Endogenous Control of Immunity against Infection: Tenascin-C Regulates TLR4-Mediated Inflammation via MicroRNA-155

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SUMMARY

Endogenous molecules generated upon pathogen invasion or tissue damage serve as danger signals that activate host defense; however, their precise immunological role remains unclear. Tenascin-C is an extracellular matrix glycoprotein that is specifically induced upon injury and infection. Here, we show that its expression is required to generate an effective immune response to bacterial lipopolysaccharide (LPS) during experimental sepsis in vivo. Tenascin-C enables macrophage translation of proinflammatory cytokines upon LPS activation of toll-like receptor 4 (TLR4) and suppresses the synthesis of anti-inflammatory cytokines. It mediates posttranscriptional control of a specific subset of inflammatory mediators via induction of the microRNA miR-155. Thus, tenascin-C plays a key role in regulating the inflammatory axis during pathogenic activation of TLR signaling.

INTRODUCTION

The danger theory of immunity changed the way we think about host defense; defining the immune system as the primary response not only against infection but also against sterile tissue injury (Matzinger, 1994). The identification of evolutionarily conserved “pattern-recognition” receptors (PRRs) and their ability to sense “danger signals” generated upon infection (pathogen-associated molecular patterns [PAMPs]) and tissue injury (damage-associated molecular patterns [DAMPs]), and in response to activate inflammatory signaling pathways, has further delineated the molecular machinery that drives immunity (Beutler, 2007; Gordon, 2002; Medzhitov and Janeway, 2002).

DAMPs have been defined as endogenous molecules specifically generated upon tissue damage. They comprise a variety of proteins, proteoglycans, and fatty acids, including intracellular molecules released from necrotic cells, extracellular matrix (ECM) fragments created by tissue destruction, and ECM molecules whose expression is specifically induced upon tissue injury (Bianchi, 2007; Gordon, 2002). Physiologically committed to alerting the body to damage and initiating tissue repair, even in the absence of microbial invasion, DAMPs can directly induce an inflammatory response via activation of PRRs (Chen and Nunez, 2010). They do so in a manner distinct to PAMP-mediated activation of PRRs in order to drive unique cellular responses (Midwood et al., 2009; Osterloh et al., 2007; Silva et al., 2007).

However, DAMPs are also generated upon microbial infection. An emerging feature of infectious disease is that levels of endogenous danger signals typically associated with tissue injury are also rapidly elevated upon pathogen invasion. For example, the nuclear DNA-binding protein HMGB1 is promptly released from infected cells (Lotze and Tracey, 2005), and expression of the glycoprotein tenasin-C is induced upon infection, often appearing before any obvious sign of tissue damage (Midwood and Orend, 2009). Although levels of DAMPs are tightly regulated during acute inflammatory responses to infection and are cleared after the microbial threat has been eliminated, persistently high levels occur in patients with sepsis (Piccinini and Midwood, 2010; van Zoelen et al., 2009; Yang et al., 2004), the clinical manifestation of a dysregulated immune response to infection, which accounts for ~9% of the overall annual mortality in the USA (Angus et al., 2001). Consistent with these data, PAMPs can directly induce the expression of endogenous danger signals in vitro. For instance, respiratory syncytial virus infection induces intracellular Hsp72 secretion in airway epithelial cells (Wheeler et al., 2009). Furthermore, tenascin-C, S100A8/S100A9, biglycan, and HMGB1 are secreted by macrophages following stimulation with Gram-negative bacterial lipopolysaccharide (LPS) (Ehren et al., 2009; Goh et al., 2010; Schaefer et al., 2005; Wang et al., 1999). These data imply a role for DAMPs not only in mediating tissue repair upon injury but also in driving inflammation during pathogenic infection. During microbial invasion, these molecules may amplify inflammatory signals by directly activating PRRs (El Mezayen et al., 2007; van der Poll and Opal, 2008); however, their role in infection-induced inflammatory signaling remains largely undefined.

Here, we identify a unique role for the danger signal tenasin-C in regulating immunity mediated by toll-like receptor 4 (TLR4) in response to pathogenic infection. Tenasin-C is an ECM glycoprotein that is absent in most healthy adult tissues but that is specifically expressed upon tissue injury (Chiquet-Ehrismann and Chiquet, 2003) as well as during infection (Kaarteenaho-Wiik et al., 2000; Päälosaho et al., 1993). Levels are high in...
patients with sepsis (Schenk et al., 1995). In vitro, LPS activation of TLR4 transiently induces tenascin-C expression in human myeloid cells (Goh et al., 2010). Our data show that tenascin-C is required for the proinflammatory response to LPS in vivo. Targeted deletion of tenascin-C impairs TNF-α production and subsequent downstream cytokine synthesis, in response to LPS in septic mice and bone marrow-derived macrophages (BMDMs). We also provide mechanistic insights into tenascin-C-dependent signaling downstream of LPS activation of TLR4 and demonstrate that tenascin-C operates posttranscriptionally, modulating levels of TNF-α translation. Moreover, we found that tenascin-C mediates this control through the induction of miR-155, a microRNA (miRNA) whose expression is strongly induced by LPS in macrophages (O’Connell et al., 2007; Tili et al., 2007), and that positively regulates the production of TNF-α (Bala et al., 2011; Tili et al., 2007). Our study identifies tenascin-C as a regulator of the early immune response and shows that an effective and coordinated inflammatory response to pathogenic activation of TLRs requires the participation of endogenous danger signals.

RESULTS

Tenascin-C Is Required for the Proinflammatory Response to LPS In Vivo

To examine the role of tenascin-C in host defense against pathogenic infection, we induced sepsis with a sublethal dose of LPS in tnc+/+ and tnc−/− mice. The major clinical symptoms of sepsis, including weight loss, reduced mobility, uveitis, ruffled fur, and diarrhea, were evident in tnc−/− mice 1 hr after injection of LPS, in contrast to tnc+/+ mice, which showed no symptoms by 1.5 hr. Although symptoms became progressively more severe in tnc−/− mice 4 hr after injection, tnc−/− mice exhibited significantly less weight loss (p < 0.0001 at 4 hr; p = 0.0006 at 8 hr), less mobility impairment (p < 0.0001 at 4 and 8 hr), and less fur ruffling (p < 0.0001 at 4 and 8 hr) than tnc+/+ mice, with mild uveitis and diarrhea (Figures 1A, 1B, S1A, and S1B).

