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Transcriptional Regulation of the Endogenous Danger Signal Tenascin-C: A Novel Autocrine Loop in Inflammation

Fui G. Goh,[1] Anna M. Piccinini,[1] Thomas Krausgruber, Irina A. Udalova, and Kim S. Midwood

Inappropriate expression of proinflammatory mediators underpins the pathogenesis of autoimmune disease and tumor metastasis. The extracellular matrix glycoprotein tenascin-C is an endogenous activator of innate immunity that promotes the synthesis of inflammatory cytokines via activation of TLR4. Little tenascin-C is observed in most healthy adult tissues, but expression is specifically upregulated at sites of inflammation. Moreover, high levels of tenascin-C are associated with chronic inflammation and found in the tumor stroma. In this study, we show that the expression of tenascin-C is induced in immune myeloid cells activated by a variety of inflammatory stimuli, including specific TLR ligands. Its synthesis is transcriptionally regulated and requires the specific activation of AKT/PI3K and NF-κB signaling pathways. Using a bioinformatic approach, we identified a large number of conserved noncoding regions throughout the tenascin-C genomic locus that may contribute to its transcriptional regulation during inflammation. We also demonstrate that tenascin-C expression is transient during acute inflammation. In contrast, persistently high levels of expression occur in the inflamed synovium of joints from rheumatoid arthritis patients. Thus, misregulated expression of this endogenous danger signal may promote an autocrine loop of inflammation and contribute to the persistence of inflammation in autoimmune diseases or to tumor egress and invasion during metastasis. ***The Journal of Immunology*****, 2010, 184: 2655–2662.**

The induction of proinflammatory genes is a critical response to injury and infection. However, inappropriate synthesis of proinflammatory mediators leads to extensive damage of healthy tissues, and it is this persistence of inflammation that is the hallmark of autoimmune diseases, such as rheumatoid arthritis (RA) and multiple sclerosis (1). Moreover, tumor metastasis has recently been shown to be driven by proinflammatory mediators synthesized by immune myeloid cells within the tumor environment, demonstrating how the inflammatory process can be hijacked during the progression of cancer (2).

During inflammation, the extracellular matrix (ECM) acts as a scaffold for cell infiltration and a reservoir for cytokines and growth factors. In addition, some ECM molecules can also act directly as inflammatory stimuli by inducing the expression of proinflammatory genes. These ECM molecules make up a class of endogenous danger signals or damage-associated molecular patterns (DAMPs). Their expression is specifically induced upon tissue injury, for example, ECM molecules, such as tenascin-C (3), biglycan (4), and fibronectin containing extra domain A (5); or they are generated by damage to tissue, for example, small m.w. fragments of hyaluronic acid (6) or heparan sulfate (7). DAMPs are vital for initiating the immune response in the absence of infection and serving as a warning sign of potential infection. However, they may also drive the perpetuation of inflammation by virtue of a vicious cycle in which increasing tissue damage creates increasing levels of proinflammatory mediators (reviewed in Refs. 8–11). As such, these danger signals are key targets in preventing inappropriate inflammation (reviewed in Refs. 12–14). However, the molecular mechanisms that regulate tissue levels of endogenous danger signals remain unclear.

Tenascin-C is a proinflammatory ECM glycoprotein. It induces cytokine synthesis in primary human macrophages and synovial fibroblasts via activation of the pattern recognition receptor, TLR4 (3). It can also stimulate cytokine and matrix metalloproteinase synthesis in murine synovial fibroblasts via activation of α_9 integrins (15). Little or no tenascin-C is found in most healthy adult tissues, but expression is specifically and rapidly induced in response to tissue injury. High tenascin-C levels are also associated with chronic inflammation in autoimmune diseases, such as RA and Crohn's disease, as well as in the tumor stroma (reviewed in Refs. 16 and 17).

Emerging evidence points to an important role for tenascin-C in driving pathological inflammation in RA. Immunohistochemical analyses revealed little tenascin-C expression in joints from healthy individuals or in non inflamed joints from patients with osteoarthritis (OA) (18–20). However, high levels of tenascin-C are localized to pathogenic foci in the synovium of RA patients (15, 18–20), where expression correlates with inflammatory cell infiltration (19). High levels of tenascin-C are also expressed in cartilage (20) and synovial fluid (21–23) from RA patients. Addition of exogenous tenascin-C stimulates cytokine synthesis in synovial membranes isolated from the joints of RA patients, an ex vivo model of human disease (3). In

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Abbreviations used in this paper: CNS, conserved noncoding sequence; DAMP, damage-associated molecular pattern; ECM, extracellular matrix; LTA, 5,6-oxido-7,9,11,14-eicosatetraenoic acid; MDDC, monocyte-derived dendritic cell; MDM, monocyte-derived macrophage; OA, osteoarthritis; Pol II, polymerase II; poly(I:C), polyinosine-polycytidylic acid; RA, rheumatoid arthritis.

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addition, tenascin-C expression is upregulated at both the mRNA and protein levels in synovial tissues in mouse models of joint inflammation (3, 15), and tenascin-C is also arthritogenic in vivo when injected intra-articularly into mice (3). Furthermore, its activation of TLR4 is essential for driving persistent joint inflammation in vivo (3). These data implicate tenascin-C as a key proinflammatory mediator in the RA joint. However, the origin of the high levels of tenascin-C in the joint is not known, and it is not clear how synovial tissue levels of this DAMP are regulated during inflammation.

In this study, we show that tenascin-C expression correlates with disease activity in human rheumatoid joints. We also demonstrate that the high level of tenascin-C synthesis observed in diseased joints is driven by specific subsets of synovial cells, including immune cells of the myeloid lineage. We show that although tenascin-C expression is persistently activated in chronic inflammation, it is subject to tight transcriptional control during acute inflammation; expression is induced upon immune cell activation and then is effectively and rapidly downregulated. Finally, we provide the first insights into the molecular machinery that exerts this control over endogenous tenascin-C expression in human myeloid cells during inflammation. Understanding how tenascin-C expression is regulated during an acute immune response will reveal how this process can be subverted during chronic inflammation and may provide the means to modulate tissue levels of this danger signal.

