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TNF α induces sustained signaling and a prolonged and unremitting inflammatory response in synovial fibroblasts

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Abstract

Objective—The non resolving character of synovial inflammation in rheumatoid arthritis (RA) is a conundrum. To identify the contribution of fibroblast-like synoviocytes (FLS) to the perpetuation of synovitis, we investigated the molecular mechanisms that govern the TNF α -driven inflammatory program in human FLS.

Methods—FLS obtained from synovial tissues of patients with RA or osteoarthritis were stimulated with TNF α and assayed for gene expression and cytokine production by qPCR and ELISA. NF- κ B signaling was evaluated using Western blotting. Histone acetylation, chromatin accessibility, and NF- κ B p65 and RNA polymerase II (Pol II) occupancy at the *IL6* promoter were measured by chromatin immunoprecipitation and restriction enzyme accessibility assays.

Results—In FLS, TNF α induced prolonged transcription of *IL6* and progressive accumulation of IL-6 protein over four days. Similarly, induction of CXCL8/IL-8, CCL5/RANTES, MMP1 and MMP3 mRNA after TNF α stimulation was sustained for several days. This contrasted with the macrophage response to TNF α , which characteristically involved a transient increase in the expression of pro-inflammatory genes. In FLS, TNF α induced prolonged activation of NF- κ B signaling and sustained transcriptional activity indicated by increased histone acetylation, chromatin accessibility, and p65 and Pol II occupancy at the *IL6* promoter. Furthermore, FLS expressed low levels of the feedback inhibitors ABIN3, IRAK-M, SOCS3 and ATF3 that terminate inflammatory responses in macrophages.

Conclusions—TNF α signaling is not effectively terminated in FLS, leading to an uncontrolled inflammatory response. The results suggest that prolonged and sustained inflammatory responses by FLS, in response to synovial TNF α , contribute to the persistence of synovial inflammation in RA.

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Author contributions A.L., Y.Q., J.C., G.G. and K.H.P.M designed and performed experiments, interpreted data and wrote the manuscript. G.D.K. and L.B.I oversaw the project, designed and supervised experiments, conceptualized the project, interpreted data and wrote the manuscript. G.D.K. had access to all primary data and is responsible for its integrity.

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Keywords

fibroblast-like synoviocytes; rheumatoid arthritis; signal transduction; TNF α ; chromatin

Introduction

Rheumatoid Arthritis (RA) is characterized by synovitis, systemic manifestations and significant morbidity and mortality (1, 2). RA pathogenesis is a multistep process consisting of a “pre-clinical phase”, involving the generation of autoantibodies, an “initiation phase”, where synovial inflammation emerges, and a “perpetuation phase”, which is dominated by non-resolving synovial inflammation and joint destruction (3). In each step, there is interplay between environmental, genetic, hormonal and stochastic factors (1, 4). The T-cell-centric pathogenetic model of RA was challenged by the discovery that cytokines derived from macrophages (M ϕ) and fibroblast-like synoviocytes (FLS), such as TNF α and IL-6 respectively, play a central role in RA pathogenesis (5). The current pathogenetic model is the so-called “integrated model” that implicates, within the synovium, cross-talk between T and B lymphocytes, M ϕ and FLS, involving cell-to-cell interactions and soluble factors which drive the initiation and perpetuation of synovial inflammation (1).

Synovial inflammation in RA is chronic and, even upon aggressive immunosuppression, long-term remission is rarely achieved (6). One potential explanation is that continuous presence of arthritogenic antigens or emergence of neoantigens sustains autoimmune responses against synovial elements (7). An alternative, but not mutually exclusive scenario is that the anatomic components of the synovium, such as stromal cells, are “hypersensitive” to systemic or local inflammatory stimuli. This model posits that, in contrast to epithelial surfaces, where homeostatic mechanisms effectively control responses to exogenous or endogenous inflammatory factors, joint synovium mounts an exaggerated or unremitting response. Susceptibility of synovial tissue to inflammation triggered by diverse factors such as injury, infection, immune complexes, and cytokines is supported by data from animal models (8-13).