On the molecular level, sepsis is driven by a “cytokine storm” comprising elevated circulating levels of proinflammatory cytokines induced in response to LPS. Analysis of plasma levels of key inflammatory mediators of sepsis showed that tnc−/− mice synthesize significantly less TNF-α (p < 0.0001) and IL-6 (p < 0.0001) compared to tnc+/+ mice 1.5 hr after LPS injection, consistent with the lack of clinical symptoms in the absence of tenascin-C. By 4 hr, there was significantly less TNF-α (p = 0.001) in tnc−/− mice compared to tnc+/+ mice. Plasma TNF-α and IL-6 dropped by 8 hr similarly in both tnc−/− and tnc+/+ mice. Furthermore, tnc−/− mice fail to induce release of HMGB1, a late mediator essential for organ damage in sepsis (Wang et al., 1999), which was significantly increased in tnc+/+ mice 8 hr after LPS injection (Figure 1C).

Tissue damage following systemic LPS injection results from immune cell recruitment to susceptible sites, most notably, rapid neutrophil infiltration to lungs and liver. Immunohistochemical staining of neutrophil elastase in lungs and liver from tnc+/+ mice 1.5 hr after LPS injection demonstrated substantial neutrophil infiltration, which was significantly reduced in tnc−/− mice, an effect also evident at 4 and 8 hr after injection (Figures 1D, S1C, and S1D).

To determine whether tenascin-C expression is elevated in mice with LPS-induced sepsis, we quantified plasma tenascin-C in tnc+/+ mice after LPS injection. Immunoblot analysis and ELISA revealed increased tenascin-C in mice upon administration of LPS. Notably, the kinetics of tenascin-C expression was similar to the early proinflammatory cytokines TNF-α and IL-6, peaking 1.5 hr after LPS injection and decreasing over time (Figures 1E–1G). Together, these results indicate that tenascin-C expression is required for the proinflammatory response to LPS in vivo.

Bone Marrow Engraftment Rescues TNF-α Synthesis in Tnc−/− Mice

TNF-α was the cytokine most affected by tenascin-C ablation. To determine if the reduced plasma levels of TNF-α observed in tnc−/− mice are mediated by defects in their hematopoietic cell population, we performed allogeneic bone marrow transplantation. Whole bone marrow cells isolated from tnc+/+ or tnc−/− male mice were injected into sublethally irradiated tnc+/+ or tnc−/− female recipient mice. Tnca−/+/+, tnc+/−/−, tnc+/−/−, and tnc−/−/− chimeric mice were generated. All mice displayed equally high donor engraftment as shown by quantitative RT-PCR analysis of the mouse testis-specific Y-encoded protein (TSPY) gene in erythrocyte-depleted peripheral blood cells from recipient mice (Figure 2A). These data indicate that the majority of bone marrow cells are from the donor.

Analysis of tenascin-C mRNA in peripheral blood cells from tnc+/−/− mice 1.5 hr after LPS injection showed significantly reduced expression of tenascin-C compared to tnc+/−/− mice. The tenascin-C mRNA observed in tnc+/−/− mice is likely produced by residual bone marrow cells of the tnc+/− recipient. Replenishment of tnc−/− mice with tnc+/+ bone marrow resulted in significantly more tenascin-C expression compared to tnc−/−/− mice (Figure 2B).

TNF-α levels in plasma from tnc+/−/− chimeric mice were significantly lower (p = 0.0033) than those from tnc+/−/− chimeric mice 1.5 hr after LPS injection (Figure 2C). Accordingly, significantly more TNF-α (p < 0.0001) was found in plasma from tnc+/−/− chimeric mice compared to tnc−/−/− chimeric mice (Figure 2D). Eight hours after LPS injection, TNF-α was reduced almost to basal levels in each group. Collectively, these data indicate that tenascin-C derived from bone marrow-derived cells drives TNF-α synthesis during LPS-induced sepsis and that bone marrow-derived cells from tnc−/− mice exhibit defects in TNF-α synthesis in vivo.

LPS-Activated BMDMs Express Tenascin-C

We next investigated whether primary BMDMs obtained from tnc+/+ mice express tenascin-C in response to LPS over time. Nonstimulated cells showed low basal levels of tenascin-C expression, which significantly increased upon stimulation with LPS. Tenascin-C mRNA started to increase at 1 hr and peaked between 4 and 8 hr, returning to basal levels by 24 hr (Figure 3A), as observed in human myeloid cells (Goh et al., 2010). Increased tenascin-C mRNA correlated with tenascin-C protein synthesis.
Figure 1. *Tnc*<sup>−/−</sup> Mice Are Less Susceptible to LPS-Induced Sepsis

(A and B) Body weight (A) and mobility (B) loss in *Tnc*<sup>+/+</sup> and *Tnc*<sup>−/−</sup> mice upon LPS injection, monitored over 8 hr. Data in (A) are presented as percent loss relative to noninjected mice (n = 17 (A) or 10 (B) mice per group; mean ± SEM). ****p < 0.0001 and ***p < 0.001 compared to noninjected mice (A). Data are from three (A) or two (B) independent experiments.

(C) Time course analysis of TNF-α, IL-6, and HMGB1 in plasma from *Tnc*<sup>+/+</sup> and *Tnc*<sup>−/−</sup> mice after LPS injection by ELISA (n = 3–5 mice per group; mean ± SEM). ****p < 0.0001, ***p < 0.001, and ** p < 0.01. Data are representative of three independent experiments.

(D) Immunohistochemical analysis of neutrophil elastase+ cell infiltration in lungs from *Tnc*<sup>+/+</sup> and *Tnc*<sup>−/−</sup> mice 0, 1.5, 4, and 8 hr after LPS injection (n = 4 LPS-injected and 3 noninjected mice per genotype). Scale bars, 100 μm. Data are representative of three independent experiments.

(E and F) Immunoblot analysis of plasma tenascin-C (TN-C) in *Tnc*<sup>+/+</sup> (one to three) and *Tnc*<sup>−/−</sup> (four to six) mice 8 hr (E) and 0, 1.5, 4, and 8 hr (F) after LPS injection. Results are representative of three independent experiments.