Materials and Methods

Patient specimens

Synovial membranes were obtained from patients with RA or OA undergoing joint replacement surgery as described previously (24). The study was approved by the local trust ethics committee, and waste tissue (synovium after joint replacement surgery) was obtained only after receiving signed informed consent from the patient and ensuring tissue anonymity to protect patient identity. Synovia were either fixed in 10% (v/v) buffered formalin for immunohistochemical analysis or processed for cell isolation.

Immunohistochemical analysis

Fixed synovial membranes were processed to paraffin, and tissue sections (4 μm) were cut and stained with rabbit anti-human tenascin-C polyclonal Ab (191011; Chemicon International, Temecula, CA).

Cell isolation

RA or OA membrane cells (representing a mixed population of all synovial cell types) were isolated from synovial membranes. Synovial fibroblasts, myeloid cells, and lymphoid cells were isolated from this mixed population of RA membrane cells as described previously (25). Immediately after isolation, total synovial cells, fibroblasts, myeloid cells, and lymphoid cells were cultured at 1×10^5 cells/well in RPMI 1640 containing 10% (v/v) FBS and 100 U/ml penicillin/streptomycin (PAA Laboratories, Pasching, Austria) in 96-well tissue culture plates for 24 h before assessing tenascin-C protein levels by Western blotting and ELISA and mRNA levels by quantitative RT-PCR as described below. Levels of inflammatory cytokines were assessed by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Primary human myeloid cell culture and activation

Human monocytes isolated from peripheral blood (London Blood Bank, London, U.K.) were differentiated into monocyte-derived macrophages (MDMs) or monocyte-derived dendritic cells (MDDCs) (26, 27) and cells plated (1×10^6 cells/ml) in RPMI 1640 containing 5% (v/v) FBS and 100 U/ml penicillin/streptomycin for 24 h before stimulation with 10 ng/ml LPS (ultrapure LPS from Escherichia *coli* 0111:B4; InvivoGen, Wiltshire, U.K.) for 1, 4, 8, or 24 h at 37°C before assessment of tenascin-C protein and mRNA levels as described below. MDDCs were also stimulated with 500 ng/ml 5,6-oxido-7,9,11,14-eicosatetraenoic acid (LTA), 1 μg/ml PAM3, 25 μg/ml polyinosine-polycytidylic acid [poly(I:C)], 1 μg/ml flagellin, and 1 μg/ml R848 (all from InvivoGen) for 24 h at 37°C before assessment of tenascin-C protein levels as described below.

HEK cell culture and stimulation

HEK293 cell lines stably expressing human TLR4/CD14/MD-2 (InvivoGen) were cultured (1×10^6 cells/ml) in DMEM containing 10% (v/v) FBS (PAA Laboratories), 10 μg/ml blasticidin, and 50 μg/ml HygroGold (InvivoGen). Unless stated otherwise, cells were stimulated with 0.1, 1, or 10 μg/ml LPS for 24h before assessment of tenascin-C protein levels as described below.

Assessment of tenascin-C expression

Total RNA was extracted from cells using a QiaAmp RNA Blood mini kit (Qiagen, Hilden, Germany). cDNA was synthesized from equivalent amounts of total RNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and 18-mer oligo dTs (Eurofins MWG Operon, Ebersberg, Germany). Gene expression was analyzed by two-standard curve or ΔΔCT methods where appropriate based on quantitative real-time PCR with TaqMan primer set human tenascin-C (Hs01115663-m1), fibronectin (Hs01549940_m1), TNF (Hs00174218-m1), and human ribosomal protein endogenous control (RPLPO) (4310879E) (Applied Biosystems, Foster City, CA) in a Corbett Rotor-gene 6000 machine (Corbett Research, Cambridge, U.K.). The relative abundance of tenascin-C and TNF normalized to RPLPO in the RA samples was analyzed by using two-standard curve method with identical top concentrations of 1 ng/μl and subsequent serial dilutions of 1/50 for all target genes. Tenascin-C protein was detected in cell supernatants by direct ELISA using Ab AF3558 (R&D Systems) and by SDS-PAGE and Western blotting using Ab MAB1908 (Millipore, Bedford, MA).

Assessment of cytokine synthesis

Cell supernatants were examined for the presence of the cytokines TNF-α, IL-6, and IL-8 by ELISA (R&D Systems) according to the manufacturer's instructions. Absorbance was read on a spectrophotometric ELISA plate reader (Labsystems Multiscan Biochromic, Vantaa, Finland) and analyzed using the Ascent software program (Thermo Labsystems, Altrincham, U.K.).

Chromatin immunoprecipitation

MDDCs were stimulated for 0, 0.5, 2, and 4 h with LPS before chromatin immunoprecipitation analysis as described previously (28). Cross-linked samples were immunoprecipitated using the RNA Pol II Ab sc-899× (Santa Cruz Biotechnology, Santa Cruz, CA) and underwent quantitative PCR using a primer pair specific to the transcription start site of tenascin-C (forward, 5′-gcaaatgggttccttccctggccga-3′, and reverse, 5′-aaggatgtctggaggcgaggcgt-3′).

Inhibitors

MDDCs or HEK cells were stimulated for 4 or 24 h with 10 ng/ml or 1 μg/ml LPS, respectively, in the presence or absence of DMSO, cyclohexamide (10 μg/ml) (Sigma-Aldrich, St. Louis, MO), actinomycin D (0.5 μg/ml), LY294002 (10 μM), wortmannin (100 nM), SH5 (1 μM), SB203580 (10 μM), SP600125 (10 μM), UO126 (10 μM) (Calbiochem, San Diego, CA), or TPCA1 (5 μM) (a gift from GlaxoSmithKline, Research Triangle Park, NC). Tenascin-C mRNA and protein levels were assessed at 4 or 24 h, respectively. Viability of the cells was not significantly affected throughout each experiment as examined by MTT assay (Sigma-Aldrich). Kinase inhibitors were used at the lowest dose that gave maximal inhibition of phosphorylation in human MDDCs assessed by Western blotting using phospho-specific Abs to each target.