RA FLS were described as “transformed” cells, sharing morphologic features with tumor cells, such as resistance to apoptosis, potentially due to somatic mutations in p53 (14). RA FLS display and retain an invasive capacity against articular cartilage (15). FLS express adhesion molecules and receptors for cytokines and TLR ligands that mediate their activation during synovial inflammation (16). In RA, FLS are the major synovial producers of IL-6, a key pathogenic cytokine (17, 18). Upon activation, FLS produce a constellation of cytokines, growth factors, chemokines, adhesion molecules, co-stimulatory molecules and tissue destructive factors (16, 19, 20). FLS mediate synovial recruitment, retention, organization, activation and survival of inflammatory cells, enhance synovial neoangiogenesis, and induce osteoclastogenesis and cartilage degradation (14, 21).

TNF α is a key driver of synovial inflammation in 50-70% of RA patients. Macrophages are likely the major source of synovial TNF α (5, 18). However, M ϕ display a transient inflammatory response to TNF α due to homeostatic mechanisms that terminate inflammatory signaling and impose a chromatin-mediated barrier that suppresses inflammatory gene expression (22). Following initial exposure to TNF α , M ϕ develop resistance to subsequent challenge with inflammatory stimuli, including TNF α , IL-1 and TLR ligands (23). This raises the question of which are the major cells that respond to synovial TNF α to sustain inflammation. Experiments with transgenic mice that express TNF α suggest that FLS, which are in close proximity to M ϕ , are the major responders to TNF α (13). In this study we found that, in stark contrast to M ϕ , TNF α -stimulation of FLS

resulted in a sustained inflammatory response characterized by prolonged expression of cytokines, chemokines and MMPs. Prolonged gene expression in FLS was associated with sustained NF- κ B signaling, prolonged transcriptional activity indicated by increased histone acetylation, chromatin accessibility, and NF- κ B and Pol II occupancy at the *IL6* promoter, and ineffective induction of feedback mechanisms that restrain inflammatory signaling and gene expression in macrophages. These results suggest that ineffective termination of inflammatory signaling in FLS contributes to the persistence of synovial inflammation.

Materials and Methods

Patients

Synovial tissues were obtained from RA or osteoarthritis (OA) patients who underwent total knee replacement (protocol approved by our hospital's Institutional Review Board). The diagnosis of RA and OA was based on the American College of Rheumatology criteria (24, 25).

Cell purification

Synovial tissue fragments were incubated with dispase for 90 min at 37 °C and cells were allowed to adhere to tissue culture dishes and passaged every 3-5 days. 3-4 passages yielded a relatively homogeneous population of FLS.

CD14⁺ cells were purified from healthy volunteers' PBMCs using anti-CD14 magnetic beads (Miltenyi Biotec).

Cell Culture

FLS and CD14⁺ cells were cultured in alpha-MEM (plus 10% FBS). The following reagents were used as indicated: TNF α (10ng/ml) and M-CSF (20ng/ml) (PeproTech), etanercept (10 μ g/ml) and anakinra (10 μ g/ml) (AMGEN), infliximab (10 μ g/ml) (Janssen Biotech) and human IgG (10 μ g/ml) (Sigma Aldrich), anti-gp130 (5 μ g/ml) and mouse IgG_{2a} (5 μ g/ml) (R&D Systems), CP690,550 (10 μ M), Bay-11 (10 μ M), IKK Inhibitor II (50 μ M), IKK Inhibitor XII (10 μ M) and MG132 (10 μ M) (Calbiochem), TAPI-1 (10 μ M) (Peptides International) and dimethyl sulfoxide was used as a vehicle control.

ELISA and FACS

We measured IL-6 and TNF α in culture supernatants (0.5 $\times$ 10⁶ FLS in 3ml medium and 2 $\times$ 10⁶ M ϕ in 1ml medium) with sandwich ELISA. For flow cytometry, mAbs to human TNFRSF1A (p55) and TNFRSF1B (p75), and isotype controls (R&D Systems) were used.

Real-time quantitative RT-PCR (qPCR)

RNA was extracted from 0.5 $\times$ 10⁶ FLS, 1 μ g was reverse transcribed and qPCR was performed.

Immunoblotting

Lysates from 10⁵ FLS were fractioned on polyacrylamide gels, transferred to polyvinylidene fluoride membranes and incubated with antibodies against I κ B α , Akt, p65, Lamin B1, and p38. Densitometry was performed (ImageJ software).

