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Aberrant Toll Receptor Expression and Endotoxin Hypersensitivity in Mice Lacking a Functional TGF-β1 Signaling Pathway

Nancy McCartney-Francis,[1] Wenwen Jin, and Sharon M. Wahl

TGF-β1 plays a central role in maintaining normal immune function and deficiency of this potent immunosuppressive molecule is linked to uncontrolled inflammation, cachexia, and multiorgan failure as seen in the TGF-β1 null mouse. Infiltration of inflammatory cells into vital organs of the null mouse is accompanied by increased gene expression of inflammatory cytokines, including TNF-α and IL-1β, as well as inducible NO synthase, each regulated by NF-κB. Treatment with the proteasome inhibitor MG132 to prevent NF-κB activation dramatically reduced NO production and expression of inflammatory cytokines. This inflammatory phenotype with NF-κB activation in the TGF-β1 null mouse, in the absence of any identifiable pathogen, suggested activation of innate immune responses. Because Toll-like receptors (TLR) are essential in the activation of innate immunity, we examined inflamed tissue from TGF-β1 null and wild-type mice for expression of TLR4, the receptor that interacts with bacterial cell wall LPS to initiate an NF-κB-dependent signaling pathway, leading to gene transcription of inflammatory mediators. Increased TLR4 mRNA expression observed in TGF-β1 null mice as well as in mice lacking the TGF-β transcription factor Smad3 was associated with LPS hyperresponsiveness leading to increased expression of inflammatory cytokines and NO and endotoxemia. Furthermore, mice lacking both TGF-β1 and a functional TLR4 were resistant to endotoxin shock. Constitutive and/or environmental activation of TLR4 and downstream elements, in the absence of TGF-β suppression, may impact on innate and adaptive immunity and contribute to massive uncontrolled inflammation. ***The Journal of Immunology,*** **2004, 172: 3814–3821.**

Transforming growth factor β1 is a key regulator of inflammation and deficiency of this molecule, as in the TGF-β1 null mouse, leads to extensive inflammation and death within 3–4 wk (1, 2). The shared, albeit less severe, pathophysiology in mice lacking a functional TGF-βR (TGF-βRII dominant negative) (3–5) or the TGF-β transcription factor Smad3 (6) highlights the essential function of TGF-β. In the TGF-β1 null mouse, the infiltration of mononuclear cells into vital organs is accompanied by the overexpression of IFN-γ and inducible NO synthase (iNOS)[2] and the resulting toxic levels of NO contribute to the lethal phenotype of the TGF-β1 null mice (7, 8). Key to the regulation of inflammation and the expression of iNOS and other proinflammatory mediators is the activation of NF-κB, a critical transcription factor involved in the activation of a large number of target genes (9). Whereas NF-κB normally exists as an inactive protein complexed with the inhibitor protein IκB in the cytosol, stimulation by cytokines or bacterial Ags initiates its activation, resulting in the phosphorylation, ubiquitinization, and proteasomal degradation of IκB and ultimately translocation of active NF-κB into the nucleus where upon binding to DNA, transcription is induced (10). Rapid activation of NF-κB is vital to host defense against microbial invasion (11). However, persistent activation leading to overproduction of inflammatory mediators may result in tissue injury, organ failure, and death. NF-κB activity is markedly increased in inflamed tissue and is thought to play a critical role in the pathogenesis of inflammatory and autoimmune diseases such as inflammatory bowel disease (12) and rheumatoid arthritis (13, 14), making this factor a potential target for therapeutic intervention.

The initiation of the rampant inflammatory response in the TGF-β1 null mouse, in the absence of any identifiable pathogen, is unknown, but becomes unregulated due to the absence of the suppressive activity of TGF-β1. The observation that mice deficient not only in TGF-β1, but also mice lacking the TGF-β transcription factor Smad3 develop spontaneous inflammatory lesions (6) suggests the possibility that uncontrolled innate immune mechanisms may be triggered by environmental or endogenous stimuli.