(G) ELISA of plasma tenascin-C in *Tnc*<sup>+/+</sup> mice 0, 1.5, 4, and 8 hr after LPS injection (n = 7; mean ± SEM). Data are from two independent experiments. Related to Figure S1.
Nonstimulated BMDMs produced no detectable tenascin-C; however, cell-associated tenascin-C significantly increased after LPS activation, peaking 8 hr after stimulation (Figure 3B). Tenascin-C was also secreted into the medium where its levels significantly increased 24 hr after stimulation (Figure 3C). These data demonstrate that tenascin-C expression in BMDMs is transiently induced by LPS.

To verify that tenascin-C ablation does not affect macrophage development in vitro and in vivo, we assayed tnc+/− and tnc−/− BMDMs or freshly isolated peritoneal macrophages for CD11b, F4/80, and CD68 expression. Each maturation marker was equally highly expressed in tnc+/+ and tnc−/− cells (Figures S2A–S2O).

**Tenascin-C Posttranscriptionally Regulates LPS-Mediated Synthesis of Specific Cytokine Subsets in BMDMs**

To further examine the mechanism behind impaired proinflammatory cytokine synthesis in tnc−/− mice upon LPS treatment, we investigated the effects of tenascin-C on cytokine synthesis in BMDMs. There was no significant difference in IL-1β, IL-12, and TGF-β1 secretion between tnc+/+ and tnc−/− BMDMs. However, BMDMs obtained from tnc−/− mice secreted significantly less TNF-α, IL-6, and CXCL1, and significantly more IL-10, upon stimulation with LPS than tnc+/+ cells (Figure 4A).

The expression of TNF-α, IL-6, CXCL1, and IL-10 mRNA was induced in tnc+/+ BMDMs by LPS stimulation and was not significantly affected by tenascin-C ablation (Figure 4B). No difference in IL-12 and TGF-β1 mRNA expression was observed in tnc+/+ and tnc−/− BMDMs (data not shown). These results suggest that regulation of cytokine synthesis by tenascin-C occurs at a posttranscriptional level.

To ensure that the effect of tenascin-C on cytokine synthesis is not due to abnormal macrophage activation in vitro or in vivo, we analyzed the expression of activation markers in tnc+/+ and tnc−/− BMDMs or freshly isolated peritoneal macrophages. Equal expression of MHC II, CD40, or CD86 and the LPS receptor TLR4/MD2 was observed in tnc+/+ and tnc−/− BMDMs (Figures S3A–S3J) or peritoneal macrophages (data not shown).

**Tenascin-C Promotes Translation of Proinflammatory Cytokines**

Impaired proinflammatory cytokine production without any reduction in cytokine mRNA levels in tnc−/− cells led us to investigate whether tnc−/− BMDMs exhibit abnormal intracellular cytokine levels as a consequence of deficient secretion. There was no significant difference in cellular TNF-α, IL-6, and CXCL1 levels in LPS-stimulated tnc+/+ and tnc−/− BMDMs (Figure 5A). Cytokine levels in the cellular fraction did not account for the defective cytokine secretion by tnc−/− BMDMs, as shown by the sum of the amounts of cellular and secreted protein fractions (Figure 5B). These data demonstrate that proinflammatory cytokine cellular trafficking occurs normally in tenascin-C null BMDMs.

We next investigated whether tenascin-C affects proinflammatory cytokine translation. Brefeldin A, an inhibitor of ER-to-Golgi protein transport, resulted in intracellular accumulation of TNF-α, IL-6, and CXCL1 in tnc+/+ cells at 24 hr. In contrast, intracellular accumulation of TNF-α was abrogated in tnc−/− BMDMs. Significantly less IL-6 and CXCL1 also accumulated within the Golgi in tnc−/− BMDMs (Figure 5C). Together, these results provided the first clue that tenascin-C may be required for effective translation of LPS-induced proinflammatory cytokines in BMDMs.
Tenascin-C Drives LPS-induced miR-155 Expression

Protein translation is regulated on several levels, among which miRNAs are key regulators of protein synthesis. Recent data have highlighted the importance of miRNA-mediated control of LPS-induced inflammatory signaling pathways (O’Neill et al., 2011); therefore, we investigated whether tenascin-C affects miRNA activity. miRNA expression in tnc+/− and tnc−/− BMDMs stimulated with LPS for 8 hr was analyzed with an array containing 375 miRNAs. We found 35 miRNAs that were reproducibly up- or downregulated by LPS (Figure 6A). A number of miRNAs have emerged as important regulators of early-response genes such as miR-155, miR-125b, and let-7i (Figure 6B) as well as late-response genes, including miR-155 and miR-125b (Figure 6C). In tnc−/− BMDMs, LPS strongly induced miR-155 expression, which was significantly inhibited in tnc−/− cells (Figures 6A and 6B). Using quantitative RT-PCR, we confirmed these results and showed that miR-155 expression was rapidly induced by LPS, peaking at 24 hr and progressively declining in tnc−/+ BMDMs. Conversely, LPS-induced miR-155 expression in tnc−/− BMDMs was inhibited and did not recover (Figure 6D). Furthermore, analysis of the primary transcript pri-miR-155 in the same samples revealed no significant difference between tnc−/− and tnc−/+ BMDMs, which suggests that tenascin-C posttranscriptionally regulates LPS-induced miR-155 expression. In line with studies showing that miR-155 expression is induced immediately after LPS stimulation (O’Connell et al., 2007) and contributes to TNF-α production (Bala et al., 2011; Tili et al., 2007), changes in TNF-α levels and miR-155 expression significantly correlated in LPS-stimulated tnc−/+ BMDMs. Accordingly, low TNF-α levels correlated with low miR-155 expression in tnc−/− BMDMs (Figure 6E). This effect was specific because IL-6, CXCL1, and IL-10 synthesis did not correlate with miR155 expression (p = 0.233; data not shown). To determine whether these data are relevant in vivo, we examined the expression of LPS-induced miR-155 in the spleen of tnc−/+ and tnc−/− mice upon LPS injection. Tnc−/− mice expressed significantly less miR-155 than tnc−/+ mice (Figure 6F). Taken together, these data suggest that tenascin-C is required for the induction of miR-155 by LPS in BMDMs and in septic mice.