Restriction Enzyme Accessibility Assay (REA)

Isolated nuclei were incubated (30 min, 37°C) with Sspl (50U) (New England Biosciences) and digested genomic DNA was purified. Equal amounts of purified DNA were digested to completion with BstXI (50U, overnight, 37°C), precipitated, and analyzed by Southern blot

using radiolabeled probe specific for the *IL6* gene (+51 to +614 relative to the transcription start site).

Chromatin-immunoprecipitation assay (ChIP)

Cells were treated with 1% formaldehyde to crosslink chromatin. Fixed cells were incubated with lysis buffer and sonicated using a Bioruptor® device (Diagenode UCD400). 5% of sonicated cell lysates was saved as input. Chromatin was immunoprecipitated using antibodies to Histone 3 (H3), Histone 4 (H4), acetylated H4 (Millipore), NF- κ B p65 subunit (Abcam) and RNA Polymerase II (Santa Cruz). Crosslinks were reversed, DNA was purified and enrichment of the target DNA was measured by real time PCR.

Statistical Analysis

Results are expressed as mean $\pm$ SEM and GraphPad Prism Analytical Software Version 5.93 for Windows was used. When comparing between 3 or more groups, differences were tested using one-way or two-way analysis of variance (ANOVA) followed by the Bonferroni test. When comparing between 2 groups, the two-tailed, paired Student *t* test was used.

Results

FLS display sustained inflammatory responses to TNF α

We investigated the molecular mechanisms that govern the TNF α -driven production of pro-inflammatory and tissue-destructive mediators by FLS. Cells were cultured in the presence or absence of TNF α (10ng/ml). TNF α was added on the first day and was not replenished. Upon TNF α stimulation, FLS secreted copious amounts of IL-6 (Figure 1A). IL-6 production was sustained and continuously increased over time, reaching > 200 ng/ml at later time points (Figure 1A). We next addressed whether this sustained pattern of IL-6 production results from continuous transcription. We found an increasing pattern of IL-6 mRNA expression in TNF α -stimulated FLS during the first 48h, which remained at these high levels until day 4 (Figure 1B; the data are presented normalized relative to GAPDH mRNA (% GAPDH), which was not altered after TNF α stimulation). In addition, we measured levels of active transcription of *IL6* using primers specific for the fourth intronic region of the *IL6* gene (amplifying primary transcripts). As shown in Figure 1C, there was a robust and prolonged induction of *IL6* primary transcripts by TNF α . Thus, continuous transcription of *IL6* correlates with the sustained pattern of IL-6 protein production. These results suggest that, in FLS, TNF α induces prolonged transcription of *IL6*.

Next, we investigated the kinetics of induction, in FLS, of additional TNF α targets. These included the chemokines CXCL8/IL-8 and CCL5/RANTES, which recruit effector cells to inflamed joints, and the metalloproteinases MMP1 and MMP3, which mediate cartilage degradation. There was a prolonged induction pattern by TNF α , similar to IL-6, for all the above genes (Figure 1D and data not shown), suggesting that FLS display a sustained inflammatory and tissue destructive program in response to TNF α . We observed a similar pattern of sustained TNF α -induced inflammatory gene expression in RA and OA FLS, although, consistent with the literature (26), there was higher expression in a subset of RA FLS (Supplementary Figure 1 and data not shown). For the experiments described below, only RA FLS were used.

M ϕ display a transient inflammatory response to TNF α

We wished to directly compare in our system TNF α responses of FLS and macrophages. Synovial macrophages spontaneously produce TNF α and induce an autocrine IFN-STAT1 loop that would confound this analysis (27), so instead we analyzed TNF α responses in blood-derived macrophages. TNF α stimulation of macrophages resulted in a robust

induction of TNF α , IL-1 β and CXCL8/IL-8 mRNA (Figure 2A-C). In stark contrast to FLS, the TNF α -induced inflammatory program in M ϕ was transient, returning to baseline expression levels by 3-48 hours (Figure 2A-C). Interestingly, in M ϕ the induction of IL-6 mRNA by TNF α was also transient (Figure 2D), with the maximal amounts of IL-6 mRNA approximately 50-fold lower than those induced in FLS (compare Figures 2D and 1B). IL-6 protein in culture supernatants was < 0.5 ng/ml by ELISA at all time points after TNF α stimulation of M ϕ (Supplementary Figure 2), which was substantially lower than IL-6 in FLS culture supernatants (> 200 ng/ml, Figure 1A). Our observations suggest that there are kinetic, qualitative and quantitative differences in the responses of M ϕ and FLS to TNF α .