Immune recognition of microbial constituents represents an essential feature of host defense against pathogenic organisms and is mediated primarily through pattern recognition receptors, of which Toll-like receptors (TLRs) are key participants (15, 16). TLRs recognize structural motifs referred to as pathogen-associated molecular patterns on invading microorganisms and interactions of pathogens with TLRs initiate innate immune responses to eliminate the pathogens. One TLR molecule, TLR4, binds LPS from Gram-negative bacteria, triggering signaling pathways that lead to the activation of NF-κB (17) and ensuing gene expression of proinflammatory antimicrobial mediators (10). C3H/HeJ mice have an impaired ability to respond to LPS due to a $Pro^{712}His$ substitution mutation in the cytoplasmic signaling domain of the *tlr4* gene (18, 19) and this resistance to endotoxin renders these mice highly susceptible to Gram-negative infections and sepsis (20). Because the inflammatory syndrome observed in the TGF-β1 null mice resembles septic shock in terms of the overexpression of inflammatory mediators, cachexia, and multiorgan failure, we

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[2] Abbreviations used in this paper: iNOS, inducible NO synthase; TLR, Toll-like receptor; FW, forward; RV, reverse; HPRT, hypoxanthine phosphoribosyltransferase; WT, wild type; KO, knockout.

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evaluated NF-κB activation and TLR4 gene expression in these mice and their wild-type (WT) littermates before and after exposure to exogenous LPS. Upon examination of the TGF-β1 null mice, we found increased activation of NF-κB coincident with increased expression of inflammatory mediators. Furthermore, elevated expression of TLR4 coincided with the inflammatory sequelae and accentuated receptor function was demonstrated by enhanced LPS responses. Mice lacking both TGF-β1 and a functional TLR4 are resistant to LPS-induced inflammatory sequelae. The uncontrolled activation of the innate immune response in the absence of TGF-β suggests cross-talk between the TGF-β and TLR signaling pathways.

Materials and Methods

Animals

TGF-β1 null (−/−) mice on a mixed C57BL/6, 129/Sv background were obtained by interbreeding TGF-β1 heterozygous mice which had been generated by disruption of the *tgf*-β1 gene by homologous recombination in murine embryonic stem cells (1). Smad3 null ($Smad3^{ex8/ex8}$) breeding pairs were generously provided by Dr. C. Deng (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health) (6). C3H/HeJ mice were obtained from Charles River Breeding Laboratories (Wilmington, MA). Because C3H/HeJ mice have a point mutation, which inactivates the *tlr4* gene, these mice were interbred with TGF-β1 heterozygous mice to generate TGF-β1 null:C3H/HeJ mice. The genotypes of the mice were determined by PCR analysis of tail biopsies. TGF-β1 null and TGF-β1 null:C3H/HeJ mice were maintained on standard mouse chow supplemented with dough diet (Bioserv, Frenchtown, NJ). All animal experiments were performed in accordance with institutional guidelines and with approval from the National Institute of Dental and Craniofacial Research Animal Care and Use Committee.

Semiquantitative RT-PCR

Total RNA was isolated from heart and lung tissue of TGF-β1 null and WT littermate mice with TRIzol reagent (Invitrogen, Gaithersburg, MD). RNA (2 μg) was reverse transcribed and the cDNA was amplified by semiquantitative PCR as described previously (21) using appropriate oligonucleotide primers and predetermined conditions. The primers (5′–3′) included the following (forward (FW), reverse (RV)): hypoxanthine phosphoribosyltransferase (HPRT) (162 bp): FW, GTTGGATACAGGCCAGACTTT GTTG; RV, GATTCAACTTGCTCTCATCTTAGGC; iNOS (270 bp): FW, TTGGGTCTTGTTCACTCCACGGAG; RV, ATTCTGTGCTGTC CCAGTGAGGAG; TNF-α (692 bp): FW, ATGAGCACAGAAAGCAT GATCCGC; RV, CCAAAGTAGACCTGCCCGGACTC; IL-1β (563 bp): FW, ATGGCAACTGTTCCTGAACTCAACT; RV, CAGGACAGG TATAGATTCTTTCCTTT; and TLR4 (479 bp): FW, GTGATCTACTC GAGTCAGAATG; RV, GGTCCAAGTTGCCGTTTCTTG.

Samples were heated at 94°C for 10 min and amplified using cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 1–2 min (HPRT, 27 cycles; TLR4, 26 cycles; TNF-α, 31 cycles; IL-1β, 29 cycles; iNOS, 35 cycles). PCR products were electrophoresed in a 1.8% agarose gel and ethidium bromide-stained bands were scanned and quantitated using a digital imaging system (Alpha Innotech, San Leandro, CA). For comparison of samples, all densitometric values were normalized to the housekeeping gene HPRT.