Tenascin-C Controls TNF-α Release via the Induction of miR-155

To determine whether reconstituting miR-155 expression rescues LPS-induced TNF-α production in the absence of tenascin-C, we overexpressed the mature miRNA in tnc−/− BMDMs. Cells were transiently transfected with a negative control precursor or miR-155 precursor, which mimics the endogenous precursor miR-155. Transfection of miR-155 precursor, but not control precursor, resulted in increased expression of mature miR-155. Stimulation of transfected tnc−/− BMDMs with LPS for 8 hr led to increased miR-155 expression, at a level similar to that induced by LPS in control tnc−/+ BMDMs (Figures 7A and S4A). We then analyzed the effect of miR-155 overexpression on cytokine release in tnc−/− BMDMs upon LPS stimulation. In the absence of LPS, low-basal cytokine synthesis was detected in untransfected and transfected cells. TNF-α production in tnc−/− BMDMs transfected with miR-155 precursor and stimulated with LPS for 8 hr was significantly higher than in LPS-activated tnc−/− cells transfected with control precursor and was equal to that of control tnc−/+ BMDMs (Figures 7B and S4B). Thus, miR-155 overexpression rescued TNF-α release in tnc−/− BMDMs in response to LPS. Together, these data provide evidence that tenascin-C posttranscriptionally controls the production of TNF-α in response to LPS via miR-155.

DISCUSSION

Since 1994, Polly Matzinger’s danger model, which suggested that the immune system is designed to combat danger mediated by infection and injury, rather than to merely recognize nonself, has revolutionized our understanding of how the innate immune system responds to pathogens. The danger model highlights the importance of recognizing nonself through the detection of nonself molecules and the activation of innate immune cells. This recognition leads to the production of cytokines, such as TNF-α, which play a critical role in orchestrating an effective immune response. Recent studies have shown that miRNAs are key regulators of protein synthesis and can mediate the expression of cytokines and other immune response genes. In this study, we investigated the role of tenascin-C in LPS-induced miR-155 expression and found that tenascin-C posttranscriptionally regulates the expression of miR-155.

Figure 3. Tenascin-C Expression Is Transiently Induced by LPS

(A) Quantitative RT-PCR analysis of tenascin-C mRNA in BMDMs stimulated for 1, 1.5, 4, 8, 24, and 48 hr with 100 ng/ml LPS. Results are presented relative to those of untreated BMDMs. Data are from seven independent experiments (mean ± SEM). ***p < 0.001 and **p < 0.01 compared to nonstimulated cells.

(B) ELISA of cellular tenascin-C in BMDMs stimulated for 1.5, 4, 8, 24, 48, and 72 hr with 100 ng/ml LPS. Results are normalized to the cell number. Data are from six independent experiments (mean ± SEM). ***p < 0.001 compared to nonstimulated cells.

(C) ELISA of secreted tenascin-C by BMDMs stimulated for 1.5, 4, 8, 24, 48, and 72 hr with 100 ng/ml LPS. Data are from six independent experiments (mean ± SEM). *p < 0.05 compared to nonstimulated cells.

See also Figure S2.
Figure 4. Tenascin-C Posttranscriptionally Regulates the Synthesis of Specific Cytokine Subsets in BMDMs

(A) ELISA of TNF-α, IL-6, CXCL1, IL-10, IL-1β, IL-12, and TGF-β1 secreted by tnc+/+ and tnc−/− BMDMs stimulated for 1.5, 4, 8, 24, and 48 hr with 100 ng/ml LPS. Data are from six to nine independent experiments (mean ± SEM). ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05.

(B) Quantitative RT-PCR analysis of TNF-α, IL-6, CXCL1, and IL-10 mRNA in tnc+/+ and tnc−/− BMDMs stimulated for 0.5, 1, 1.5, 4, 8, 24, and 48 hr with 100 ng/ml LPS. Results are presented relative to those of untreated BMDMs. Data are from four to five independent experiments (mean ± SEM).

See also Figure S3.
immune system works (Matzinger, 1994). Both PAMPs and DAMPs act as danger signals that trigger inflammation through activation of PPRs, including TLRs (Gordon, 2002). Established drivers of sterile inflammation, DAMPs have also been reported to be specifically induced upon infection (Ehren et al., 2009; Merline et al., 2011; Piccinini and Midwood, 2010; Wheeler et al., 2009). However, little is known about their role in infection-induced TLR signaling. This study identified how control of LPS-mediated TLR4 signaling is exerted by the proinflammatory ECM glycoprotein tenascin-C. In particular, we show that tenascin-C is an early LPS-responsive gene that is required for the proinflammatory response to LPS in experimental sepsis. Furthermore, we demonstrate that tenascin-C derived from bone marrow-derived cells posttranscriptionally controls TNF-α levels via regulation of miR-155 expression.

The induction of tenascin-C expression upon infection in vivo (Pållysha et al., 1993; Schenk et al., 1995; Titta et al., 1992) and its transcriptional regulation by LPS in human myeloid cells (Goh et al., 2010) prompted us to investigate whether this DAMP plays a role in pathogenic inflammation. Using an experimental model of LPS-induced systemic inflammation, we showed that circulating tenascin-C increased rapidly in septic tnc−/− mice, peaking 90 min after LPS injection, long before the induction of HMGB1 release, an established tissue injury marker in sepsis (Wang et al., 1999). These data support specific tenascin-C upregulation by LPS-induced TLR4 signaling rather than tissue damage-driven release of tenascin-C. Indeed, we demonstrated that tenascin-C is expressed at both mRNA and protein level in LPS-activated BMDMs, the major cellular players in sepsis. In response to bacterial endotoxins, macrophages promptly release proinflammatory cytokines, including TNF-α, which is both crucial for effective innate immunity and a key pathologic contributor to sepsis (Tracey et al., 1987). However, septic tnc−/− mice had significantly less circulating TNF-α, a phenomenon that was reversed by allogeineic transplantation of bone marrow from tnc+/+ mice. Consistent with low-circulating TNF-α, less plasma IL-6 and reduced neutrophil infiltration to lungs and liver were observed in tnc−/− mice after LPS injection compared to tnc+/+ mice. Furthermore, tnc−/− mice failed to induce release of HMGB1, a late mediator of sepsis that is released by activated macrophages partly through a TNF-α-dependent mechanism (Chen et al., 2004). These data suggest that tenascin-C is an early-response gene that regulates LPS-induced TLR4-mediated inflammation in vivo operating upstream of TNF-α and HMGB1. This is further supported by our in vitro findings that tnc−/− BMDMs secreted significantly less TNF-α, IL-6, and CXCL1, upon stimulation with LPS than tnc−/− cells. This effect was specific because it was not observed in other cytokine subsets, including IL-1β, IL-12, or TGFβ1. Furthermore, tenascin-C appears to specifically control the switch from anti- to proinflammatory cytokine programs downstream of TLR4 activation. In the absence of tenasin-C, not only do BMDMs induce submaximal TNF-α
Figure 6. Tenascin-C Is Required for Induction of miR-155 in Activated BMDMs and in Septic Mice

(A) Microarray analysis of miRNA profile in tnc+/+ and tnc−/− BMDMs stimulated for 8 hr with 100 ng/ml LPS. The heatmap shows suppression (green) and induction (red) of expression as fold change on a log2 scale, relative to nonstimulated BMDMs (n = 3 mice per genotype).