Continuous TNF α signaling is required for sustained inflammatory response in FLS

The sustained pattern of TNF α -induced inflammatory gene expression described above for FLS suggests that TNF α may induce an autocrine cascade of factors that maintain gene expression after the initial TNF α signal resolves. Alternatively, ongoing direct signaling by TNF α may be required. We next performed experiments to distinguish between these two possible mechanisms that can sustain gene expression. As shown in Figure 3A, in FLS culture supernatants, concentrations of exogenous TNF α protein (added at 10ng/ml) decayed over time, likely due to consumption by the cells and protein degradation, to reach a concentration of < 4 ng/ml after two days of culture; FLS did not produce significant amounts of endogenous TNF α , as expected (16). Addition of the MMP inhibitor TAPI-1, to prevent TNF receptor shedding, did not change the levels of TNF α (Fig 3A), suggesting that the decrease in TNF α was not secondary to interference with the ELISA assay by soluble TNF receptors. Next, we investigated whether the late phase of gene expression requires the ongoing presence of the low residual amounts of TNF α , and tested the role of other cytokines that may act in our system in an autocrine or paracrine manner. Strikingly, elevated expression of IL-6 mRNA was abrogated when the TNF α blocker etanercept was added 2 or 3 days after the initial stimulation with TNF α (Figure 3B). Concordantly, we found that addition of the TNF α blocker infliximab on day 2 prevented further accumulation of IL-6 protein (Figure 3C). These results suggest that the late phase of inflammatory response in FLS was dependent on ongoing signaling by residual low amounts of TNF α . Next, we investigated whether other cytokines may cooperate with the low amounts of TNF α to sustain the late phase of the inflammatory response in our system. We added several inhibitors that successfully block inflammation and cytokine production in RA clinical trials, including the anti-TNF α monoclonal antibody infliximab, the IL-1 receptor antagonist anakinra, and the Jak-STAT signaling inhibitor CP690550. In addition, we tested a gp130 blocking antibody, which inhibits the effects of IL-6 and other gp130 signaling cytokines. All inhibitors and their controls were added on day 3 after the initial TNF α stimulation and cells were harvested the next day. TNF α -induced IL-6 mRNA was significantly suppressed by etanercept or infliximab, but the other inhibitors had minor (CP690550) or no effect (Figure 3D). Similar results were found for CXCL8/IL-8, CCL5/RANTES and MMP1 mRNA (data not shown). The findings that FLS retain a prolonged responsiveness even to low amounts of TNF α , suggest that the late phase inflammatory response in FLS requires the continued presence and signaling by TNF α .

In FLS, TNF α induces prolonged NF- κ B signaling that is required for IL6 transcription

Activation of the canonical NF- κ B signaling pathway typically follows cell stimulation with TNF α . In addition, activation of NF- κ B pathway has been observed in RA synovium and the detrimental role of this pathway in synovitis has been suggested by studies in animal models (28). After TNF α stimulation, I κ B α protein, which inhibits NF- κ B signaling by retaining NF- κ B proteins in the cytoplasm, was rapidly degraded and then returned to baseline levels within 2-3 hours (Figure 4A upper panels and Supplementary Figure 3). These results are consistent with rapid activation of NF- κ B signaling followed by

termination through the resynthesis of I κ B α , as has been previously established in many cell types (29). Strikingly, in FLS there was a second phase of I κ B α protein degradation beginning at 6h after TNF α stimulation (Fig. 4A upper panels and Supplementary Figure 3) that was maintained throughout the timeframe of our experiments (Fig. 4A lower panels and Supplementary Figure 3). p38, whose expression did not change, was used as loading control as previously described (23). In addition, TNF α stimulation resulted in the expected rapid (within 1-3 hours) increase in NF- κ B p65 nuclear localization (Fig 4B upper panel). Consistent with the sustained pattern of I κ B α degradation, p65 nuclear localization in TNF α -stimulated FLS was sustained throughout the timeframe of our experiments (96 hours) (Fig. 4B). Sustained p65 nuclear localization was dependent on continuous TNF α signaling, as it was abrogated by the addition of infliximab (on day 3) or an IKK inhibitor (on day 4, for 3 hours) (Fig. 4B lower panel, lanes 3-6). Next, several inhibitors of IKKs (Bay-11, IKK inhibitor II and XII) or the proteasome inhibitor MG132 were added 3 days after TNF α stimulation to acutely terminate NF- κ B signaling. Interruption of TNF α -mediated NF- κ B signaling for 3 hours resulted in a substantial decrease in *IL6* primary transcripts (Fig. 4C).

