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Modulation of TNF-Induced Macrophage Polarization by Synovial Fibroblasts

Laura T. Donlin,* Arundathi Jayatilleke,*† Eugenia G. Giannopoulou,*,# George D. Kalliolias,*‡ and Lionel B. Ivashkiv*‡,§

Mesenchymal stromal cells have emerged as powerful modulators of the immune system. In this study, we explored how the human macrophage response to TNF is regulated by human synovial fibroblasts, the representative stromal cell type in the synovial lining of joints that become activated during inflammatory arthritis. We found that synovial fibroblasts strongly suppressed TNF-mediated induction of an IFN-β autocrine loop and downstream expression of IFN-stimulated genes (ISGs), including chemokines CXCL9 and CXCL10 that are characteristic of classical macrophage activation. TNF induced the production of soluble synovial fibroblast factors that suppressed the macrophage production of IFN-β, and cooperated with TNF to limit the responsiveness of macrophages to IFN-β by suppressing activation of Jak–STAT signaling. Genome-wide transcriptome analysis showed that cocultured synovial fibroblasts modulate the expression of approximately one third of TNF-regulated genes in macrophages, including genes in pathways important for macrophage survival and polarization toward an alternatively activated phenotype. Pathway analysis revealed that gene expression programs regulated by synovial fibroblasts in our coculture system were also regulated in rheumatoid arthritis synovial macrophages, suggesting that these fibroblast-mediated changes may contribute to rheumatoid arthritis pathogenesis. This work furthers our understanding of the interplay between innate immune and stromal cells during an inflammatory response, one that is particularly relevant to inflammatory arthritis. Our findings also identify modulation of macrophage phenotype as a new function for synovial fibroblasts that may prove to be a contributing factor in arthritis pathogenesis. The Journal of Immunology, 2014, 193: 000–000.

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stromal cells of the mesenchymal lineage have emerged as powerful modulators of the immune system (1). Traditionally viewed as constituents of connective tissue passively providing structural and homeostatic support, stromal cells such as fibroblasts indeed shape innate and adaptive immune responses involved in host defense, tumor immunity, and autoimmunity (1, 2). The influential capacity of stromal cells has been widely demonstrated in cancer, for which the stroma impacts tumor growth, metastasis, and even drug resistance (3–5). In most settings, the immunomodulatory activity of the mesenchymal

Abbreviations used in this article: EGF, epidermal growth factor; IFIT, IFN-γ-induced protein with tetratricopeptide repeats 1; IFNAR, IFN-α receptor; IGF1, insulin-like growth factor 1; ISG, IFN-stimulated gene; MX1, IFN-induced GTP-binding protein Mx1; NKG7, NK cell granule protein 7; RA, rheumatoid arthritis; RNAseq, RNA sequencing; SLE, systemic lupus erythematosus.

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inflammatory cytokines such as IL-1 and chemokines such as IL-8, CXCL9, and CXCL10 (18-20), although TNF can also engage feedback inhibitory pathways (21). The pathogenic capacity of TNF has been established by the efficacy of anti-TNF therapies in rheumatoid arthritis (RA) (9, 22). Alternatively, elevated IFN levels, typically measured as an “IFN signature” gene expression profile, likely contribute to loss of tolerance in systemic lupus erythematosus (SLE) (23-26). It has been proposed that TNF and IFN can directly counteract each other and that deregulation of this interplay contributes to autoimmunity (23, 27-29). In RA, where chronic TNF stimulation is common, an IFN signature has been detected in the synovial tissue and PBMCs from a subset of patients (30). In contrast to SLE, TNF contributes to the IFN signature in RA synovial macrophages, including expression of M1 chemokines such as CXCL9 and CXCL10, which are abundant in RA synovium and can lead to recruitment and activation of inflammatory cells within joints (19, 27, 31–34). Although type I IFNs contribute to inflammatory M1 macrophage activation (19, 35), they also have protective effects in autoimmune diseases (36). Thus, understanding how their production and function are regulated may lend insight into their role in RA pathogenesis and suggest approaches to modulate IFN responses in a manner beneficial to patients.

Our laboratory previously demonstrated that TNF-stimulated macrophages secrete IFN-β, which acts in an autocrine and paracrine fashion to induce IFN-stimulated genes (ISGs) (31, 37). We also previously reported that the TNF-induced IFN response in macrophages is inhibited by RA synovial fluids (31). Because synovial fibroblasts secrete many of the synovial fluid components, we tested whether synovial fibroblasts regulate the TNF-induced IFN response in macrophages. In cocultures designed to recapitulate cellular cross-regulation that occurs in inflamed synovial tissue, we demonstrate that synovial fibroblasts secrete factors that suppress the IFN response in TNF-stimulated macrophages and affect macrophage activation by modulating Myc- and growth factor–regulated genes, which are important for macrophage survival and polarization toward an alternatively activated (M2) phenotype. We suggest that modulation of TNF-induced macrophage program by synovial fibroblasts results in a mixed M1/M2 macrophage phenotype in inflammatory settings like RA, which may contribute to RA pathogenesis.