Plasmids, transfections, and infections

The sequences and restriction maps of the NF-κB expression constructs in the pENTR vector (Invitrogen) modified to contain the CMV promoter and IRES-linked GFP are available upon request. The constructs were recombined into pAD/PL DEST vector (Invitrogen) for adenovirus production and delivery into MDDCs. The construct expressing mutant IκBα S32,36A has been described previously (29). HEK cells were transfected with equal amounts of expression plasmids (empty vector [pENTR] or RelA or mutant IκBα S32,36A) using Lipofectamine 2000 (Invitrogen). MDDCs were infected with adenovirus expressing empty vector (pENTR) or RelA or mutant IκB S32,36A. Forty-eight hours posttransfection or infection, cells were left untreated or were stimulated with 1 μg/ml (HEKs) or 10 ng/ml (MDDCs) LPS for 4 or 24 h before assessing tenascin-C levels by quantatative PCR or ELISA. Short interfering RNA-mediated protein knockdown in HEK cells was performed using On-target plus SMART pool reagents (Dharmacon, Lafayette, CO) and Lipofectamine RNAiMAX (Invitrogen).

Bioinformatic analysis

Genomic sequences were obtained using the publicly available UCSC hg18 human genome assembly (http://genome.ucsc.edu/). The ECR Browser (http://ecrbrowser.dcode.org/) was used to analyze sequence conservation in the tenascin-C genomic locus. The nucleotide sequences were inspected with JASPAR transcription factor binding sites searching software

(http://jaspar.cgb.ki.se/) for the presence of putative NF-κB sites (JASPAR matrix MA0061 with profile score threshold of 0.75) (30).

Statistical methods

Mean, SD, SEM, and statistical significance were calculated using GraphPad version 3 (GraphPad, San Diego, CA). Multiple group means were analyzed by one-way ANOVA, followed by the Dunnett or the Tukey's multiple comparison test where appropriate. A two-tailed, unpaired *t* test or Mann-Whitney *U* test was used for experiments involving only two groups and a two-tailed, paired *t* test to compare two paired groups. To quantify the association between two variables a Spearman correlation with 95% confidence interval was used. Values of $p < 0.05$ were considered significant.

Results

Tenascin-C expression correlates with disease activity in RA synovia

Within the RA synovium, tenascin-C has been reported to be specifically but widely distributed; it localizes to the invading pannus, superficial and deep cells of the synovial layer, subsynovial tissue, blood vessels, lymphoid aggregates, and areas of fibrosis (15, 18–20). We confirmed this pattern of localization by immunohistochemical staining of synovial membranes isolated from RA patients undergoing joint replacement. Tenascin-C was observed in the synovial and subsynovial layers around blood vessels and in areas of cellular infiltration (Fig. 1*A*). No staining was observed in negative control sections stained with nonspecific rabbit serum or where the primary Ab was omitted (data not shown). We also quantified the amount of tenascin-C synthesized by RA synovial membrane cells. Total cells isolated from the synovia obtained from RA patients exhibited significantly higher levels of tenascin-C protein than cells isolated from noninflamed synovia obtained from OA patients (Fig. 1*B*, 1*C*). The predominant form of tenascin-C upregulated in RA synovia was 320 kDa in size (Fig. 1*C*). During the progression of RA, inflammatory cytokines, such as IL-6, IL-8, and TNF drive, prolonged inflammation and joint destruction (31). High levels of tenascin-C protein in RA synovia significantly correlated with high levels of IL-6 (Fig. 1*D*), IL-8, and TNF (data not shown).

These data are consistent with published reports that show widespread localization of tenascin-C at specific loci throughout inflamed synovia and further demonstrate quantitatively higher levels of tenascin-C in inflamed versus noninflamed synovia. These data also provide evidence that high levels of tenascin-C protein in the RA joint correlate with high levels of markers of disease activity. Taken together, these results confirm that tenascin-C is an important pathological factor in RA.

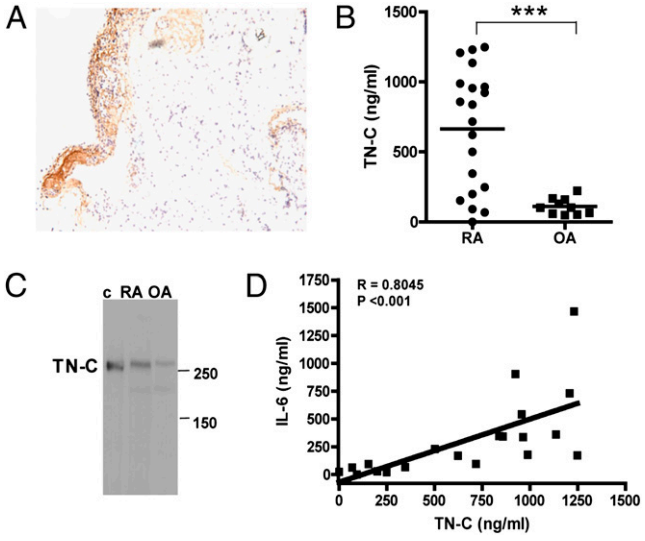

FIGURE 1. Localization and quantification of tenascin-C in RA synovium. *A*, Immunohistochemical localization of tenascin-C in the inflamed RA synovium (original magnification ×10). *B*, Tenascin-C protein in a mixed population of cells isolated from RA or OA synovial membranes (RA, $n = 20$; OA, $n = 10$; ***$p < 0.001$). *C*, Representative Western blot of one RA and one OA patient (c, purified recombinant human tenascin-C). *D*, Correlation of tenascin-C protein with the synthesis of IL-6 in a mixed population of cells isolated from RA synovial membranes ($n = 20$).