The above results suggest that the late phase of TNF α -induced *IL6* expression in FLS is dependent on sustained NF- κ B signaling. This could be explained by sensitization of FLS to TNF α by upregulation of TNF receptors or proximal signaling components, or by ineffective termination of signaling by feedback inhibitory homeostatic mechanisms. The potential sensitization of FLS to TNF α in our culture system was investigated using flow cytometry to measure cell surface expression of the two TNF Receptors, p55 and p75. There was a significant down-regulation of p55 receptor at 72h of TNF α -stimulation in FLS, and no up-regulation of p75 was observed upon early or prolonged TNF α stimulation (Fig. 4D and Supplementary Figure 4A). There was also no increase in TNF signaling components TRADD, TRAF2 or RIP1 as measured by immunoblotting (Supplementary Figure 4B). These results suggest that sensitization of TNF receptors and upstream signaling components did not occur, and led us to investigate feedback regulatory mechanisms instead.

Feedback regulatory mechanisms that control inflammatory responses in macrophages are not effectively induced in FLS

Our results suggest that TNF α -induced inflammatory responses are not effectively terminated in FLS by homeostatic mechanisms that deactivate inflammatory signaling in other cell types such as macrophages. The inflammatory response in M ϕ is rapidly down-regulated by inhibitory molecules including IRAK-M, ABIN3, A20, SOCS, SHIP1, and ATF3 which inhibit inflammatory signals or repress transcription of inflammatory genes, such as *IL6* (22). Interestingly, we observed that the TNF α -induced levels of *ABIN3*, *IRAK-M*, *SOCS3* and *ATF3* expression were considerably higher in M ϕ compared to FLS (Figure 5). In addition, baseline IRAK-M and ATF3 mRNA was substantially higher in M ϕ than in FLS (Figure 5B and 5D). These data suggest that relatively low expression of ABIN3, IRAK-M, SOCS3 and ATF3 in FLS may enable the sustained inflammatory response observed in TNF α -stimulated FLS.

TNF α induced a sustained increase in chromatin accessibility, histone acetylation and NF- κ B p65 and Pol II recruitment at the *IL6* promoter in human FLS

In macrophages, closing of chromatin accessibility at the *IL6* promoter after prolonged stimulation with TLR ligands or TNF α is an important mechanism for abrogating expression of *IL6* (23, 30, 31). Such epigenetic mechanisms work in tandem with feedback inhibition of signaling to ensure post-induction suppression of IL-6 production (22). We used restriction enzyme accessibility assays (REAs) to measure changes in chromatin

accessibility at the *IL6* promoter after TNF α stimulation of FLS. An accessible or “open” chromatin state is vulnerable to nuclease cleavage, which results in smaller size DNA cleavage products, while inaccessible or “closed” chromatin is more resistant to cleavage. We measured accessibility at the SspI restriction endonuclease site in the *IL6* promoter. Cleavage at the SspI site upstream of the transcription start site was substantially increased in FLS by TNF α stimulation during the early phase (3h), as measured by decreased amounts of uncut chromatin (Figure 6A, left upper panel, lanes 1-2), with a concomitant increase in amounts of SspI cut products (Figure 6A, left lower panel, lanes 1-2). Remarkably, increased cleavage by SspI was detected during the late phase (days 1, 2 and 4 after TNF α stimulation) (Figure 6A, left lower panel, lanes 3-6 and middle lower panel, lanes 1-2). In stark contrast, M ϕ stimulated with TNF α for 4 days displayed no detectable cleavage by SspI (Figure 6A, middle panels, lanes 3-4); as a positive control, a 3 hour LPS stimulation increased SspI cleavage as expected (Figure 6A, right panels). These results correlate with the sustained pattern of *IL-6* mRNA expression in TNF α -stimulated FLS and the transient pattern observed in M ϕ , and suggest that a sustained increase in chromatin accessibility contributes to sustained *IL6* transcription.