RNase protection assay

The expression of IL-1β and TNF-α receptors was determined by the RNase protection assay (BD PharMingen, San Diego, CA) using the mouse mCR4 template to synthesize a ^{32}P-radiolabeled RNA probe as described by the manufacturer. Total RNA (5 μg) was hybridized overnight at 56°C to the radioactive RNA probe (6.8×10^5 cpm/μl) and after RNase treatment to eliminate the single-stranded, nonhybridized RNA species, the protected RNA-RNA hybrids were ethanol precipitated, washed with 70 and 90% ethanol, air dried, resuspended in loading buffer, and electrophoresed on a 6% denaturing acrylamide gel. After drying, the gel was exposed to a phosphor screen and radioactivity was quantified by a PhosphorImager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Results are reported as an RNA index, which represents the ratio of cytokine receptor mRNA to GAPDH housekeeping gene.

Western blot analysis

Heart tissue was homogenized in lysis buffer (150 mM NaCl, 50 mM Tris (pH 7.5), 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 2 mM EDTA) containing protease and phosphatase inhibitors (100 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml soybean trypsin inhibitor, and 20 μM sodium vanadate). Proteins were separated by electrophoresis on a 7.5–10% SDS-polyacrylamide gel, electrotransferred onto nitrocellulose membrane and blocked in 1% chicken albumin (Sigma-Aldrich, St. Louis, MO) at room temperature for 1 h. The membrane was incubated with Abs directed against NF-κB (p65 and p50). After washing, the blots were incubated with protein A HRP (Amersham Pharmacia Biotech, Piscataway, NJ) or avidin-HRP (Neutralite; Southern Biotechnology Association, Birmingham, AL) for 1 h at room temperature and developed with an ECL detection system (Renaissance ECL, New England Nuclear, Boston, MA).

EMSA

Cellular proteins were isolated from heart tissue by homogenization in lysis buffer (20 mM Tris (pH 7.6), 120 mM NaCl, 1% Nonidet P-40, 10% glycerol, 10 mM Na pyrophosphate, 100 mM NaF, 2 mM sodium orthovanadate, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 5 μg/ml leupeptin). Extracted proteins (5 μg) were incubated with polynucleotide kinase-radiolabeled DNA probe containing the NF-κB binding site (0.05 pmol, E3291; Promega, Madison, WI) in reaction buffer (10 mM Tris (pH 7.5), 1 mM DTT, 1 mM EDTA, 4% glycerol, 0.08 mg/ml salmon sperm DNA, with a final concentration of 0.08 M NaCl) for 30 min at room temperature. The reaction mixture was electrophoresed in a 6% nondenaturing gel containing 0.25 M Tris-borate-EDTA, which was then vacuum dried and analyzed by a PhosphorImager. To assess specificity, 50-fold excess of unlabeled oligonucleotide was used to block binding to the DNA probe. For supershift assays, 1 μl of NF-κB p50 or p65 Ab (0.2 μg; Santa Cruz Biotechnology, Santa Cruz, CA) was preincubated with the protein extracts for 30 min at 4°C before addition of the NF-κB probe. Alternatively, cellular proteins were analyzed for activated NF-κB using an ELISA-based p65 transcription factor assay (TransAM; Active Motif, Carlsbad, CA).

In vivo studies

To study NF-κB-dependent responses, mice were injected i.p. every 3 days from day 10 until day 18 with the proteasome inhibitor MG132 (Z-leu-leu-leu-CHO, dissolved in DMSO and then diluted 1/200 in sterile saline for injections; Calbiochem, La Jolla, CA) at a dosage of 0.3 μg/g body weight (22). In some cases, another proteasome inhibitor, lactacystin (0.3 μg/g body weight; Peptide Institute, Osaka, Japan) or an inhibitor of IκBα phosphorylation (Bay 11-7082, (E)2-[(4-methylphenyl)sulfonyl]-2-propenenitrile, 20 μg/g body weight; Calbiochem) (23) was administered using the same injection schedule. Control mice were injected with an equal amount of 0.5% DMSO. To study the effect of the proteasome inhibitor on survival, injections were continued every 3 days. No adverse effects of the inhibitor or DMSO were noted as WT mice treated with MG132 or DMSO alone had similar weight gains as untreated WT mice. Also, survival of DMSO-treated TGF-β1 null mice was comparable to PBS-treated or untreated null mice. For LPS responses, mice were injected i.p. with a sublethal dose of LPS (12.5 μg LPS/g body weight) (24). LPS from *Escherichia coli* strain K-235 was obtained from Sigma-Aldrich (L-2018). Mice were euthanized after 2, 4, and 6 h and blood was collected by cardiac puncture for plasma, and lung tissue was collected and frozen for RNA and protein studies. For survival studies, mice were injected with 35 μg LPS/g body weight and monitored for 6 days.