Identification of tenascin-C–producing cells within RA synovia

To determine whether cells resident in the synovium act as a source of tenascin-C, we quantified tenascin-C mRNA in mixed populations of cells extracted from inflamed synovial membranes of RA patients. High levels of tenascin-C mRNA comparable to levels of the largely abundant ribosomal gene *RLPO* were consistently observed in these cells, and tenascin-C mRNA was significantly more abundant than TNF mRNA relative to levels of *RPLO* (Fig. 2*A*).

The RA synovium comprises a mixture of cells of many different lineages, including fibroblast-like cells, myeloid cells, and lymphocytes. To determine which cell types among these are responsible for synthesizing tenascin-C, we purified fibroblasts, myeloid cells, and lymphocytes from the total population of cells isolated from RA membranes and analyzed levels of tenascin-C mRNA in each. Both myeloid cells and fibroblasts were major contributors of tenascin-C synthesis, whereas mRNA levels in lymphoid cells were relatively low (Fig. 2*B*). These data are consistent with published data demonstrating tenascin-C expression in synovial fibroblasts (19) but also show for the first time that myeloid cells synthesize significant levels of tenascin-C mRNA within the RA synovium.

Transient induction of tenascin-C expression in acute inflammation

Tenascin-C expression specifically colocalizes to areas of immune myeloid cell activation during the acute innate immune response upon infection and injury, in addition to the tumor stroma and in autoimmune diseases, such as RA (17). As such, these cells may be a major source of tenascin-C at global sites of inflammation, not just in the RA synovium. However, nothing is currently known about how tenascin-C expression is induced and regulated in cells of the myeloid lineage.

To further examine tenascin-C expression in immune myeloid cells during inflammation, we used a model of acute inflammation. Primary human monocytes, MDMs, and MDDCs were activated by the bacterial cell wall component LPS. Nonactivated cells expressed little tenascin-C, but expression significantly increased in response to stimulation with LPS. Levels of mRNA peaked between 4 and 8 h, returning to basal levels by 24 h (Fig. 3*A*). Although the absolute levels of mRNA induction varied between different donors

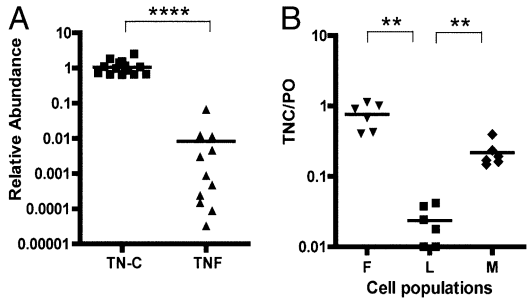

FIGURE 2. Quantification of tenascin-C mRNA in specific subsets of synovial cells. *A*, Tenascin-C and TNF mRNA in a mixed population of cells isolated from RA synovial membranes normalized to levels of RLPO ($n = 11$; ****$p < 0.0001$). *B*, Tenascin-C mRNA in specific populations of cells isolated from RA synovial membranes; fibroblasts (F), lymphoid cells (L), and myeloid cells (M), normalized to levels of RLPO ($n = 6$; **$p < 0.01$). F, fibroblast; L, lymphoid cell; M, myeloid cell.

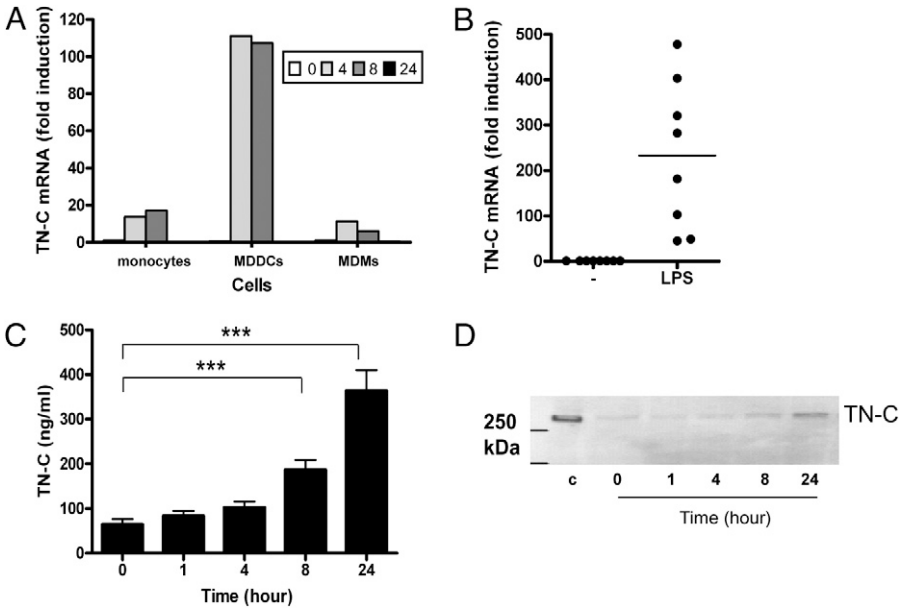

FIGURE 3. Tenascin-C expression is induced in activated myeloid cells. *A*, Tenascin-C mRNA in myeloid cells from a single donor stimulated with LPS (10 ng/ml) for 0, 4, 8, or 24 h, normalized to ribosomal protein PO levels and to the level of gene expression in nonactivated monocytes, which were assigned the value of 1 (ΔΔCt method). *B*, Tenascin-C mRNA in MDDCs from eight different donors without (−) or with LPS stimulation (LPS) for 4 h. *C*, Tenascin-C protein in MDDCs after stimulation with LPS for 0, 1, 4, 8 or 24 h. Data shown are the mean values from four independent donors (± SEM; ***$p < 0.001$). *D*, Representative Western blot of tenascin-C in supernatants of MDDCs (c, purified human tenascin-C).