Chromatin accessibility can be increased by depletion of histones/nucleosomes that exposes DNA or by histone acetylation that weakens DNA-histone interactions. Thus, we examined the effects of TNF α stimulation on histone occupancy and acetylation at the *IL6* locus. Stimulation with TNF α for 72 hours resulted in decreased H4 levels at the *IL6* promoter (Fig 6B, left panel), and the remaining histones showed increased acetylation as measured by the acetylated H4/total H4 ratio (Figure 6B, right panel). H4 acetylation was not detected at the Hemoglobin B (*HBB*) promoter, which served as negative control. Induction of a more accessible chromatin state would facilitate binding of transcription factors and Pol II; indeed, we observed a striking increase in NF- κ B p65 (Fig. 6C) and Pol II (Fig. 6D) occupancy at the *IL6* promoter 72 hr after TNF α stimulation. These data further corroborate that TNF α induces sustained NF- κ B signaling and *IL6* transcription. Overall, our results suggest that TNF- α -induced sustained signaling by the NF- κ B pathway, coupled with prolonged chromatin accessibility at the *IL6* promoter, result in prolonged *IL6* transcription.

Discussion

Our study reveals fundamental differences between FLS and M ϕ in the kinetics, quality and quantity of their TNF α -induced inflammatory program. Whereas M ϕ display a transient inflammatory response, TNF α -stimulated FLS exhibit a prolonged inflammatory response. Strikingly, in FLS, we found very low expression of the negative regulators of inflammatory responses ABIN3, IRAK-M, SOCS3 and ATF3 in combination with sustained activation of the canonical NF- κ B pathway. In addition, in FLS, TNF α induced sustained histone modifications and increased chromatin accessibility at the *IL6* promoter. This removal of a chromatin barrier augments cell responsiveness to ongoing TNF α signaling by facilitating recruitment of NF- κ B and Pol II to the *IL6* promoter. These findings provide a molecular explanation for the well-known capacity of FLS to produce, upon stimulation, large quantities of IL-6, and suggest that sustained expression of inflammatory genes by FLS contributes to unremitting synovial inflammation in RA.

From a teleological point of view, the differences we detected between FLS and M ϕ probably reflect their distinct functions. The main function of FLS is to create the structural scaffold of synovium and produce extracellular matrix, lubricin and cartilage nutrients (16). FLS reside in a sterile internal environment and are not normally exposed to environmental antigens or microbes, and thus under physiological conditions do not require homeostatic mechanisms utilized by immune cells, such as macrophages, that are often exposed to microbes and their products. On the other hand, macrophages need to limit their responses to

inflammatory factors in order to prevent local and systemic toxicity caused by high levels of inflammatory mediators. They possess remarkable plasticity, and a capacity for a wide spectrum of tasks varying from rapid inflammatory response to homeostatic functions (32). During inflammation, M ϕ normally exhibit a biphasic response, initially promoting acute inflammation, while later dampening inflammation and triggering tissue repair (22). Thus, it is important for the proper function of macrophages to rapidly adapt to the distinct requirements of defense and homeostasis. From a mechanistic perspective, one path that M ϕ use to accomplish these divergent functions is to become tolerant to further inflammatory stimulation via regulatory mechanisms that either terminate the input of inflammatory signaling or repress inflammatory genes by epigenetic modifications (22). On the other hand, our data show that FLS do not become tolerant to continuous inflammatory stimulation, but instead display sustained responsiveness, even to low amounts of TNF α . The low expression of ABIN3, IRAK-M, SOCS3 and ATF3 in FLS support the hypothesis that in FLS there are insufficient “brakes” to turn off the response to TNF α , whereas in M ϕ the inflammatory response is tightly controlled via cooperation of a series of signaling and epigenetic “brakes”.