Materials and Methods

Cell culture

Human CD14+ monocytes were purified from whole-blood cell preparations from healthy donors as previously described (37) and differentiated into macrophages for 1–2 d in 10 ng/ml M-CSF (PeproTech) and RPMI 1640 medium (Life Technologies) supplemented with 10% defined HBS (Hyclone). Cells were stimulated with 20 ng/ml recombinant human TNF and simultaneously with 10 ng/ml M-CSF (PeproTech) for mRNA and protein analyses, respectively. Infliximab (Innogenetix Biotech), human IgG (Sigma-Aldrich), human IL10/IL10R (R&D Systems), and human gap1p30 (R&D Systems) Abs were added at 5 μg/ml.

Human synovial fibroblasts derived from deidentified synovial tissues of RA or osteoarthritis (OA) patients undergoing total joint arthroplasty (protocol approved by Institutional Review Board at the Hospital for Special Surgery) were digested and passaged as described previously (13). The diagnoses of RA were based on the American College of Rheumatology criteria (38).

For coculture experiments, in vitro–differentiated macrophages were replated directly onto adherent synovial fibroblasts, with the fibroblast:macrophage ratio at 1:2.5, respectively. For Transwell culture experiments, synovial fibroblasts adhered to polyester chambers with 0.4-μm pores (Corning) were suspended above the wells containing macrophages. Supernatants collected from synovial fibroblasts cultured with or without TNF for 16 h were added to macrophage cultures (0.8 ml supernatant into 1.2 ml standard culture media). Synovial fibroblast supernatants were fractionated by Amicon Ultra Centrifugal Filters (50-, 30-, 10-, and 3-kDa membranes).

Real-time PCR

RNA obtained using RNAeasy kit (Qiagen) with DNase treatment was reverse transcribed into cDNA (Fermentas) and analyzed by real-time quantitative PCR (Fast SYBR Green; Applied Biosystems) on a 7500 Fast Real-Time PCR System (Applied Biosystems). Gene–specific primer sequences for CXCL9, CXCL10, IFNβ, IFN-induced protein with tetra- tricopeptide repeat 1 gene (IFIT1), IFN-induced GTP-binding protein Mx1 gene (Mx1), GAPDH, CXCL5, and IL1b were as previously described (37), whereas the primers for the NK Cell Granule Protein 7 gene (NKG7) consisted of the following sequences: F:GATCCAGACCTTTCTTCCTCTC, R:CAACGCTTAAACTCCT. Expression levels were normalized to GAPDH and either calculated relative to the uninduced macrophage monocyte sample or as a percentage of the TNF- or IFN-induced macrophage monocyte sample.

Western blots

Western blot analyses were performed using standard procedures with the additional step of adding Pefabloc (Sigma-Aldrich) to macrophage cultures before cell lysis to prevent STAT protein degradation. Abs used for Western blots include phospho-STAT1 (Y701) (number 9171; Cell Signaling Technology), phospho-STAT2 (Y689) (number 07-224; Millipore), STAT1 (number sc-346; Santa Cruz Biotechnology), STAT2 (number 610188; BD Biosciences), and p38 (number sc-535; Santa Cruz Biotechnology).

RNA-seq

Total RNA was first extracted using RNAeasy mini kit (Qiagen). Tru-seq sample preparation kits (Illumina) were then used to purify poly-A transcripts and generate libraries with multiplexed barcode adaptors. All samples passed quality control analysis on a Bioanalyzer 2100 (Agilent Technologies). Paired-end reads (50 × 2 cycles, ~75 × 10^6 reads/sample) were obtained on an Illumina HiSeq 2500 in the Weill Cornell Medical College Genomics Resources Core Facility. The TopHat program (39) was used to align the reads to the UCSC Hg19 human reference genome, while the Cufflinks program (40) allowed for measurements of transcript abundance (represented by fragments per kilobase of exon model per million mapped reads). The RNA sequencing (RNAseq) data have been deposited to the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) and assigned accession number GSE57723.

The Ingenuity Pathway Analysis program (Ingenuity Systems, www.ingenuity.com) was used to analyze the genes in TNF-induced macrophages that were differentially regulated by fibroblasts ≥2-fold. The Upstream Regulator analytic was used to predict upstream regulators whose change in expression or function could explain the observed gene expression changes. The number of genes indicates the extent to which a target gene is regulated by an upstream regulator and genes differentially regulated in the current data set. The upstream regulators listed in our results were limited to proteins and endogenous small molecules. A z-score of higher absolute value suggests the majority of target genes for that upstream regulator were altered in a direction consistent with either inhibition or activation of the upstream regulator (an absolute value of ≥2 is considered significant), for which a negative or positive value represents the predicted inhibition or activation of the target genes for an upstream regulator and genes differentially regulated in the current data set. Upstream regulators listed in our results were limited to proteins and endogenous small molecules. A z-score of higher absolute value suggests the majority of target genes for that upstream regulator were altered in a direction consistent with either inhibition or activation of the upstream regulator (an absolute value of ≥2 is considered significant), for which a negative or positive value represents the predicted inhibition or activation of the target genes for an upstream regulator. Western Blot analysis was performed using standard procedures with an additional step of adding Pefabloc (Sigma-Aldrich) to macrophage cultures before cell lysis to prevent STAT protein degradation. Abs used for Western blots include phospho-STAT1 (Y701) (number 9171; Cell Signaling Technology), phospho-STAT2 (Y689) (number 07-224; Millipore), STAT1 (number sc-346; Santa Cruz Biotechnology), STAT2 (number 610188; BD Biosciences), and p38 (number sc-535; Santa Cruz Biotechnology).