(B and C) Expression of TLR-induced miRNAs in tnc+/+ and tnc−/− BMDMs (identified in A) classified as early (B) and late (C) response genes (n = 3 per genotype; mean ± SEM). **p < 0.01; ns, not significant.

(D) Time course analysis of miR-155, pri-miR-155, miR-146a, and miR-21 expression in tnc+/+ and tnc−/− BMDMs stimulated for 4–72 hr (miR-155), 0.5–48 hr (pri-miR-155), or 4–96 hr (miR-146a, miR-21) with 100 ng/ml LPS. RNA used in (A) (n = 3 mice per group) and RNA isolated from BMDMs derived from two (miR-146a, miR-21) and four (miR-155, pri-miR-155) additional mice per genotype was analyzed by quantitative RT-PCR (mean ± SEM). **p < 0.01 compared to tnc−/− BMDMs at the same time point.

(E) Correlation of miR-155 expression with TNF-α synthesis in tnc+/+ and tnc−/− BMDMs nonstimulated or stimulated with 100 ng/ml LPS for 4, 8, and 24 hr (n = 5 mice per genotype; R = 1.0; p < 0.05).

(F) Quantitative RT-PCR analysis of miR-155 expression in spleen from tnc+/+ and tnc−/− mice 1.5 hr after LPS injection (n = 3 per genotype; mean ± SEM). *p < 0.05. Data are from two independent experiments.
levels, but they also secreted more IL-10, an anti-inflammatory cytokine that is key to dampening the inflammatory response to infection induced by TLR signaling. Thus, transient tenascin-C expression promotes an initial proinflammatory response that is later suppressed by IL-10 synthesis.

The ability to express biologically active cytokines on demand is regulated at the transcriptional, translational, and posttranscriptional level. We have demonstrated here that tenascin-C controls the synthesis of specific cytokine subsets in BMDMs at the posttranscriptional level. Neither the extent of induction nor the kinetics of expression of TNF-α, IL-6, CXCL1, and IL-10 mRNA was significantly affected by tenascin-C. This provides evidence that the impaired synthesis of proinflammatory cytokines is not at a gene transcription level. However, exocytosis of cytokines to their site of action, which is mediated predominantly by classic secretory pathways in activated macrophages (Lacy and Stow, 2011), can also be the focus of a regulatory event. By quantifying cytokines in the cellular fraction, we found no significant difference in the intracellular cytokine levels between LPS-activated tnc+/+ and tnc−/− BMDMs. Thus, cellular trafficking of cytokines does not account for reduced cytokine release in the absence of tenascin-C. However, when protein transport from the ER to the Golgi was prevented by brefeldin A during stimulation of tnc−/− BMDMs with LPS, intracellular accumulation of TNF-α was abrogated. IL-6 and CXCL1 accumulation within the Golgi in tnc−/− BMDMs was significantly reduced when compared to tnc+/+ BMDMs.

Together, these data suggest that tenascin-C is required for effective translation of LPS-induced proinflammatory cytokines in BMDMs.

A key finding of our study is the observation that tenascin-C mediates an initial proinflammatory response to infection by posttranslationally modulating the expression of the early TLR-induced miRNA miR-155. This miRNA is processed from the nonprotein-coding transcript of the bic gene, which is extremely well conserved between humans and mice and is highly expressed in lymphoid organs (Tam, 2001). Required for the innate immune response to bacterial and viral infection, miR-155 is strongly induced by LPS and type I interferons in macrophages (O’Connell et al., 2007; Thai et al., 2007; Tili et al., 2007). In line with this, by means of a miRNA screen and validation analysis, we found that expression of both primary transcript pri-miR155 and mature miR-155 was induced in tnc−/− BMDMs immediately after LPS stimulation. However, the induction of mature miR-155 in tnc−/− BMDMs was significantly inhibited. This is underscored by the significantly lower miR-155 expression detected in vivo in the spleen of septic tnc−/− mice. Fundamental to the posttranscriptional control of gene expression, miRNAs have emerged as key regulators that fine-tune TLR signaling (O’Neill et al., 2011). Emerging evidence points to a proinflammatory role for miR-155 during TLR-mediated inflammation. It suppresses the domain-containing inositol-5’-phosphatase 1 (SHIP1), a negative regulator of TLR-induced responses, allowing the initial propagation of a proinflammatory response (O’Connell et al., 2009). Moreover, miR-155 has been proposed to increase the translation of TNF-α transcripts either by acting as the 3’ UTR of TNF-α mRNA to release its self-inhibitory effects (Tili et al., 2007) or by increasing TNF-α mRNA stability (Bala et al., 2011). In line with this, miR-155 overexpression in macrophages results in increased TNF-α production, and miR-155 downregulation results in lower TNF-α levels (Bala et al., 2011). Further evidence is provided by in vivo studies; miR-155 transgenic mice have elevated serum TNF-α and increased susceptibility to septic shock, whereas miR-155-deficient B cells fail to produce TNF-α (Thai et al., 2007; Tili et al., 2007). Consistent with this, we found a positive correlation between miR-155 expression and TNF-α production in tnc−/− BMDMs upon LPS stimulation. Interestingly, in the absence of tenascin-C, impaired TNF-α synthesis was parallel to impaired miR-155 expression. Overexpressing miR-155 in tnc−/− BMDMs fully restored their ability to release TNF-α in response to LPS. These data suggest that tenascin-C induced by LPS in BMDMs uses miR-155 to modulate TNF-α levels. The synthesis of IL-6, CXCL1, and IL-10 did not correlate with miR-155 expression, nor was the release of these cytokines rescued in tnc−/− BMDMs by miR-155 overexpression. This is in line with data that show that the effect of miR-155 on the expression of cytokines other than TNF-α is likely to be indirect, for example mediated via suppression of negative regulators of cytokine-production suppressor of cytokine signaling 1 (SOCS1) (Androulidaki et al., 2009) and/or SHIP1 (Kuwoska-Stolarska et al., 2011; O’Connell et al., 2009), which are both validated targets of miR-155, or as a result of impaired production of TNF-α, which itself induces the expression of both IL-6 and CXCL1. Finally, tenascin-C appears to specifically
control the induction of miR-155 without affecting the expression of other LPS-responsive miRNAs that have been functionally linked to TLR signaling, including miR-146a and miR-21 (Sheedy et al., 2010; Taganov et al., 2006).