Inflammatory mediator determinations in plasma

Plasma levels of NO (nitrite plus nitrate) were measured with a NO analyzer (Sievers NOA 280; Ionics Instrument Business Group, Boulder, CO) under conditions that reduce both nitrate and nitrite to NO (0.1 M VCl_3 in 1.0 M HCl at 89°C). TNF-α and IL-1β levels were measured using a cytokine multiplex bead assay (BioSource International, Camarillo, CA) according to the manufacturer's instructions.

Statistical analysis

Data are presented as mean ± SEM and analyzed using GraphPad (San Diego, CA) PRISM for significance by the one-tailed unpaired *t* test. One-way ANOVA was used to determine differences between more than two populations. Data from the multiplex cytokine bead assay were analyzed using a one-phase exponential association of a nonlinear regression curve fit.

Results

Inflammatory phenotype of TGF-β1 null mice

The lethal phenotype of the TGF-β1 null mice is characterized by overwhelming infiltration of inflammatory cells into the heart, lungs, and less frequently in liver, which compromises the structure and function of these vital organs, contributing to organ failure. When total RNA from these tissues from symptomatic (>12 days of age) null mice and WT littermates was analyzed by semiquantitative RT-PCR for TNF-α and IL-1β, key proinflammatory cytokines (Fig. 1, *A* and *B*), increased RNA expression of both cytokines was observed. IL-1β RNA was also elevated in TGF-β1 null liver that typically shows limited ductal inflammation. In addition, both TNF-α and IL-1β RNA were elevated in salivary and lacrimal glands, also targets of autoimmune-like infiltration (21, 25). Also contributing to the milieu of inflammatory mediators, iNOS gene expression was elevated, most dramatically in the hearts of the null mice (Fig. 1*C*) and was reflected in the circulation by increased levels of nitrite and nitrate, decomposition products of NO, in the plasma (7, 8). This dysregulation of proinflammatory cytokine and iNOS gene expression in the absence of TGF-β1 likely drives the pathology observed in the TGF-β1 null mice.

Because of the pronounced TNF-α and iNOS expression in the heart, we focused on the underlying events, which contribute to compromised function in this organ. Accompanying the increased gene expression of TNF-α and IL-1β, their cognate receptors were also elevated in the inflamed null heart (Fig. 2). RNase protection assays revealed increased receptor RNA levels for IL-1β (IL-1RI and IL-1RII) and TNF-α (TNFR p55 and p75) in heart tissue from both asymptomatic (10 days old; data not shown) and symptomatic TGF-β1 null mice (Fig. 2). The coincident expression of inflammatory cytokines and their receptors favors an autocrine and/or paracrine pathway to initiate, augment, or accelerate signaling involved in the inflammatory disease in the TGF-β1 null mice.

NF-κB-dependent signaling regulates the inflammatory phenotype of TGF-β1 null mice

One intracellular signaling molecule that is shared by TNF-α and IL-1β, as well as iNOS, is the transcription factor NF-κB. Promoters of all three genes contain at least one κB binding site and binding of NF-κB activates transcription of these genes. Examination of NF-κB signaling pathway components by multiplex RT-PCR revealed constitutive expression of both NF-κB and inhibitor protein IκB RNAs between 3 and 17 days of age and levels of RNA expression were similar in both null and WT mice (data not