Statistical analyses

A paired two-tailed Student t test was used to analyze for statistical significance. A p value < 0.05 was considered significant.

Results

Cocultured synovial fibroblasts suppress the TNF-induced IFN signature in macrophages

Consistent with our previous findings, TNF stimulation of human monocyte–derived macrophages resulted in a type I IFN signature,
measured here as increased expression of the well-established ISGs CXCL9, CXCL10, IFIT1, and MX1 (Fig. 1A, first two bars). We had previously shown that this response depends on IFN-β production and autocrine stimulation (37). Because synovial fluid suppresses the macrophage TNF-induced IFN response (31) and synovial fibroblasts secrete many synovial fluid factors, we wished to test whether cocultured synovial fibroblasts would suppress the macrophage TNF-induced IFN response. Consistent with published findings (41), TNF also induced ISG expression in RA synovial fibroblasts in monoculture but to a much lesser extent than in macrophages (Fig. 1A, bars 3 and 4, with the same data plotted with split y-axis in Supplemental Fig. 1). Notably, consistent with our previous experiments using synovial fluids (31), cocultured synovial fibroblasts strongly suppressed the TNF-induced IFN response in macrophages (Fig. 1A, bar 6 versus bar 2, Supplemental Fig. 1). This included suppression of the IFN-inducible chemokines CXCL9 and CXCL10 as well as the antiviral factors IFIT1 and MX1 (Fig. 1A). Synovial fibroblasts also modestly suppressed basal levels of ISG expression (Supplemental Fig. 1). Importantly, expression of genes induced by TNF with similar kinetics but not dependent on IFN, including NKG7, IL1B, and CXCL5, were not significantly suppressed by cocultured fibroblasts (Fig. 1B; data not shown). Thus, fibroblast-like cells from RA synovium control specific components of the TNF response in macrophages, including expression of classic ISGs.

To gain an understanding of whether the observed impact of synovial fibroblasts on the macrophage TNF response was specific for RA synovial fibroblasts, we tested synovial fibroblasts derived from OA patients and the IMR90 lung fibroblast cell line in similar coculture experiments. RA, OA, and IMR90 fibroblasts comparably suppressed the macrophage TNF-induced production of CXCL10 (Supplemental Fig. 2) and other ISGs (data not shown). Thus, the observed inhibitory effect is not specific for RA synovial fibroblasts. It is important to note that the experimental design necessitates that fibroblasts cocultured with macrophages also are exposed to and activated by TNF, suggesting the results may reflect not a tissue- or disease-specific function of fibroblasts but rather a function of fibroblasts that becomes apparent under TNF-stimulated inflammatory conditions, such as is often found in RA. These data collectively suggest that although macrophages can produce and respond to type I IFNs during a TNF response, neighboring fibroblasts inhibit the IFN response.

TNF-stimulated synovial fibroblasts produce soluble mediators that inhibit the TNF-induced IFN signature in macrophages

In our coculture experiments, synovial fibroblast-mediated inhibition of the macrophage IFN response may have resulted from direct cell contact and/or secreted fibroblast factors. To determine whether cell contact was required, the fibroblasts were cultured above and separate from the macrophages in porous Transwell chambers. This separated arrangement also allowed for isolation and analysis of the cell populations individually. In this Transwell system, the TNF-induced IFN response in macrophages also was suppressed by the cocultured synovial fibroblasts (Fig. 2A). These data suggest that cell contact is not required, but rather, soluble fibroblast-derived products inhibit the macrophage TNF-induced IFN response.

Mesenchymal stromal cell–derived immunomodulatory factors are commonly secreted in response to inflammatory stimuli (1, 2). In our coculture and Transwell systems, the synovial fibroblasts were stimulated by TNF along with the macrophages. Thus, we next sought to determine whether TNF stimulation of the synovial fibroblasts was required for production of the soluble inhibitory factors. Supernatant media collected from TNF-treated synovial fibroblasts were analyzed by quantitative PCR. Genes analyzed are listed above each graph. Gene expression values represent the mean of four experiments; error bars represent SE. In each experiment, macrophages were derived from a unique blood donor and the synovial fibroblasts were generated from a unique patient, such that n = 4 for both cell types. The amount of transcript was normalized to GAPDH levels in the sample, and all samples for a given gene are shown relative to the TNF-induced macrophage monoculture sample, which was set to 100. The mean level of mRNA encoded by the indicated genes relative to the internal standard GAPDH mRNA (percentage of GAPDH) in the TNF-induced samples was CXCL9, 10%; CXCL10, 600%; IFIT1, 60%; MX1, 50%; NKG7, 1%; CXCL5, 750%; and IL1B, 40%. **p < 0.001.