(Fig. 3*B*), the kinetics of tenascin-C expression in all three cell types from all donors was identical. The highest level of induction was observed in MDDCs, and this was consistently ~10-fold higher than in monocytes and MDMs. Expression of the ECM glycoprotein fibronectin was not induced by LPS in any cell type (data not shown), consistent with published data (32). Increased tenascin-C mRNA correlated with tenascin-C protein synthesis. Nonactivated MDDCs secreted low levels of tenascin-C, which significantly increased between 8 and 24 h after stimulation (Fig. 3*C*). Tenascin-C was secreted as a single band of molecular mass 320 kDa (Fig. 3*D*), similar to the predominant form observed in RA samples.

These data demonstrate that tenascin-C expression in immune myeloid cells is transiently induced in acute inflammation. This is in stark contrast to the sustained expression we observed in chronically inflamed tissues, such as the RA synovium, indicating that abnormal regulation of the tenascin-C gene occurs in pathological inflammation. We therefore further investigated how the expression of tenascin-C is regulated in immune myeloid cells to begin to understand how this may be compromised in RA.

Tenascin-C expression is transcriptionally regulated during inflammation

To examine whether expression of tenascin-C in myeloid cells occurred at the level of gene transcription, we examined recruitment of RNA polymerase II (Pol II) to the transcription start site of tenascin-C in MDDCs stimulated with LPS. Pol II recruitment was observed as early as 30 min and steadily increased up to 4 h post-LPS stimulation (Fig. 4*A*). Furthermore, tenascin-C mRNA expression was significantly inhibited by actinomycin D (data not shown), confirming the role of transcriptional regulation in modulating the levels of tenascin-C expression. We also detected a significant reduction of tenascin-C in cycloheximide treated cells (Fig. 4*B*), indicating that its expression requires de novo protein synthesis. These data place tenascin-C in the group of secondary inflammatory response genes (that includes IL-6 and IL-12), which require new protein synthesis for both transcription and nucleosome remodeling (33).

Specific inflammatory stimuli induce tenascin-C expression in primary human MDDCs

We investigated the mechanism of tenascin-C expression in primary human MDDCs, which expressed the highest levels of tenascin-C upon activation by LPS. LPS stimulates myeloid cell surface TLR4 (34). Tenascin-C expression could also be induced by stimulation of TLR1/2 by LTA, TLR2/6 by PAM3, and TLR5 by flagellin but not by stimulation of TLR3 by poly(I:C) or TLR7/8 by R848 (Fig. 5). These data suggest that in addition to LPS, expression of tenascin-C can be induced by activation of other types of specific exogenous pathogenic stimuli, suggesting the involvement of common signaling pathways.

Activation of PI3K/AKT and NF-κB–mediated signaling pathways drives tenascin-C expression

Distinct patterns of gene expression are induced upon TLR activation. This is mediated by selective activation of specific signaling pathways and transcription factors (1). Among the major pathways activated during inflammation and shared by TLR1/2, TLR2/6, TLR4 and TLR5, are those driven by PI3K/AKT, MAPK, or NF-κB signaling (35). We used inhibitors of each of these three pathways to determine which, if any, are required for transcription of the tenascin-C gene upon activation of MDDCs.

The induction of tenascin-C expression by LPS was significantly inhibited by the PI3K-specific inhibitors LY294002 (Fig. 6*A*) and wortmannin (data not shown) and by the AKT inhibitor (SH5) at

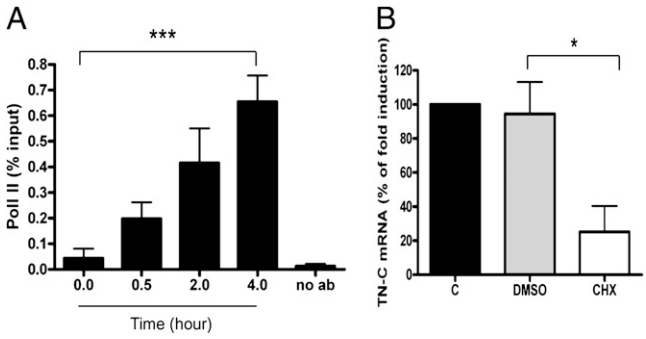

FIGURE 4. Tenascin-C expression is transcriptionally regulated by LPS. *A*, Chromatin immunoprecipitation analysis of Pol II recruitment in MDDCs following stimulation with LPS. Data shown are the mean of three independent donors (± SEM; ***$p < 0.001$). *B*, Tenascin-C mRNA in MDDCs stimulated with LPS for 4 h with no additional treatment (c) or with addition of DMSO or cyclohexamide (CHX). Data are expressed as the percentage of stimulated control cells (c) and are the mean values from three different donors (± SEM; *$p < 0.05$). c, control cell; CHX, cyclohexamide.

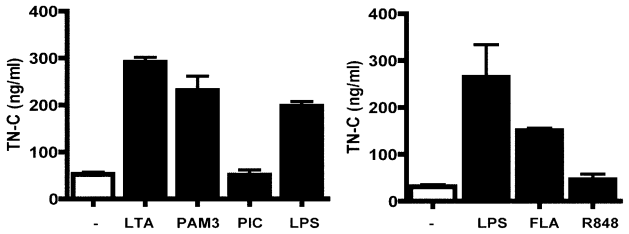

FIGURE 5. Induction of tenascin-C expression by specific inflammatory stimuli in MDDCs. Tenascin-C protein in MDDCs either left unstimulated (−) or after stimulation with LTA, PAM3, LPS, poly(I:C) (PIC), flagellin (fla), or R848 for 24 h. Data are shown as the average of triplicate values from a single representative experiment from a total of three independent donors (± SD). fla, flagellin; PIC, poly(I:C).

both the protein (Fig. 6*A*) and mRNA level (data not shown). DMSO had no significant effect on either tenascin-C mRNA or protein. Inhibition of MAPKs using the p38 inhibitor SB203580, the JNK inhibitor SP600125 and downstream inhibition of ERK1 and 2 using the MEK inhibitor UO126 did not significantly affect tenascin-C expression (Fig. 6*A*). However, TPCA1, an inhibitor of IκB kinase 2 (36) significantly inhibited tenascin-C protein (Fig. 6*A*) and mRNA (data not shown) synthesis. Taken together, these data suggest that activation of PI3K/AKT and NF-κB signaling pathways contribute to the induction of tenascin-C expression in activated MDDCs but that signaling by MAPKs is not required. Moreover, overexpression of the NF-κB subunit RelA in nonactivated primary human MDDCs enhanced tenascin-C mRNA (Fig. 6*B*) and protein (data not shown) synthesis, whereas expression of a dominant-negative (superrepressor) mutant of IκB inhibited LPS-induced tenascin-C expression by ~75% (Fig. 6*C*).