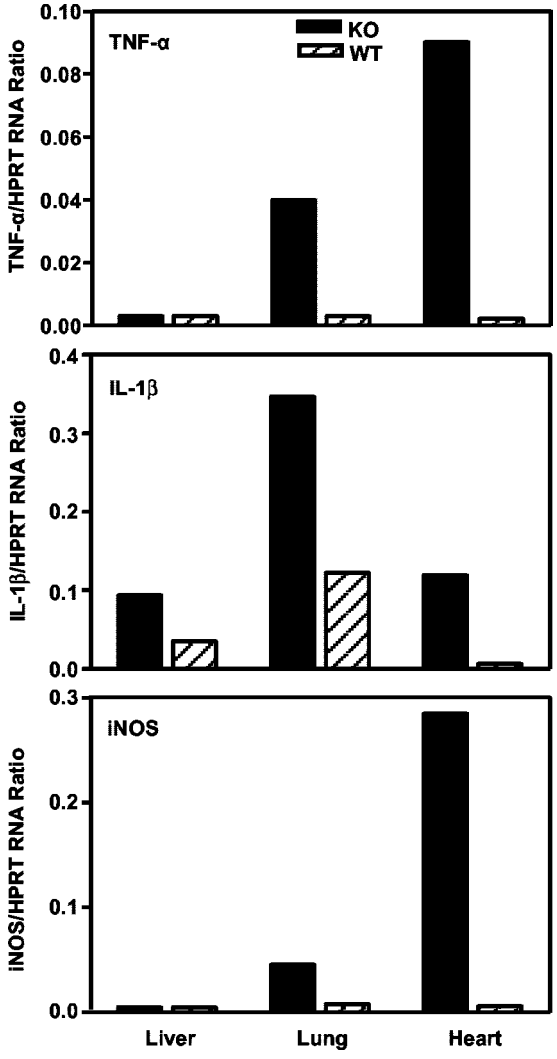

FIGURE 1. Up-regulation of inflammatory mediator mRNA in TGF-β1 null mice. Total RNA from multiple tissues of TGF-β1 null mice (KO) and WT littermates (21 days old) was analyzed by semiquantitative RT-PCR for inflammatory mediators. TNF-α (*A*), IL-1β (*B*), and iNOS (*C*) mRNAs were elevated in heart, lung, and liver of the null mice.

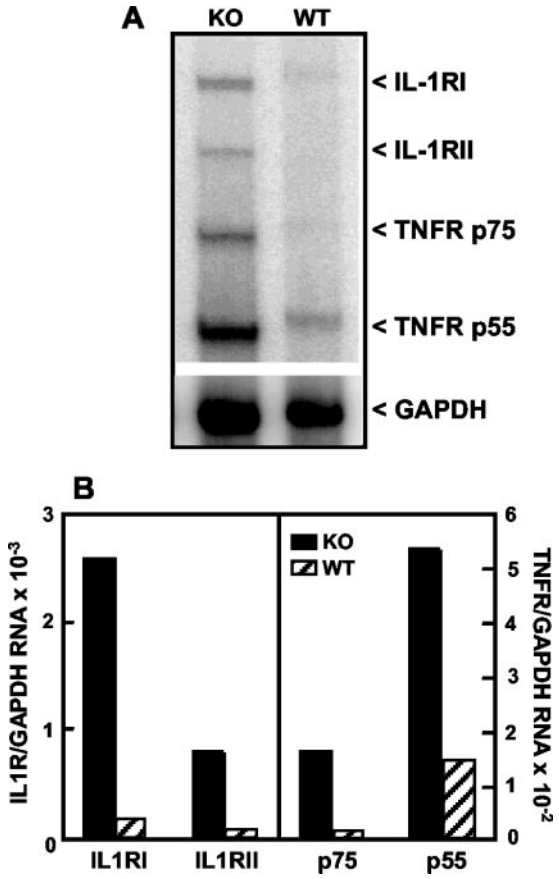

FIGURE 2. Cytokine receptor RNA expression is increased in heart tissue of TGF-β1 null mice. *A*, Total RNA from heart tissue of TGF-β1 knockout (KO; $n = 5$) and WT ($n = 5$) littermates was analyzed by a multiprobe RNase protection assay. Representative KO and WT mice (28 days old) are shown. *B*, Bands representing the two IL-1β receptors, IL-1RI and IL-1RII, and TNF-α receptors, p75 and p55, were quantitated by a PhosphorImager and normalized to GAPDH.

