing translocation treated by Filippakopoulos et al. who generated a specific cell permeable competitive inhibitor that prevented binding of the BET bromodomain member BRD4, forcing differentiation of a previously incurable cancer [71].

Conclusion

Synovial fibroblasts are key effectors in the pathogenesis of RA. Aspects of the mechanisms involved in the triggering of their abnormal phenotype have begun to be deciphered. Drugs aimed at these cells either via targeting of markers for specific pathologically important subpopulations or modulating epigenetic programming of fibroblasts may be added to the therapeutic arsenal in RA in the near future. Just as in the field of cancer, combinations of epigenetic and conventional antirheumatic therapies may both improve efficacy and modify disease persistence.

Correspondence: Maria Juarez, LMS (MBBS equivalent), MRCP, Cert Med Ed. Rheumatology Research Group, 3rd floor IBR, MRC Centre for Immune Regulation, University of Birmingham, Birmingham B15 2TT, United Kingdom, mariajuarezp[at]gmail.com

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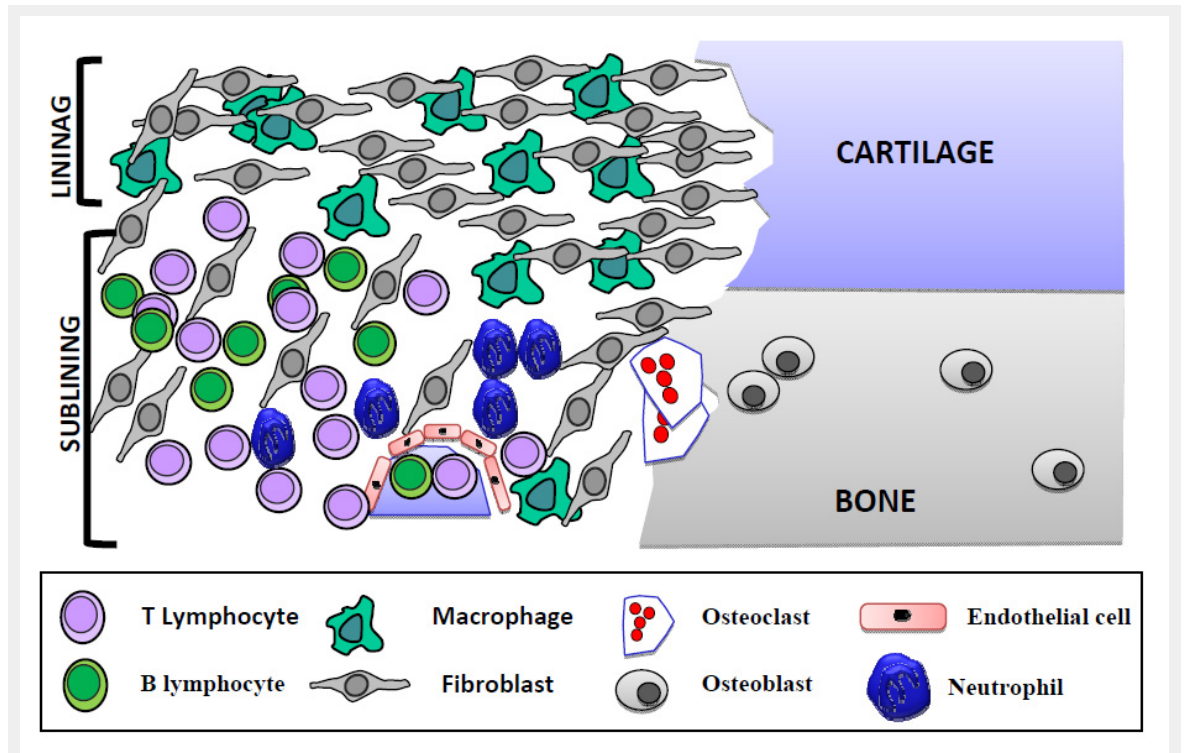
Figures (large format)