To further study the transcriptional regulation of tenascin-C, HEK293 cells overexpressing TLR4/CD14/MD-2 were stimulated with LPS. Upon activation this cell line upregulates the synthesis of proinflammatory mediators (37) and tenascin-C (Supplemental Fig. 1*A*). Induction of tenascin-C expression in HEK293s was inhibited by LY294002, wortmannin, SH5, and TPCA1 and was unaffected by p38, JNK, and MEK inhibitors (data not shown) suggesting that the same transcriptional machinery drives tenascin-C expression in both MDDCs and HEKs in response to LPS stimulation. In addition, expression of the dominant-negative (superrepressor) mutant of IκB inhibited LPS-induced tenascin-C expression (Supplemental Fig. 1*B*) as effectively as the known NF-κB–dependent gene TNF (data not shown). Knocking the expression of the NF-κB subunit RelA down to ~15% using short interfering RNAs also inhibited TNF (data not shown) and tenascin-C expression (Supplemental Fig. 1*C*), whereas over expression of RelA stimulated TNF (data not shown) and tenascin-C expression (Supplemental Fig. 1*D*) in nonstimulated cells.

These data confirm that specific activation of both PI3K/AKT and NF-κB signaling pathways, but not MAPKs, are important for the induction of tenascin-C expression by LPS.

Identification of putative NF-κB binding sites in conserved noncoding regions of the tenascin-C gene locus

We demonstrated that tenascin-C expression in MDDCs is regulated on the transcriptional level. The human tenascin-C gene is located on chromosome 9q32-q34 (116954270–116708662) (38) where it is flanked by the DEC1 and TNFSF8 genes. It comprises 30 exons separated by 29 introns (39–41). The entire coding region (exons 2–30) is restricted to a ~7.5-kbp segment of this locus; this constitutes only 3.6% of the overall gene, which spans ~211 kbp. The exons range in size from 90 to 1410 bp and the introns from 578 to 26,827 bp (42). The proximal 220 bp upstream of the transcription start site in exon 1 is sufficient for the basal expression of tenascin-C gene reporter constructs indiscriminately in cell lines producing (SK-MEL-28 human melanoma) or not producing (hamster R1-G9 glucagonoma and human FL ovary carcinoma) endogenous tenascin-C (39). However, nothing is known about the *cis*-regulatory elements responsible for inducible transcription of the tenascin-C gene in primary human myeloid cells during inflammation.

One feature of functional regulatory elements is their strong evolutional conservation (43). We inspected the organization of the entire ~211 kbp of the tenascin-C genomic locus and identified multiple conserved noncoding sequences (CNS) throughout this region (Fig. 7*A*). Next, we examined in more detail the region 5′ upstream of the tenascin-C transcriptional start site as well as the first intron, a large 26,827-bp region that separates the untranslated

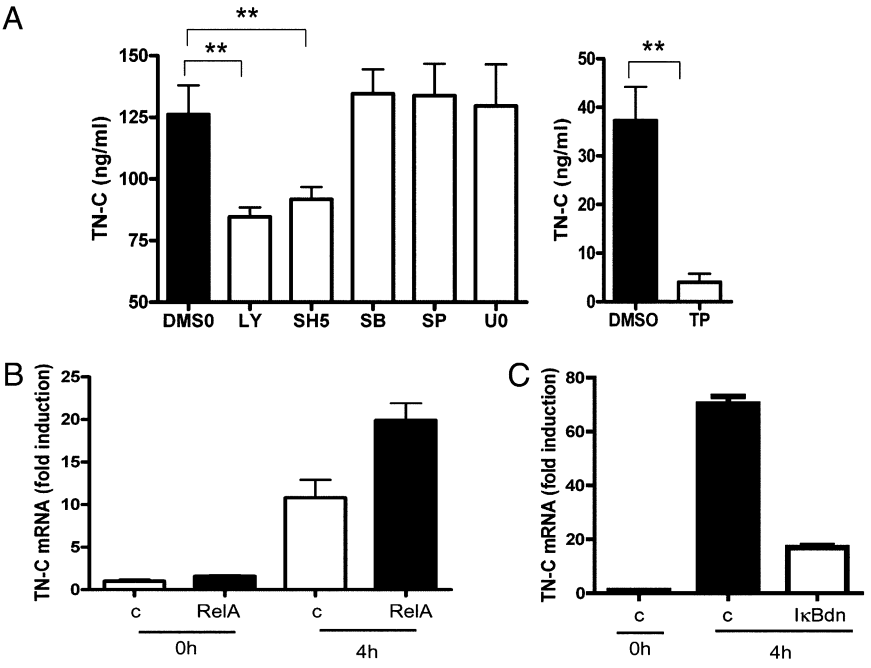

FIGURE 6. Blocking PI3K/AKT and NF-κB signaling pathways inhibits tenascin-C induction in MDDCs. *A*, Tenascin-C protein in MDDCs stimulated with LPS for 24 h in the presence of DMSO, LY294002 (LY), SH5, SB203580 (SB), SP600125 (SP), UO126 (UO), or TPCA1 (TP). Data are the mean values from three different donors (± SEM; $^{**}p \leq 0.01$). *B*, Tenascin-C mRNA in LPS-stimulated MDDCs infected with adenovirus expressing control vector (c) (□) or RelA (■) at a multiplicity of infection of 50. Data shown are the mean fold induction of mRNA compared with control at 0 h (± SD) of a representative of independent experiments from three different donors. *C*, Tenascin-C mRNA in unstimulated MDDCs (0 h) or LPS-stimulated MDDCs (4 h) infected with adenovirus expressing control vector (c) or dominant-negative IκB (IκBdn) at a multiplicity of infection of 50. Data shown are the mean fold induction of mRNA compared with control without LPS (± SD) of a representative of independent experiments from three different donors. c, control vector, IκBdn, dominant-negative IκB; LY, LY294002; SB, SB203580; SP, SP600125; TP, TPCA1; UO, UO126.