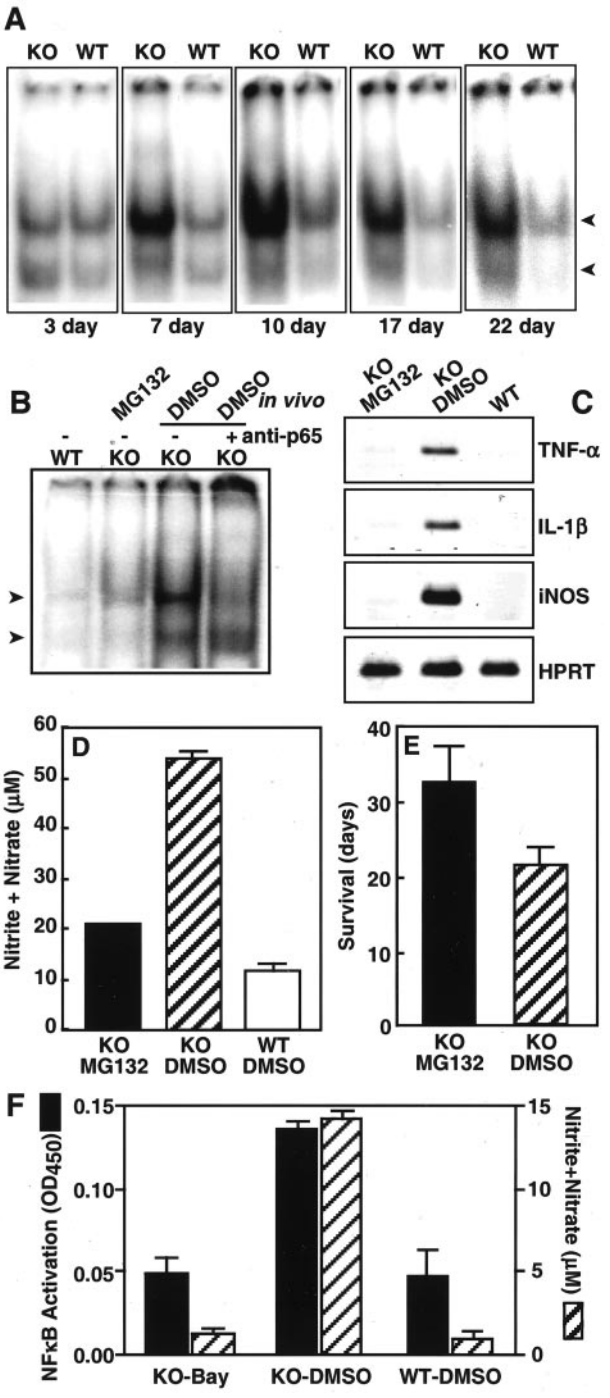

FIGURE 3. Increased activation of NF-κB in heart tissue of TGF-β1 null mice is prevented by the proteasome inhibitor MG132. *A,* Electrophoretic mobility shift analysis was performed on homogenates (5 μg protein) of heart tissue, revealing increased binding of lysates from TGF-β1 null hearts to the oligonucleotide probe containing the NF-κB site. *B,* Mice were injected i.p. every 3 days from day 10 until day 18 with the proteasome inhibitor MG132 (0.3 μg/g body weight) or DMSO (0.5% in saline) and heart tissue homogenates (5 μg protein) were analyzed by EMSA to detect NF-κB binding. Supershift of the binding complex by the addition of Ab directed against the 50 kDa (data not shown) and 65 kDa (*far right lane*) subunits of NF-κB confirmed the identification of the complex. *C,* Total RNA from heart tissue was analyzed by RT-PCR using primers specific for TNF-α, IL-1β, iNOS, and the housekeeping gene HPRT. *D,* Plasma levels of nitrite plus nitrate (n = 2–4) were measured with a NO analyzer. *E,* TGF-β1 null mice were injected every 3 days with MG132 (n = 11) or DMSO (n = 8) beginning on day 7 and monitored for survival. *F,* TGF-β1 null mice were injected every 3 days with the IκBα phosphorylation inhibitor Bay 11-7082 (n = 2) or DMSO (n = 2) beginning on day 10 through day 18. Homogenates of heart tissue (10 μg) were analyzed for NF-κB p65 activation (OD_{450}). Activation could be competitively inhibited by the WT NF-κB oligonucleotide but not a mutated NF-κB oligonucleotide (data not shown). Serum levels of nitrite plus nitrate were measured with a NO analyzer.

shown). Because NF-κB must be activated to be translocated into the nucleus to initiate transcription, we performed EMSAs using an oligonucleotide probe containing the κB binding site to detect active NF-κB. As shown in Fig. 3*A*, increased binding to the κB probe by proteins isolated from TGF-β1 null heart was observed within the first week after birth, coincident with gene expression of TNF-α, IL-1β, and iNOS (8), but before overt symptomology. Variable expression of active NF-κB was apparent throughout the life span of the null mice, likely reflecting variability between individual null mice in terms of time of onset of inflammatory disease, extent, and severity of lesions.