Figure 1

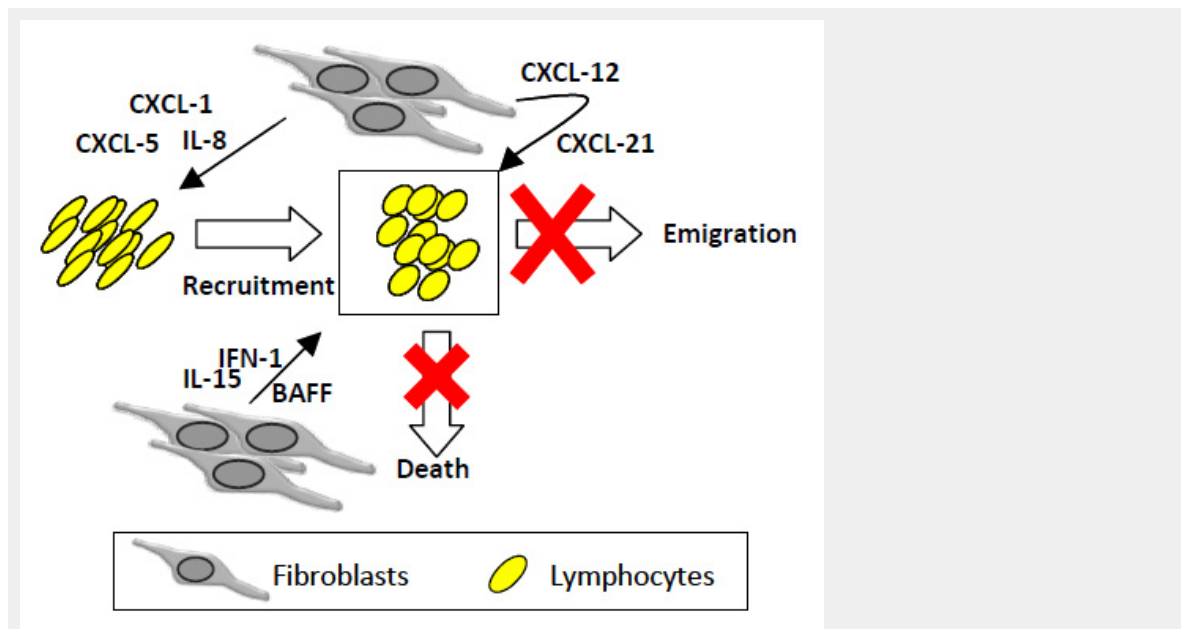Representation of the different cell types in the rheumatoid synovium.

Figure 2

Fibroblasts orchestrate chronic inflammation in RA. The maintenance of persistent leukocyte infiltrates results from a distorted homeostatic balance between leukocyte recruitment, proliferation, emigration and death. Activated RASFs produce inflammatory chemokines (IL-8, CXCL5, CXCL1) implicated in leukocyte recruitment to diseased synovium, survival factors (type I interferon, IL-15, BAFF) and constitutive chemokines (e.g. CXCL12, CCL21). The net result is the chronic accumulation, survival and retention of leukocytes at sites of disease.
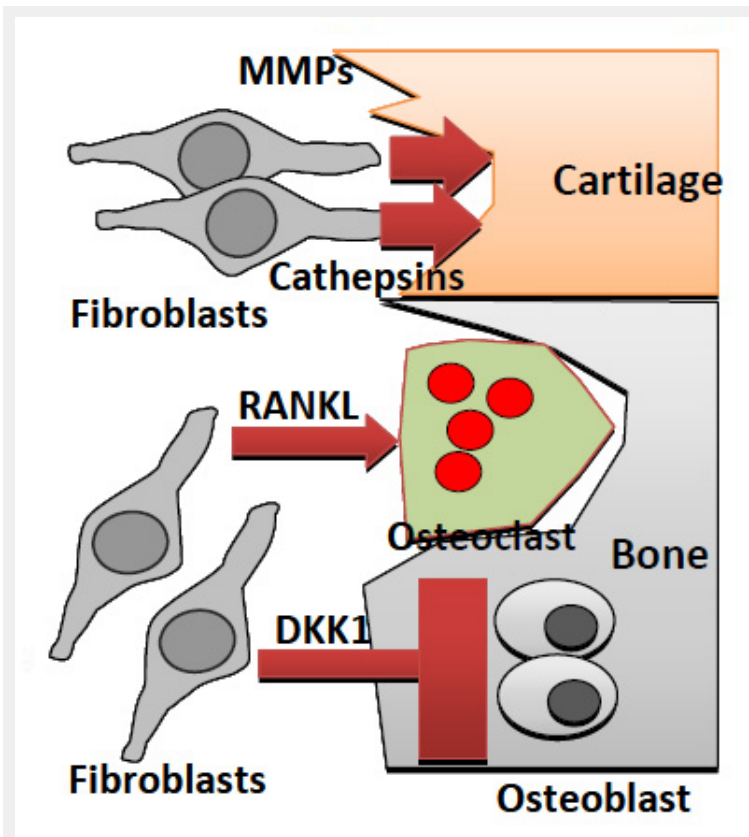
Figure 3