A major feedback loop that limits inflammatory cytokine production in macrophages is induction of IL-10 which in turn activates STAT3 (33). The STAT3-mediated feedback inhibition is not effectively induced in FLS, as these cells produce minimal IL-10, and STAT3 appears to play an activating rather than an inhibitory role in FLS (34). Our results show modest suppression of IL-6 by a Jak inhibitor, further supporting the notion that STAT3 does not suppress inflammatory cytokine production in FLS. Thus, the absence of an effective IL-10-STAT3-mediated inhibitory axis also likely contributes to the sustained pattern of inflammatory gene expression we observed in TNF α -stimulated FLS. In contrast to IL-10, TNF α induces IFN β in FLS, resulting in Jak-STAT signaling and expression of CCL5/RANTES and CXCL10/IP-10 that are suppressed by the Jak inhibitor CP690550 (35).

Several signaling pathways, activated directly or indirectly, downstream of TNF receptors mediate the pleiotropic effects of TNF α on FLS. The classical NF- κ B pathway has been linked primarily with the production of inflammatory mediators and is critical for FLS survival (28). Activation of JNK pathway contributes to the induction of tissue degrading enzymes (36), whereas the induction of PI3K δ by TNF α results in the activation of Akt, which in turn leads to increased growth and survival of FLS (37). Notably, a plethora of regulatory molecules have been described to impact the balance and activity of these pathways in FLS, modulating (amplifying, attenuating or reprogramming) the effects of TNF α during homeostasis or synovial inflammation. In this context, it has been suggested that the low expression, in RA FLS, of clusterin, which inhibits NF- κ B by stabilizing I κ B proteins, may amplify the induction of NF- κ B and IL-6 by TNF α (38). In contrast, TWEAK, a member of the TNF superfamily, may attenuate TNF α -mediated IL-6 production via the activation of RelB (39). In addition, during synovitis there is induction of the autotaxin-LPA axis, which enhances the positive effects of TNF α on survival, growth, migration and production of inflammatory cytokines, chemokines and MMP9 in FLS (40). Interestingly, ablation of this axis switches the effects of TNF α on FLS from pro-survival to pro-apoptotic (41). Another potential modulator of the effects and signaling of TNF α on FLS is the p21-activated kinase 1 (PAK1) that amplifies the production of MMPs via the activation of JNK (42). These various TNF-induced signaling events cooperate to induce the full pathogenic phenotype of RA FLS.

The role of IL-6 in RA, as pathogenic and treatment target, is now well established (43). Tocilizumab which blocks the IL-6 receptor, a monoclonal antibody which neutralizes circulating IL-6, and Jak-inhibitors which block cytokine signaling, all target the IL-6

pathway (44-47). FLS are the major source of IL-6 in RA (18), and our study provides a model on how the cross-talk of synovial M ϕ and FLS leads to continuous production of inflammatory mediators, such as IL-6, and contributes to chronic synovitis. The model is that, in the course of synovial inflammation, there is a continuous recruitment of activated monocytes and macrophages that produce TNF α . Our results suggest that TNF α , even in low amounts, acts on neighboring FLS in a paracrine manner, inducing prolonged NF- κ B signaling and sustained chromatin remodeling at the *IL6* promoter, due to ineffective homeostatic regulatory mechanisms in FLS. The combination of these signaling and epigenetic events results in continuous transcription of *IL6*, overall contributing to the maintenance of synovial inflammation.

Complete remission of synovitis is still an unmet need for most RA patients and, even in patients fulfilling the criteria for clinical response, joint destruction may proceed, which suggests residual sub-clinical inflammation (6, 48, 49). Notably, the approved therapies for RA target primarily immune cells and only tocilizumab targets a product mainly derived from FLS. Thus, direct targeting of FLS emerges as an attractive alternative strategy for breaking the vicious circle that leads to unresolved synovitis in RA. Therapeutic targeting of FLS by blocking cadherin-11, a molecule primarily expressed on FLS within the synovium, has been proven effective in animal models of arthritis (50). Our observations suggest that blocking sustained inflammatory signaling or altering the chromatin state of inflammatory and tissue destructive genes in FLS represent additional strategies to suppress the detrimental functions of FLS in RA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. TNF α induces sustained production of IL-6, chemokines and MMPs in FLS

FLS were cultured in time course experiments in the presence or absence of TNF α (10ng/ml) that was added on the first day on culture and was not replenished. A, IL-6 protein in culture supernatants was measured by ELISA. B-C, IL-6 mRNA and *IL6* primary transcripts (primers specific for the fourth intronic region of *IL6* gene) and D, CXCL8/IL-8 mRNA, CCL5/RANTES mRNA, and MMP1 mRNA were measured by qPCR and normalized relative to GAPDH mRNA. Statistical analysis was performed using the two-way analysis of variance test (ANOVA) followed by the Bonferroni test. (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$, ****= $p < 0.0001$)