FIGURE 1. Cocultured synovial fibroblasts suppress the macrophage IFN signature induced by TNF. (A and B) Human macrophages (Mφ) and RA synovial fibroblasts (Fib) cultured alone or together (Mφ + Fib) and stimulated with TNF for 16 h were analyzed for mRNA levels by quantitative PCR. Genes analyzed are listed above each graph. Gene expression values represent the mean of four experiments; error bars represent SE. In each experiment, macrophages were derived from a unique blood donor and the synovial fibroblasts were generated from a unique patient, such that n = 4 for both cell types. The amount of transcript was normalized to GAPDH levels in the sample, and all samples for a given gene are shown relative to the TNF-induced macrophage monoculture sample, which was set to 100. The mean level of mRNA encoded by the indicated genes relative to the internal standard GAPDH mRNA (percentage of GAPDH) in the TNF-induced samples was CXCL9, 10%; CXCL10, 600%; IFIT1, 60%; MX1, 50%; NKG7, 1%; CXCL5, 750%; and IL1B, 40%. **p < 0.001.
fibroblast cultures inhibited the macrophage TNF-induced IFN response, whereas supernatants from unstimulated synovial fibroblasts did not significantly alter the response (Fig. 2B). These data indicate that stimulation of synovial fibroblasts with TNF leads to production of factors that cross-regulate the macrophage TNF program, specifically inhibiting the ensuing IFN response.

The Transwell and supernatant experiments demonstrated that a soluble factor produced by the fibroblasts confers the suppressive effect. To gain a better understanding of the biochemical nature of the factor, we fractionated fibroblast supernatants by molecular mass. Fractions containing molecules >50 kDa, consistent with the size of macromolecules, significantly inhibited the TNF-induced CXCL10 expression to a similar extent as the complete supernatant (Fig. 2C, left panel). Furthermore, heat denaturation of the fibroblast supernatants partially reduced the suppressive activity, suggesting a protein component to the inhibitory activity (Fig. 2C, right panel). However, smaller molecular mass fractions retained some inhibitory activity, and only 50% of the inhibitory activity was abrogated by boiling, suggesting that small molecules resistant to denaturation by boiling can contribute to inhibitory activity. Last, we directly tested whether blocking the function of candidate soluble factors that have immunosuppressive activity and are known to be present in synovial fluids could reverse the suppressive activity of fibroblast culture supernatants. However, neither blockade of IL-10 nor the IL-6 family of cytokines that signal via the gp130 receptor subunit had any effect on fibroblast-mediated suppression of macrophage IFN responses (Fig. 2D). We also tested whether TGF-β, a well-characterized anti-inflammatory factor, mediated the suppression by fibroblasts. However, TGF-β was surprisingly required for TNF-induced IFN response (data not shown), thus in fact functioning as a positive regulator of this pathway. Taken together, the results suggest that TNF stimulates synovial fibroblasts to produce soluble factor(s) of >50 kDa in size, likely including a protein component, that acts in trans on macrophages to suppress TNF-induced IFN responses.

**Figure 2.** Synovial fibroblasts stimulated by TNF produce soluble mediators responsible for restricting the macrophage TNF-induced IFN response. (A) Quantitative PCR mRNA analysis of human macrophages stimulated by TNF for 16 h in the presence of synovial fibroblasts suspended in porous Transwell chambers. Mφ (Fib), Transwell cultures of macrophages with synovial fibroblasts, in which only the macrophage mRNA was analyzed. (B) Quantitative PCR mRNA analysis of macrophages stimulated by TNF for 16 h in the presence of supernatant media. Supernatants were collected from independent cultures of synovial fibroblast treated with TNF (Fib<sub>TNF</sub> Supe) and without TNF (Fib Supe) for 16 h. (C) Quantitative PCR mRNA analysis of macrophages cultured in a similar manner to (B) except that the supernatants from TNF-treated fibroblasts were fractionated by molecular mass (left panel) or boiled (right panel) and individually incubated with the macrophages. (D) Quantitative PCR mRNA analysis of macrophages treated with neutralizing Abs and incubated alone or with synovial fibroblasts in a Transwell chamber (Fib). mRNA levels were normalized to GAPDH and made relative to the TNF-induced macrophage monoculture sample, which was set to 100. The mean level of mRNA encoded by the indicated genes relative to GAPDH (% GAPDH) for the TNF-induced samples in the Transwell cultures in (A) was CXCL10, 300%; IFIT1, 20%; and in the supernatant experiments in (B)–(D) was CXCL10, 1400%; and IFIT1, 40%. Mean of n = 3 in (A), n = 4 in (B), and n = 2 in (C) and (D), error bars represent SE. ***p < 0.0001, *p < 0.02.
responsiveness of macrophages to type I IFNs. Strikingly, synovial fibroblast supernatants blocked induction of CXCL10 and IFIT1 by exogenously added IFN-β (Fig. 3B, condition 3 versus condition 5). Although a portion of this inhibition resulted from residual TNF activity in the supernatants (Fig. 3B, condition 4 versus condition 5), blockade of TNF by the neutralizing Ab infliximab demonstrated that fibroblast factors alone significantly inhibited macrophage responsiveness to IFN-β (Fig. 3B, condition 4 versus condition 2). This suggests that synovial fibroblast products can suppress responses downstream of the receptor for type I IFNs, referred to as the IFN-α receptor (IFNAR). Taken together, these data demonstrate that synovial fibroblast products inhibit the TNF-induced IFN signature in macrophages via two distinct but complementary mechanisms, namely by inhibiting IFN-β production and restricting IFNAR function.