We previously found high tenascin-C levels in inflamed rheumatoid joints, where it is specifically expressed by synovial fibroblasts and myeloid cells (Goh et al., 2010). We showed that tenascin-C induces cytokine synthesis in both human and murine macrophages and in rheumatoid arthritis (RA) synovial fibroblasts via activation of TLR4 (Midwood et al., 2009). Tenascin-C also induces cytokine synthesis in human chondrocytes in a TLR4-dependent manner (Patel et al., 2011). Other studies report that murine myeloid cells require α9-integrin expression to be activated by tenascin-C (Kanayama et al., 2009, 2011). These data suggest that a number of receptors can play a role in tenascin-C-driven inflammation. We found that the fibrinogen-like globule of tenascin-C activates TLR4 (Midwood et al., 2009), whereas α9-integrin was activated by the third fibronectin type III-like repeat of tenascin-C (Kanayama et al., 2011). Data from these studies, carried out in the absence of cell-activating levels of LPS, imply that within one tenascin-C molecule exist at least two proinflammatory domains with distinct mechanisms. Data presented here showed that upon cell stimulation with LPS, tenascin-C sustains TNF-α synthesis by controlling miR-155 expression. It is not yet known how tenascin-C regulates miR-155 levels. Secreted tenascin-C may activate TLR4, α9-integrin, or other cell surface receptors. However, addition of soluble tenascin-C at a concentration comparable to that made by LPS-stimulated tnc+/+ BMDMs (<10 ng/ml) did not rescue defects in tnc−/− BMDM cytokine synthesis. Moreover, high concentrations of soluble tenascin-C (up to 1 μg/ml) inhibited cytokine synthesis (Figure S4C). These data indicate that, rather than amplifying activation of TLR4 by LPS, adding high concentrations of exogenous tenascin-C prevents LPS activation of TLR4. This may reflect competition for the same receptor by the two stimuli. Together, these results indicate that addition of soluble tenascin-C is not sufficient to rescue LPS-mediated cytokine synthesis in BMDMs derived from tenascin-C-deficient mice. Thus, ligation of a receptor by soluble tenascin-C at the cell surface may not be responsible for stimulating miR-155 expression and sustaining cytokine production during LPS activation. It may be that receptor activation requires tenascin-C assembly into a cell-associated matrix; this may function as a scaffold essential for receptor ligation or for recruitment of potential coreceptor(s). Alternatively, in light of emerging evidence highlighting a role for the 3’ UTR of matrix molecule mRNAs in controlling miRNA function (Jeyapalan and Yang, 2012; Lee et al., 2009), noncoding regions of the tenascin-C gene may drive miRNA biogenesis.

These data raise a number of questions, foremost of which is why tenascin-C should exhibit multiple proinflammatory modes of action. The answer may lie in the role of tenascin-C in driving both pathogenic and sterile inflammation. During acute inflammatory responses to LPS, tenascin-C acts swiftly to sustain cytokine synthesis at the posttranscriptional level, enabling a robust, immediate response to microbial threat. Upon sterile tissue injury, however, the ability of tenascin-C to drive de novo cytokine synthesis, via activation of TLR4 or integrins, ensures effective tissue repair but that sterile inflammation also contributes to chronicity in diseases such as RA.

Both tenascin-C and miR-155 come into play during infection, promoting innate immune responses that culminate in the synthesis of proinflammatory cytokines. Physiologically low and specifically regulated, their expression is transiently induced by LPS in innate immune cells in a NF-κB-dependent manner (Bala et al., 2011; Goh et al., 2010; Rai et al., 2008). Their dysregulation, however, can lead to both cancerous phenotypes (Cain and Croce, 2006; Chiquet-Ehrismann and Chiquet, 2003) and inflammatory and autoimmune diseases, such as sepsis (Schenk et al., 1995; Tili et al., 2007) and RA (Midwood et al., 2009; Stanczyk et al., 2008). Pharmacological inhibition of LPS-induced miR-155 expression by glucocorticoids in macrophages has been reported by Zheng et al. (2012). Notably, tenascin-C expression was also inhibited by glucocorticoids in bone marrow stromal cells and fibroblasts (Ekbloom et al., 1993). Furthermore, both tenascin-C and miR-155 have been shown to affect the ability of the innate immune system to prime adaptive immune responses. In particular, they promote dendritic cell-mediated polarization of Th17 lymphocytes during inflammation (O’Connell et al., 2010; Ruhmann et al., 2012).

In conclusion, our study has identified how tenascin-C control of miR-155 levels regulates the inflammatory axis upon pathogenic activation of TLR4. These data highlight how endogenous danger signals are required for optimal cytokine synthesis in response to PAMP activation of TLRs. This finding provides insight into the positive regulation of TLR4 signaling and DAMP function, which goes beyond sterile inflammation and defines DAMPs additionally as key controllers of nonsterile inflammation. Understanding which molecular inputs are required for optimal induction of cytokines upon TLR activation may refine our strategies to manipulate excessive inflammation.