Figure 2. TNF α induces a robust but transient inflammatory response in human M ϕ
 CD14+ cells from healthy donors' blood were differentiated in vitro into M ϕ by stimulation with M-CSF (20ng/ml) for 48h. M ϕ were cultured in time course experiments in the presence or absence of TNF α (10ng/ml) that was added the first day on culture and not replenished. TNF α (A), IL-1 β (B), CXCL8/IL-8 (C), and IL-6 mRNA (D) were measured by qPCR and normalized relative to GAPDH mRNA. Statistical analysis was performed using the two-way analysis of variance test (ANOVA) followed by the Bonferroni test. (*=p<0.05, ***=p<0.0001)

Figure 3. The sustained inflammatory response requires ongoing TNFα signaling

FLS were stimulated with TNFα on day 0. A, TNFα protein was measured with ELISA in culture supernatants ($\pm$ TAPI-1). B, Etanercept or hIgG (Control) was added on days 0, 1, 2, or 3. Cells were harvested on day 4 and IL-6 mRNA was measured with qPCR. Statistical analysis was performed using the two-tailed, paired Student *t* test. C, IL-6 protein was measured with ELISA in culture supernatants at 48h and 72h with or without Infliximab (IFX) added at 48h. D, On day 3, etanercept, infliximab, anakinra, anti-gp130 or CP690550 were added and IL-6 mRNA were measured on day 4 with qPCR. Dashed line represents TNFα-stimulated control FLS that were set to 100%. Statistical analysis was performed using the one-way analysis of variance test (ANOVA) followed by the Bonferroni test or the two-tailed, paired Student *t* test. (*= $p < 0.05$, ***= $p < 0.001$, ****= $p < 0.0001$, n.s.= not significant)

Figure 4. The prolonged induction of IL-6 by TNF α is dependent on sustained NF- κ B signaling FLS were cultured in the presence or absence of TNF α (10ng/ml). I κ B α (A) and nuclear p65 (B) protein were measured with western blot and bands were quantified by densitometry (right panels). C, DMSO, Bay-11, IKK inhibitors II/XII, or MG132 were added on culture day 3 for 3h and *IL6* primary transcripts (IL-6 PT) were measured with qPCR. Statistical analysis was performed using the two-tailed, paired Student *t* test (**= $p < 0.01$). D, Expression levels of p55 or p75 protein on the cell surface were measured by FACS. Representative results of three independent experiments are shown.

Figure 5. Regulatory mechanisms that control TNFα-induced signaling and gene transcription in Mφ are not effectively induced in FLS

FLS and Mφ were cultured in the presence or absence of TNFα (10ng/ml) in time course experiments. ABIN3 (A), IRAK-M (B), SOCS3 (C) and ATF3 (D) mRNA were measured with qPCR (Mφ: n=6, FLS: n=5). mRNA amounts were normalized relative to GAPDH mRNA, which was comparable in FLS and Mφ. ND: Not Done. Statistical analysis was performed using the two-way analysis of variance test (ANOVA) followed by the Bonferroni test. (*=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001)

Figure 6. TNF α in FLS, induces sustained chromatin remodeling, histone acetylation, p65 and Pol II recruitment on the *IL6* gene promoter

FLS and M ϕ were cultured in the presence or absence of TNF α (10ng/ml) in time course experiments. A, Nuclei from control and TNF α - or LPS-treated cells were digested with SspI (restriction enzyme accessibility assay for *IL6* promoter). Purified genomic DNA was analyzed by Southern blot. Representative results from two independent experiments are shown. B-D, 72h after TNF α stimulation, FLS were harvested and ChIP assay was performed with anti-Histone 4 (H4), anti-acetylated H4 (H4Ac) (B), anti-p65 (C), or anti-Polymerase II (Pol II) (D) antibodies. The enrichment of immunoprecipitated proteins is shown as % of input and levels of H4Ac were normalized to total H4 levels at the promoters of *IL6* and Hemoglobin B (*HBB*) (negative control). Representative results of three independent experiments are shown.