Fibroblast products suppress TNF-mediated induction of Jak–STAT signaling and protein effectors of the IFN response

Rapid induction of ISGs by type I IFNs is mediated by STAT1 and STAT2 proteins, which become activated by tyrosine phosphorylation upon IFNAR engagement and function as transcriptional activators of ISGs (36). In addition, when cells are exposed to IFNs for longer time periods, such as those in our experiments, STAT1 and STAT2 expression increases, and this increased expression contributes to the increased pool of tyrosine phosphorylated STAT1 and 2 and increased transcriptional activity (36, 37, 42). As predicted, overnight stimulation of macrophages alone with TNF-induced expression and tyrosine phosphorylation of STAT1, which we showed previously is mediated by autocrine IFN-β (Fig. 4A) (37). TNF also weakly increased STAT2 expression while robustly increasing STAT2 tyrosine phosphorylation. Addition of synovial fibroblasts to these transwell cocultures reduced TNF-induced phosphorylation of STAT1 and STAT2, as well as total protein levels for STAT1 and STAT2 (Fig. 4A). These data indicate that synovial fibroblasts suppress induction of an IFN response in macrophages by inhibiting an IFN-β–mediated autocrine loop and downstream Jak–STAT signaling. This inhibition was biologically relevant because synovial fibroblasts suppressed expression of several ISGs (Fig. 1) and strongly inhibited TNF-induced production of the potent proinflammatory chemokine CXCL10 when cocultured with macrophages (Fig. 4B). Taken together, these data suggest that fibroblast factors interfere with the potential of TNF to induce an effective IFN response program.

TNF precisely controls the macrophage IFN response by both inducing and restricting IFN responsiveness

The results in Fig. 3B suggested that overnight exposure to exogenous TNF cooperates with soluble factors secreted by fibroblasts to suppress responses to exogenous IFN-β. We then tested whether TNF alone could partially induce hyporesponsiveness to type I IFNs in macrophages in the absence of fibroblast-derived
added exogenous IFN-α pretreatment suppresses macrophage responsiveness to IFNs, we determined whether TNF or other mechanisms induced by inflammatory cytokines such as those that encode type I collagens. For macrophages cultured alone, the RNaseq analysis demonstrated that TNF induced or repressed expression of 1629 and 1628 genes by >2-fold, respectively. The RNaseq results for <220 genes regulated by TNF was confirmed by quantitative real-time PCR and Nanostring nCounter mRNA expression analyses (data not shown). Importantly, among the 1629 TNF-inducible macrophage genes, coculturing with fibroblasts attenuated expression for 366 of these genes (by 2-fold; the heat map in Fig. 6B depict the differential pattern and levels of expression changes, the Ingenuity Pathway Analysis program was used to analyze the genes in TNF-induced macrophages that were differentially regulated by synovial fibroblasts >2-fold; the heat maps in Fig. 6B depict the differential pattern and levels of expression for genes repressed (left panel) or upregulated (right panel) by fibroblasts in TNF conditions. The Ingenuity Pathway Analysis Upstream Regulator analytic was used to predict upstream molecules whose change in expression or function could be responsible for a subset of the observed gene expression changes (Fig. 6C). Reassuringly, two of the three highly significant upstream regulators predicted to be inhibited by synovial fibroblasts

Transcriptome-wide analysis of the synovial fibroblast-mediated modulation of the macrophage TNF response

To address whether synovial fibroblasts selectively regulate macrophage IFN responses or whether they more broadly impact the TNF-induced macrophage phenotype, we performed genome-wide transcriptomic analysis using high-throughput RNaseq. Human primary macrophages were stimulated with TNF and cultured either alone or in the presence of synovial fibroblasts. Transwell cultures were used to allow for isolation of pure macrophage populations (absence of significant fibroblast cell contamination was verified by monitoring expression of fibroblast-specific genes and stream inhibitors predicted to be inhibited by synovial fibroblasts responsible for a subset of the observed gene expression changes).