**EXPERIMENTAL PROCEDURES**

**Mice**

Littermate tnc+/- and tnc−/− mice were from heterozygous breeding pairs on a 129/SvJ background (Saga et al., 1992). All mice were male aged 8–12 weeks, except for bone marrow transplantation, which were female aged 6–8 weeks. For the generation of BMDMs, bone marrow was flushed from tibia and femurs of tnc+/+ and tnc−/− mice, and erythrocytes were lysed with Red Blood Cell Lysis Buffer (Sigma-Aldrich). The resulting cells were cultured in DMEM (PAA) supplemented with 20% FCS (GIBCO), 1% Antibiotic-Antimycotic solution (PAA), and 50 μM β-mercaptoethanol (Invitrogen) containing 100 ng/ml recombinant murine M-CSF (PeproTech). After 7 days, adherent cells were washed, replated, and stimulated with LPS alone (100 ng/ml; Enzo Life Sciences) or with brefeldin A (1 μg/ml; Sigma-Aldrich). To avoid cell toxicity, we treated BMDMs with brefeldin A for the last 8 hr of incubation with LPS. For the generation of thioglycolate-elicited peritoneal macrophages, tnc+/+ and tnc−/− mice were injected intraperitoneally with 1 ml thioglycollate (3% w/v; Sigma-Aldrich). Peritoneal exudates were collected 5 days after with PBS, and cells were analyzed by FACS. For analysis of LPS-induced sepsis, tnc+/- and tnc−/− mice were injected intraperitoneally with LPS from E. coli 055:B5 (8 mg/kg; Sigma-Aldrich). Mice were observed for 8 hr. Body weight, mobility, uveitis, respiration, fur ruffling, and diarrhea were recorded at 0, 1.5, 4, and 8 hr after LPS injection. Mobility impairment was graded using an arbitrary scale where 0 = none, 1 = mild (reduced and slower movement), 2 = moderate (movement upon stimulation), and 3 = severe (no movement). Mice were killed after 1.5, 4, or 8 hr, and plasma and tissues were collected. All procedures were approved by the UK Home Office.
Immunoblot
different areas of the total tissue section.

Immunohistochemical Analysis
Formalin-fixed tissues from tnc−/− and tnc+/− mice were paraffin embedded, cut into 4 μm sections, and stained for neutrophil elastase (MCA7771G; AbD Serotec). Immunostaining specificity was confirmed by omitting the primary antibody or using a nonimmune rat IgG2a (MCA1212; AbD Serotec). Neutrophil elastase* cell number was determined in counting cells in five different areas of the total tissue section.

Immunoblot
Plasma samples from tnc−/− and tnc+/− mice were resolved by SDS-PAGE and analyzed by western blotting using goat anti-tacin-3 polyclonal Ab (AF3358; R&D Systems).

Bone Marrow Transplantation
Each tnc−/− and tnc+/+ female recipient mouse was exposed to a single dose of 9 Gy at 80 cGy/min radiation in a γ irradiator (Nordion) and, 3 hr later, intravenously injected with whole bone marrow cells isolated from one tnc−/− or tnc+/+ male mouse resuspended in 0.2 ml PBS. Transplanted mice received antibiotic orally for 11 weeks (2.5% Baytril; Bayer). The degree of donor engraftment was assessed as described previously by Wang et al. (2002).

RNA Extraction and Quantitative Real-Time PCR
Total RNA was extracted from cells (1 x 10^6) using a RNeasy Mini Kit (QIAGEN). cDNA was synthesized from equivalent amounts of RNA with AffinityScript reverse transcriptase and oligo(dT) primer (Stratagene). Quantitative real-time PCR was performed in a Corbett Rotor-gene 6000 machine with TaqMan primers and probe designed to detect the TSPY gene (forward 5'-TCCTTGCCCTCTCATTCTTAAC-3'; reverse 5'-GAGAACCACCGTGTGATTGATG-3'; probe F6AM-TCCGGAT CAGAGTGGCTTACCCAGG-TAMRA; Applied Biosystems). Standard curves were obtained by mixing male and female DNA from nontreated control mice. Samples were standardized against a mouse GAPDH genomic primer/probe set (Applied Biosystems). Samples were calibrated against DNA from male-nontreated control mouse.

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SUPPLEMENTAL INFORMATION
Supplemental Information includes Extended Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.cirep.2012.09.005.

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EXTENDED EXPERIMENTAL PROCEDURES

Clinical Evaluation of Septic Mice
Mice were observed during a period of 8 hr upon LPS injection. Pictures of mice were taken to record uveitis at 0, 4 and 8 hr after LPS injection. Uveitis was considered mild when eyes were watery, moderate when one or two eyes displayed pus and severe when one or two eyes were closed. Fur ruffling was recorded at 0, 1.5, 4 and 8 hr after LPS injection and was scored as 0, 1, 2 or 3 for absent, mild, moderate or severe, respectively.

Flow Cytometry
For surface staining, peritoneal macrophages and BMDMs were stained for 20 min at 4°C with anti-F4/80-PE (Caltag Medsystems), anti-CD11b-APC, anti-MHC II (I-A/I-E)-PE, anti-CD40-APC, anti-CD80-PE, anti-CD86-FITC, anti-TLR4/MD-2-APC and appropriate isotype controls (eBioscience). For intracellular staining, cells were fixed with 4% paraformaldehyde in PBS for 20 min, were made permeable with PBS containing 2% (vol/vol) BSA, 2mM EDTA, 0.02% NaN₃ and 0.05% (vol/vol) saponin and were stained with anti-CD68-APC (AbD Serotec). Samples were analyzed on a FACSCanto II (BD Bioscience) and data were analyzed with FlowJo software (TreeStar).

Treatment of BMDMs with Soluble Tenascin-C
To assess whether addition of exogenous tenascin-C to tnc⁻/⁻ BMDMs can rescue defects in cytokine synthesis in response to LPS, recombinant human tenascin-C, was synthesized and purified as described previously (Midwood et al., 2009), or commercially purified human tenascin-C (IBL, Japan) was used. 1x10⁵ tnc⁺/+ and tnc⁻/⁻ BMDMs were stimulated for 24 hr with 100ng/ml LPS without or with 1 ng/ml to 1 µg/ml recombinant purified tenascin-C. Cell supernatants were analyzed by ELISA to quantify secreted TNF-α and IL-6 (R&D). Viability of the cells throughout the experimental time period was examined by the MTT cell viability assay (Sigma-Aldrich). Cell viability was not affected by treatment with tenascin-C. Addition of tenascin-C to tnc⁻/⁻ and tnc⁺/+ BMDMs in the absence of LPS stimulated cytokine synthesis (data not shown).