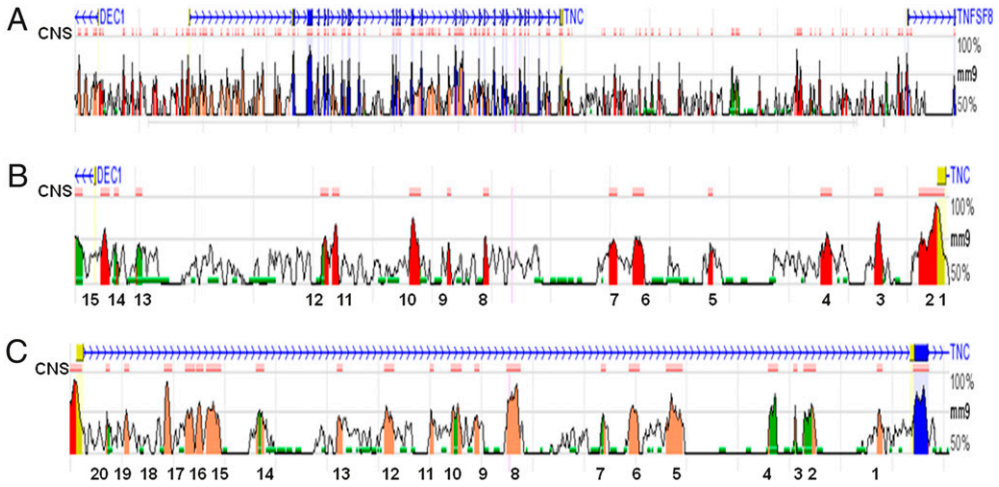

FIGURE 7. Organization of the tenascin-C genomic locus. Evolutionarily conserved regions in the whole tenascin-C genomic locus (*A*), within the 5′ upstream region of the tenascin-C gene (*B*), or located in the first intron of the tenascin-C gene (*C*) mapped in relation to known genes within alignments of the human and mouse genomes. Conserved noncoding sequences (CNS) were defined as noncoding sequences of ≥100 bp with 75% of identity between the genomes. Conserved noncoding sequences are depicted as pink boxes above the gene sequence (*A–C*) and are numbered below the gene sequence (*B* and *C*). Color key: exons, blue; introns, salmon; UTR, yellow; intergenic, red; and transposons and simple repeats, green.

first exon from the translational start site in exon 2. This intron has previously shown to contribute to the cell-specific expression of tenascin-C in human melanoma and carcinoma cell lines (39). We identified 15 conserved noncoding sequences in the 5′ upstream region (Fig. 7*B*, pink boxes), whereas 20 conserved noncoding sequences were located in the first intron of the gene (Fig. 7*C*, pink boxes). To elucidate which conserved noncoding sequences may be involved in NF-κB regulation of tenascin-C expression during inflammation, we computationally mapped putative NF-κB binding sites within each conserved noncoding sequence: 15 were identified in the 5′ upstream region (Table I) and 18 in the first intron (Table II). These data indicate that a large number of putative *cis*-regulatory elements exist within the tenascin-C gene.

Discussion

Exerting tight control over tissue levels of inflammatory mediators is key to an effectively regulated immune response. ECM molecules that are specifically upregulated in response to tissue damage act as danger signals that drive inflammation to mediate tissue repair. Although many studies support a role for the inappropriate synthesis of these DAMPs in driving chronic inflammation (12, 13), little is known about how their expression is induced or regulated. This study has identified a novel autocrine mode of action for the proinflammatory ECM glycoprotein tenascin-C that is tightly controlled during acute inflammation but that proceeds unchecked during destructive joint inflammation. In particular, we show that primary human immune myeloid cells are a major source of tenascin-C during inflammation. Furthermore, the high levels of tenascin-C expression in MDDCs imply that, in addition to its established function in stimulating innate immunity, it may also play a role in mediating adaptive immunity.

We have demonstrated that tenascin-C expression in MDDCs is regulated at the level of gene transcription. Little is known about the mechanisms of tenascin-C transcription. Gene reporter studies have demonstrated that the proximal 220 bp upstream of the transcription start site plays a role in basal transcription (39). The activity of this basal promoter can be both positively and negatively modulated by noncoding sequences; the untranslated first exon contains two discrete regions that repress or enhance transcription of the reporter in human glioblastoma cells lines (44). In addition, as with many ECM genes, including collagen type I (45) and aggrecan (46), cell-specific transcription of tenascin-C is directed by the cooperation of intronic

Table I. *Putative NF-κB binding sites in the 5′ region of the tenascin-C gene*

CNS No.	Locus	Relative Score	Sequence	Strand
1	116920308	0.7857	GAGGCGTCCC	R
1	116920361	0.7963	GGGCTGTTCC	R
1	116920465	0.8234	GGGAATTCCT	R
2	116920745	0.7944	GGGGGTTTCA	F
3	116921904	0.771	TGGGCTTTCT	R
3	116921986	0.7602	GGGAAGTTTT	F
6	116928610	0.7613	GGAGCTTTGC	F
6	116928748	0.7805	GAGGATTCAC	F
7	116929323	0.7507	GGGAGTGCTC	F
7	116929376	0.8431	TGGATTTCCC	F
7	116929425	0.8244	GGGGATTATC	R
9	116934041	0.7612	CAGACTTTCC	R
10	116934902	0.82	GGGGAGACCC	F
10	116935028	0.8221	GAGGTTTCCC	R
12	116937562	0.7898	GGAAGATTCC	R

CNS, conserved noncoding sequence.