**FIGURE 4.** Synovial fibroblast products suppress the production of type I IFN response protein effectors in the presence of TNF. (A) Western blot analysis of human macrophages stimulated by TNF for 16 h in the presence of synovial fibroblasts suspended in porous Transwell chambers. Mb (Fib), Transwell cultures of macrophages with synovial fibroblasts, in which only the macrophage lysate was analyzed. Blots represent one of three experiments, with the average densitometry value and SE for phospho-STAT1 from all three experiments plotted to the right. ***p < 0.0005. (B) ELISA protein measurements for the CXCL10/IP10 chemokine found in supernatants from human macrophages and synovial fibroblasts cultured alone or in Transwell cultures (as in A) and treated with TNF at day 0. Data points represent the average of three independent experiments, with error bars representing the SE.

factors. We performed a time course of TNF stimulation of macrophages in the absence of fibroblasts, followed by recombinant IFN-β treatment. As expected (37), after TNF exposure, the expression of ISGs peaked between 6 and 12 h, with the donor and returned to baseline ~18 h after TNF stimulation, consistent with a feedback mechanism that limits the IFN response (Fig. 5A; data not shown). To determine whether TNF pretreatment suppresses macrophage responsiveness to IFNs, we added exogenous IFN-β at this 18-h time point. As would be expected in the absence of TNF pretreatment, IFN-β strongly induced CXCL10 and IFIT1 expression in control cells (Fig. 5A, dotted lines). In contrast, the response to exogenous IFN-β was attenuated in TNF-pretreated macrophages (Fig. 5A, solid lines). Furthermore, although exogenous IFN-β induced relatively high levels of STAT1 and STAT2 phosphorylation in control cells (Fig. 5B, lane 3), TNF pretreatment for one day dampened this response (Fig. 5B, lane 4). This attenuation is consistent with negative feedback of IFNAR signaling, which is mediated by signaling inhibitors such as suppressor of cytokine signaling proteins or other mechanisms induced by inflammatory cytokines such as TNF or IL-1 (36, 43, 44). Thus, in macrophages, although TNF initially induces an autocrine IFN-β response, it also overtime limits IFN signaling to induce a tempered response. Our coculture studies demonstrate that synovial fibroblasts can inhibit the early and direct induction of IFNBI expression by TNF and also enhance the late-phase TNF effect of dampening IFNAR signaling.
In macrophages, TNF induces an IFN signature but also limits IFN responsiveness. (A) Quantitative PCR mRNA analysis of macrophages cultured with and without TNF for 18 h and subsequently stimulated with IFN-β for 3 h. Graphs depict a representative experiment from three independent donors. Gene expression changes were normalized to GAPDH and calculated relative to uninduced. (B) Western blot analysis of macrophages treated with or without TNF for 24 h, followed by IFN-β for 15 min. Blots represent one of three experiments.

Under TNF-stimulated conditions were related to type I IFN activity (IFNA2; z-score = −4.7, p = 4 × 10^{-17} and IFN-β; z-score = −2.4, p = 3 × 10^{-12}) (Fig. 6C, left table), thus confirming that fibroblasts broadly suppressed IFN responses in macrophages. Interestingly, the only other upstream regulator predicted to be significantly inhibited due to the presence of fibroblasts was MYC (z-score = −3.5, p = 2 × 10^{-11}) (Fig. 6C, left table), a transcription factor that contributes to an alternatively activated macrophage phenotype (45). The network of genes known to be regulated by MYC and differentially expressed >4-fold in the presence of fibroblasts is shown in Fig. 6D, left panel. Conversely, upstream regulatory factors predicted to exhibit increased activity in TNF-induced macrophages because of the presence of fibroblasts included the growth factors EGF, hepatocyte growth factor, TGF-β1, CSF1 (C-M-CSF), CSF2 (GM-CSF), and platelet-derived growth factor; the small molecules cholesterol, PGE2, and tretinoin (retinoic acid); and the cytokines IL-4 and IL-13 (Fig. 6C, right table). The network of genes known to be regulated by EGF and differentially expressed >4-fold in the presence of fibroblasts is shown in Fig. 6D, right panel. Cumulatively, these factors and downstream signaling pathways would contribute to increased macrophage survival and thereby potentially contribute to pathogenesis, with a subset (M-CSF, IL-4, and IL-13) promoting features of alternative (M2) activation.