Statistical Analysis
Statistical analysis was performed using Student’s t test or two-way ANOVA with Bonferroni posttest where appropriate; (Prism 5; GraphPad Software).
Figure S1. Tnc−/− Mice Develop Less-Severe Symptoms upon LPS Injection, Related to Figure 1

(A) Representative images of the eyes of tnc+/+ and tnc−/− mice 0, 4 and 8 hr after LPS injection. Data are representative of three independent experiments.

(B) Fur ruffling in tnc+/+ and tnc−/− mice upon LPS injection, monitored over 8 hr. (n = 10 per genotype; mean ± SEM); **** = p < 0.0001. Data are from two independent experiments.

(C) Immunohistochemical analysis of neutrophil elastase+ cell infiltration in liver from tnc+/+ and tnc−/− mice 0, 1.5, 4 and 8 hr after LPS injection (n = 4 LPS-injected mice per genotype and 3 non-injected mice per genotype); scale bars, 100 μm. Results are representative of three independent experiments.

(D) Neutrophil elastase+ cell count in lungs and liver from tnc+/+ and tnc−/− mice 0, 1.5, 4 and 8 hr after LPS injection. Values are expressed as mean ± SD (n). Data are representative of three independent experiments.
Figure S2. BMDMs Mature Effectively in the Absence of Tenascin-C In Vitro and In Vivo, Related to Figure 3

(A–D) FACS analysis of CD11b and F4/80 surface expression in tnc\textsuperscript{+/+} and tnc\textsuperscript{−/−} BMDMs. (A) One representative plot per genotype is shown; (B) percentage of CD11b\textsuperscript{+}/F4/80\textsuperscript{+} BMDMs; (C),(D) mean fluorescence intensity (MFI) of CD11b\textsuperscript{+} (C) and F4/80\textsuperscript{+} (D) BMDMs (n = 3; mean ± SEM).

(E–G) FACS analysis of intracellular CD68 expression in tnc\textsuperscript{+/+} and tnc\textsuperscript{−/−} BMDMs. (E) One representative histogram per genotype is shown; percentage (F) and MFI (G) of CD68\textsuperscript{+} BMDMs (n = 4; mean ± SEM); ns = not significant.

(H) Quantitative RT-PCR analysis of CD11b, F4/80 and CD68 mRNA in tnc\textsuperscript{+/+} and tnc\textsuperscript{−/−} BMDMs. Results are presented relative to those of tnc\textsuperscript{+/+} BMDMs (n = 4; mean ± SEM); ns = not significant.

(I–L) FACS analysis of CD11b and F4/80 surface expression in thioglycollate-elicited peritoneal macrophages from tnc\textsuperscript{+/+} and tnc\textsuperscript{−/−} mice. (I) One representative plot per genotype is shown; (J) percentage of CD11b\textsuperscript{+}/F4/80\textsuperscript{+} macrophages; (K),(L) MFI of CD11b\textsuperscript{+} (K) and F4/80\textsuperscript{+} (L) macrophages (n = 3; mean ± SEM); ns = not significant.

(M–O) FACS analysis of intracellular CD68 expression in tnc\textsuperscript{+/+} and tnc\textsuperscript{−/−} macrophages. (M) One representative histogram per genotype is shown; percentage (N) and MFU (O) of CD68\textsuperscript{+} macrophages (n = 3; mean ± SEM). Data are from three or four (E–H) independent experiments.
Figure S3. Tenascin-C Does Not Affect LPS-Induced Activation of BMDMs, Related to Figure 4

(A–J) FACS analysis of macrophage activation marker expression in tnc<sup>+/+</sup> and tnc<sup>−/−</sup> BMDMs non-stimulated or stimulated for 24 hr with 100ng/ml LPS. (A)–(D) Analysis of MHC II and CD40 surface expression in tnc<sup>+/+</sup> and tnc<sup>−/−</sup> BMDMs +/- LPS. (A) One representative plot per genotype +/- LPS is shown; (B) percentage of MHC II+/CD40+ BMDMs +/- LPS; (C),(D) MFI of MHC II+ (C) and CD40+ (D) BMDMs +/- LPS (n = 3; mean ± SEM); ns = not significant. (E)–(G) Analysis of CD86 surface expression in tnc<sup>+/+</sup> and tnc<sup>−/−</sup> BMDMs +/- LPS. (E) One representative histogram from tnc<sup>+/+</sup> and tnc<sup>−/−</sup> BMDMs +/- LPS is shown; percentage (F) and MFI (G) of CD86+ BMDMs +/- LPS (n = 3; mean ± SEM); ns = not significant. (H)–(J) Analysis of TLR4/MD-2 surface expression in tnc<sup>+/+</sup> and tnc<sup>−/−</sup> BMDMs +/- LPS. (H) One representative histogram from tnc<sup>+/+</sup> and tnc<sup>−/−</sup> BMDMs +/- LPS is shown; percentage (I) and MFI (J) of TLR4/MD-2+ BMDMs +/- LPS (n = 3; mean ± SEM); ns = not significant. Data are from three independent experiments.
Figure S4. TNF-α Production in Tnc−/− BMDMs Is Rescued by miR-155 Overexpression but Not Soluble Tenascin-C, Related to Figure 7

(A) Quantitative RT-PCR analysis of miR-155 expression in tnc+/+ untransfected BMDMs and tnc−/− untransfected, mock transfected and transfected with control precursor or miR-155 precursor BMDMs, all in the absence of any stimulation with LPS (n = 3; mean ± SEM). Data are representative of four independent experiments each with cells obtained from three mice per genotype.

(B) ELISA of TNF-α secreted by tnc+/+ untransfected BMDMs and tnc−/− untransfected, mock transfected and transfected with control precursor or miR-155 precursor BMDMs, all in the absence of any stimulation with LPS (n = 6; mean ± SEM). Data are from two independent experiments each with cells obtained from three mice per genotype.

(C) ELISA of TNF-α and IL-6 secreted by tnc+/+ and tnc−/− BMDMs stimulated for 24 hr with 100ng/ml LPS without (-) or with recombinant purified tenascin-C (TN-C) (n = 3; mean ± SEM). *** = p < 0.001 compared to tnc+/+ BMDMs stimulated with LPS. Data are representative of three independent experiments each with cells obtained from three mice per genotype. The same results were obtained when we added equivalent amounts of commercially purified human tenascin-C.