Fibroblast contribute to joint damage. Pannus tissue consists of activated RASFs that attach to and damage cartilage through production of MMPs and cathepsins. In addition, fibroblasts secrete RANKL, which promotes osteoclast differentiation and activation, leading to bone erosions. Furthermore production of DKK-1 inhibits Wnt signaling pathways that normally promote anabolic osteoblast activity, preventing repair of bone erosions. Abbreviations: MMPs: matrix metalloprotenaises; RANKL: receptor activator of nuclear factor k B ligand; DKK-1: dickkopf-1.
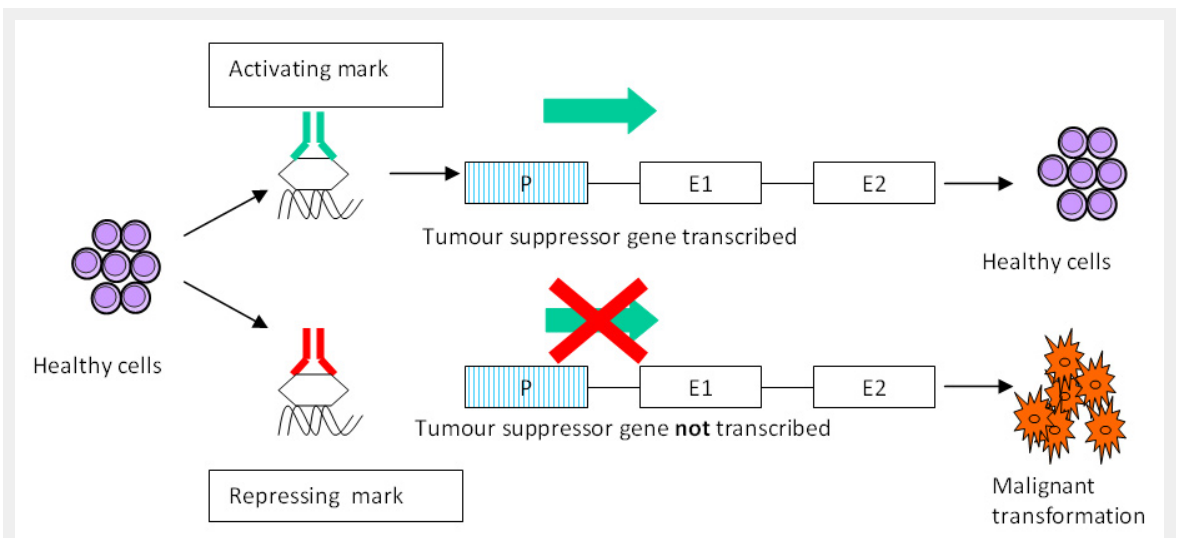

Figure 4

Schematic representation of epigenetics. Epigenetic changes mark the genome at specific sites acting as switches that determine whether a particular gene is transcribed (switch "on") or not (switch "off"). In the figure an activating mark results in normal transcription of a tumour suppressor gene and maintenance of normal cell phenotype whilst a repressing mark in the same gene results in lack of transcription and malignant transformation of that cell line. Abbreviations: P: promoter; E: exon.