To assess whether the fibroblast-regulated pathways identified in the coculture experiments corresponded to pathways regulated in RA synovial macrophages, we compared our data to that from a parallel study in which we analyzed gene expression patterns in RA synovial macrophages. Importantly, among these RA macrophage–specific genes, more than a quarter (27%) are also regulated by RA fibroblasts in TNF-induced macrophages. These data support the notion that in the RA synovium, synovial fibroblasts influence a significant portion of the cellular program of macrophages. Furthermore, through an Ingenuity Pathway Analysis of the RA macrophage data set, EGF (z-score = 3.6, p = 1 × 10^{-18}) and CSF2 (z-score = 2.7, p = 2 × 10^{-29}) are also predicted to be activated upstream regulators. Importantly, close to half (~40%) of the EGF and CSF2 target genes affected in RA macrophages were also affected by fibroblasts in our TNF-induced macrophages. Thus, it will be interesting to determine whether the EGF and CSF2 signatures in RA macrophages are derived from RA synovial fibroblast factors, as these signatures are in macrophages from our coculture experiments and ultimately whether these responses contribute to the pathologic state.

Discussion

In this study we explored how the two predominant synovial lining cell types, macrophages and synovial fibroblasts, coordinate to respond to the pathogenic RA cytokine TNF. We demonstrate that synovial fibroblasts strongly suppress induction of an IFN response in macrophages by TNF, which recapitulates the suppressive activity of RA synovial fluids (31). Suppression of macrophage IFN responses was mediated by soluble fibroblast factors whose production was induced by TNF. These fibroblast-derived factors suppressed TNF-induced expression of ISGs in macrophages by two mechanisms: 1) suppression of TNF-induced production of IFN-β by macrophages, and 2) cooperation with TNF itself to limit macrophage responsiveness to IFNs by suppressing activation of the Jak–STAT signaling pathway. Thus, macrophages exposed to TNF in the presence of synovial fibroblasts are restricted both in their capacity to produce and respond to IFN. Transcriptional analysis revealed that synovial fibroblasts regulated ~28% of the macrophage TNF response, including the IFN response and pathways downstream of important regulators of macrophage polarization and survival. Our findings identify a new function for synovial fibroblasts that may be relevant for inflammatory arthritis pathogenesis, namely the modulation of synovial macrophage phenotype.

Investigation of the pathogenic role of synovial fibroblasts in RA has focused on their ability to produce inflammatory cytokines, chemokines, and tissue degradative enzymes, their attachment to and invasion of synovial tissues, and their ability to present Ags and activate T cells and promote lymphocyte survival (10, 11, 46–48). Previous work on synovial fibroblast-macrophage interactions in the absence of inflammatory factors showed that these cells interact (“co-compact”) to form a lining-like structure that resembles that of the synovium, supporting the biological relevance of the coculture approach (49, 50). Experiments using this system showed that synovial fibroblasts promote macrophage survival, and that stimulation with TNF results in lining hyperplasia and inflammatory mediator production (49); however, the effects of cell cross-talk in an inflammatory setting were not examined. Our findings extend this approach to show substantial cross-regulation between these two cell types in the setting of inflammasome activation as modeled by TNF stimulation. One outcome of this crossregulation is suppression of IFN responses in macrophages. Importantly, RA synovial fluids similarly suppress TNF-induced IFN responses in macrophages (31), suggesting that the inflammatory synovial environment, which often includes TNF, stimulates...
FIGURE 6. Transcriptome-wide analysis of the synovial fibroblast influence over macrophage TNF responses. (A) Venn diagrams representing the overlap of macrophage genes that TNF induces (left diagram, red circle) or represses (right diagram, green circle), which in the presence of synovial fibroblast are opposingly regulated (left diagram, green circle represents macrophage genes repressed by fibroblasts upon TNF treatment; right diagram, red circle represents macrophage genes induced by fibroblasts in TNF conditions). Human blood–derived macrophages were treated for 2 d with TNF with or without synovial fibroblasts suspended above the macrophages in Transwell chambers, and the macrophage RNA was sequenced (RNAseq). TNF-regulated genes included those that became >2-fold induced or repressed in comparison with untreated macrophages. Fibroblast-regulated genes included those with >2-fold differences when comparing macrophages treated with TNF alone versus treated with TNF in the presence of fibroblasts. (B) Heat-map depiction of all macrophage genes repressed (left) or upregulated (right) by cocultured synovial fibroblasts upon TNF treatment (by at least 2-fold) in comparison with macrophages treated with TNF and cultured alone. Colored bars represent the gene expression levels (log2 of fragments per kilobase of exon model per million mapped reads), with red representing higher and green representing lower levels. (C) Proteins and small molecules (Figure legend continues)
synovial fibroblasts to secrete factors that suppress IFN responses into synovial fluids, similar to the factors secreted into culture supernatants in our model. Regulation of IFN responses by synovial fibroblasts has a homeostatic component, because suppression of IFN-induced M1 chemokines such as CXCL10 would attenuate inflammation. However, type I IFNs also have anti-inflammatory functions, such as suppression of angiogenesis and proliferation (36). Thus, the overall functional consequences of fibroblast-mediated regulation of IFN responses would depend on the balance between the pathogenic and protective functions of type I IFNs in the context of RA. The role of type I IFNs in RA has not yet been clarified, but the importance of understanding IFN responses in RA is underscored by a recent study showing that 24% of single nucleotide polymorphisms associated with autoimmune diseases, including RA, fall within regulatory elements targeted by IFN–Jak–STAT signaling in immune cells (51).