Table II. *Putative NF-κB binding sites in intron 1 of the tenascin-C gene*

CNS No.	Locus	Relative Score	Sequence	Strand
5	116901141	0.7743	GGGTCTTCGC	R
5	116900942	0.8041	AGGAATTTCT	R
5	116900774	0.8418	GGAGAATTCC	F
6	116902288	0.7621	AGTCTTTCC	F
6	116902206	0.7711	AGGGCTTTCT	F
10	116907989	0.7706	CGAGATTTCC	R
11	116908932	0.7684	TGGGCTTCAC	F
12	116910104	0.8136	GCGAGTTTCC	F
13	116911727	0.7568	GGGGATTTGA	R
14	116914274	0.7721	GGGACAATCT	R
15	116915891	0.96	GGGATTTTCC	F
16	116916321	0.9754	GGGGATTCCC	F
16	116916236	0.939	GGGGTTTTCC	F
17	116916727	0.8702	GGGGTTCTC	F
17	116916608	0.778	TGAACTTTCC	F
19	116918637	0.7815	GGGAAAGCTC	F
20	116919311	0.7663	GGGGTTCTTC	R
20	116919245	0.7804	GGAGAATACC	F

CNS, conserved noncoding sequence.

enhancer elements with the basal promoter; the first intron contributes to cell-type specific inducible expression in human melanoma cell lines (39). Our inspection of the tenascin-C genomic locus revealed that this gene spans a large area of chromosome 9, although the coding region made up a relatively low proportion of this area. We identified many highly conserved regions within the noncoding areas of the tenascin-C gene. These spanned the entire gene locus from the 5′ to the 3′ flanking genes, indicating that expression of tenascin-C may be subject to complex regulatory mechanisms by multiple sequences throughout the whole locus. This may explain how the specific temporal and tissue-specific pattern of expression reported for tenascin-C during development and in the adult (reviewed in Refs. 16 and 17) is mediated. Determining how cell-specific transcription is orchestrated within this large locus will require further systematic analysis of functional noncoding sequences.

The induction of tenascin-C in MDDCs is NF-κB dependent; this is the first transcription factor identified that is able to drive endogenous tenascin-C expression. Transfection of c-Jun into rat embryonic fibroblasts activated a construct comprising the −220 to +79-bp fragment of the basal promoter, and this required synergistic binding of NF-κB and c-Jun (47), supporting the importance of NF-κB in controlling tenascin-C transcription. We found 33 putative NF-κB binding sites in conserved noncoding sequences within the 5′ region of the tenascin-C gene and in the first intron (known to be important for cell-specific induction of the gene); putative binding sites in other conserved noncoding sequences may also exist. In addition, the requirement for de novo protein synthesis for tenascin-C mRNA expression and relatively delayed kinetics of Pol II recruitment to its transcription start site suggests a possible role for chromatin remodeling in the regulation of tenascin-C in MDDCs. Future work will focus on systematically dissecting any epigenetic mechanisms that regulate tenascin-C gene expression during acute inflammation in human myeloid cells.

In MDDCs, tenascin-C expression was induced by specific components of the bacterial cell wall, including LTA, PAM3, and LPS, as well as bacterial flagellin, indicating that tenascin-C expression can be directly induced in response to pathogenic invasion. Synthesis of tenascin-C may also be induced as a direct consequence of tissue damage or mechanical strain. Loading the anterior latissimus dorsi muscle in the chick wing (48) or the soleus muscle in the rat hind limb (49) specifically, rapidly, and reversibly induced high levels of tenascin-C mRNA. Cyclical stretch of isolated chicken fibroblasts and neonatal rat ventricular cardiac myocytes also induces tenascin-C transcription (50, 51). In addition, denatured, but not native, type I collagen has been shown to induce tenascin-C protein in fetal rat smooth muscle cells (52). Taken together, these data suggest that tenascin-C expression may be triggered as an immediate and direct result of tissue injury or infection.

Our data suggest that signaling molecules that are spatially restricted to the plasma membrane may be involved in driving tenascin-C expression. Tenascin-C synthesis was specifically induced by TLRs that can be located at the cell surface; TLR2, 4, and 5, but not to the endosome; and TLR3 or TLR8. Compartmentalization of TLRs is key to defining the stimulation of specific downstream pathways by recruitment of distinct adaptor molecules and associated factors (reviewed in Ref. 53). We also showed that only a specific subset of pathways that are activated by stimulation of TLR4 by LPS are needed to drive tenascin-C synthesis; activation of AKT/PI3K was essential, but activation of MAPK was not required. Our results highlight the fact that synthesis of individual proinflammatory mediators is induced by activation of specific signaling pathways downstream of TLR4. For example, TLR4-induced production of TNF, but not IL-6, requires the activity of p38 MAPK (54) and suppressor of cytokine signaling-1 selectively inhibits LPS-induced IL-6 production but not the production of TNF, G-CSF, or IFN (55). Unraveling the specificity of these pathways may lead to the ability to selectively modulate tissue levels of different proinflammatory mediators.

Taken together, data from this study have demonstrated that tenascin-C expression is rapidly and transiently induced in immune myeloid cells upon activation in response to tissue injury and infection. Synthesis of this proinflammatory mediator contributes to orchestrating an immune response designed to eliminate the pathogen and/or repair damaged tissue. This proceeds via an autocrine loop whereby expression of tenascin-C is induced upon activation of cell surface TLRs, and subsequent activation of TLR4 by tenascin-C drives the synthesis of proinflammatory cytokines. Under normal circumstances, tenascin-C expression is downregulated before repair is complete, consistent with controlled resolution of inflammation. However, in RA, persistent gene expression leads to high tissue levels of tenascin-C that would then drive a nonresolving loop leading to chronic inflammation. Further information about how tissue levels of DAMPs, such as tenascin-C, are controlled may yield strategies to block inappropriate inflammation in autoimmune diseases and cancer.

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Disclosures

The authors have no financial conflicts of interest.

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