One mechanism by which synovial fibroblasts suppressed macrophase IFN responses was to inhibit induction of autocrine IFN-β by TNF. Several signaling molecules that have been previously shown to suppress IFNβ expression, namely the glycoprotein synthase kinase 3 that is regulated by PI3K–Akt signaling, and the prostaglandin endoperoxide synthase 1 (PTGS1, also known as cyclooxygenase-1 or COX-1), possibly via production of nonprostaglandin lipid mediators and engagement of G protein-coupled receptors (52, 53). However, preliminary experiments using inhibitors of glycoprotein synthase kinase 3, G proteins, and cyclooxygenase proteins suggested that synovial fibroblast factors inhibit IFN-β production independently of these pathways (data not shown). Insight into which receptors and pathways are important will be provided by further biochemical characterization of fibroblast supernatants, particularly in identification of the >50-kDa factor, likely a protein, that exhibited inhibitory activity, but which will be challenging given the complexity and limited quantities of culture supernatants. A second mechanism by which synovial fibroblasts suppressed macrophase IFN responses was to cooperate with the late phase of the TNF response, which attenuates activation of Jak–STAT signaling. Overall, our findings suggest a tight functional coupling between synovial fibroblasts and macrophages in which the fibroblasts target different aspects of the TNF-induced autocrine IFN-β loop to effectively suppress ISG expression.

In addition to regulating IFN responses induced downstream of TNF, a pathway analysis of our transcriptomic data revealed that TNF-stimulated conditions, fibroblasts inhibit expression of genes downstream of MYC and augment expression of genes induced by multiple growth factors, including EGF, hepatocyte growth factor, TGF-β, CSF1 (M-CSF), CSF2 (GM-CSF), platelet-derived growth factor, IL-4, and IL-13 in nonproliferating primary human macrophages. This suggests that under TNF-stimulated coculture conditions synovial fibroblasts produce growth factors that regulate the macrophase phenotype; accordingly increased expression of vascular endothelial growth factor A (VEGFA), heparin-binding EGF-like growth factor (HBEGF), and insulin-like growth factor 1 (IGF1) was observed in the coculture RNA-seq data. Interestingly, MYC is not involved in proliferation or survival of human macrophages but instead has been implicated in the induction of a subset of genes in alternatively activated macrophages that are important for resolution of inflammation and tissue repair (45). Thus, suppression of MYC-dependent pathways and genes in synovial macrophages will alter and may partially compromise development of proresolvement macrophage phenotype. Although primary human macrophages have minimal proliferative capacity, activation of growth factor pathways will promote cell survival, thereby promoting disease pathogenesis (54, 55). In addition, other macrophase pathways predicted be upregulated in the presence of fibroblasts, in particular IL-4 and IL-13 and to some extent CSF1 (M-CSF), promote a global alternative activation phenotype (also termed M2), which is associated with tissue remodeling and resolution of inflammation (56, 57). In many inflammatory settings, M2 polarization would be considered beneficial because it can promote resolution of classcal M1-mediated inflammation. However, RA pathogenesis is characterized by an expanding inflammatory mass of synovial fibroblasts and macrophages (termed pannus), and expression of subsets of M2 genes may actually contribute to pathogenesis by promoting growth and dysregulated tissue repair. Because synovial fibroblasts simultaneously suppress expression of IFN-inducible genes such as CXCL9 and CXCL10 that are part of the classic activation (M1) phenotype, suppress MYC, but augment growth factor-mediated gene expression, the data argue that synovial fibroblasts partially shift the polarization pattern of macrophages toward an M2 phenotype, distinctively lacking the MYC-mediated component of M2 and the IFN-mediated component of M1 phenotypes. Mixed macrophase phenotypes simultaneously expressing subsets of M1 and M2 genes are common in complex inflammatory settings in vivo (18, 20, 58), and indeed, we found a quarter of the genes uniquely regulated in macrophages from RA patients also are controlled by fibroblasts in TNF-induced macrophages, suggesting the presence of fibroblast-controlled M2 components in RA macrophages. Thus, our results provide insights into mixed macrophase responses characteristic of RA and how such complex phenotypes can be generated.

In summary, we discovered a complex interplay between synovial fibroblasts and macrophages where fibroblasts modify subsets of macrophase responses to TNF, including suppression of ISG expression and modulation of select polarization features along the spectrum of classical inflammatory (M1) and alternative (M2) activation. Seminal previous work analyzing cytokine networks in cocultures of cells including fibroblasts, macrophages, and lymphocytes obtained from disaggregated RA synovial tissues...
led to the identification of TNF as an upstream regulator of cytokine production and therapeutic target in RA (22, 59). Similarly we propose that our work with a simplified synovial fibroblast-macrophage interaction system, which recapitulates the activity of RA synovial fluids, will yield not only mechanistic insights into regulation of TNF responses, but may identify new pathways and regulators that can be therapeutically targeted.

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References