To interrupt this fatal inflammatory pathway in the TGF-β1 null mice, we targeted the activation of NF-κB with a proteasome inhibitor, MG132, known to inhibit NF-κB by blocking the degradation of ubiquitinated IκB by the 26S proteasome complex (22, 26). Mice were injected i.p. every 3 days from day 10 after birth until day 18 with 0.3 μg MG132/g body weight or 0.5% DMSO in PBS. Gel shift analysis of heart tissue homogenates (day 18) revealed a reduction in NF-κB activity in TGF-β1 null mice treated with MG132 to levels more comparable to that observed in WT littermates (Fig. 3*B*). Furthermore, TNF-α, IL-1β, and iNOS mRNA levels were dramatically reduced (Fig. 3*C*), as determined by semiquantitative RT-PCR. Circulating levels of NO were also reduced nearly to the WT baseline (Fig. 3*D*). Treatment with another proteasome inhibitor, lactacystin, recently shown to block expression of LPS-induced genes in RAW 264.7 macrophages (27), also reduced plasma NO levels in the TGF-β1 null mice as compared with DMSO-treated null mice (16.33 $\pm$ 0.07 μM vs 53.3 $\pm$ 1.50 μM). Moreover, NF-κB activation was blocked by in vivo treatment with an inhibitor of IκBα phosphorylation, Bay 11-7082, resulting in a 9-fold reduction in circulating NO levels as compared with DMSO-treated null mice (Fig. 3*F*). In addition, serum levels of TNF-α were also reduced (Bay 11-7082-treated null mice: 18.8 $\pm$ 1.6 pg/ml vs DMSO-treated null mice: 61.4 $\pm$ 6.4 pg/ml, p = 0.01). Continued treatment with the proteasome inhibitor MG132 extended the life span of the TGF-β1 null mice by 150% ($p \leq 0.05$; Fig. 3E), with some mice surviving for 6–8 wk. Thus, therapeutic modulation of NF-κB activity interrupts the fatal inflammatory process in the TGF-β1 null mice by reducing expression of inflammatory mediators, resulting in prolonged life.

TLR expression is increased in TGF-β1 null mice

In many ways, the inflammatory profile displayed by the TGF-β1 null mice appears comparable to that seen in septic shock, a systemic inflammatory response to an infection, most commonly caused by Gram-negative organisms (28). Although no specific pathogen has been associated with disease in the null mice, it is conceivable that in the absence of the suppressive activity of TGF-β1, abnormal responses to enteric bacteria and/or environmental organisms may elicit such a response. Because the recently described Toll receptors serve as signaling receptors for bacterial components and activation of the receptors results in a parallel profile of inflammatory mediators (16), we evaluated the role of Toll receptors, specifically TLR4, in the activation of signaling pathways in the TGF-β1 null mice. Consistent with the cytokine and NF-κB data, TLR4 mRNA was elevated >2-fold in heart, lung, and liver of symptomatic null mice as compared with the WT

littermates (Fig. 4*A*). Even as early as 3 days after birth, TLR4 mRNA was elevated >2-fold as compared with WT mice, identifying a potential target for activation in the null mice (Fig. 4*B*). Importantly, treatment with the proteasome inhibitor MG132 not only reversed the inflammatory profile of the null mice (Fig. 3), but also reduced TLR4 mRNA expression to the level observed in the WT littermates (Fig. 4*C*), strengthening the link between TLR4 up-regulation and constitutive NF-κB activation in the null mice.

TGF-β1 null mice are hyperresponsive to endotoxin challenge

Based on the enhanced expression of TLR4 in TGF-β1null mice, we predicted that the mice would be more responsive to Gram-negative microorganisms. As a measure of this response, we injected null and WT mice with LPS and collected tissues for RNA analysis at various time intervals (Fig. 5). Dramatic increases in TNF-α and iNOS mRNA were observed in lungs of the null mice within 1.5 h following LPS challenge. TNF-α mRNA levels were increased >2-fold as compared with WT littermates and, even more striking, iNOS levels increased almost 8-fold and were sustained at this elevated level over the 6-h observation period. In comparison, sharp increases in IL-1β mRNA were observed in both null and WT mice but sustained at a higher level in the null mice. Kinetics of LPS-induced TLR4 RNA differed between null and WT mice with TLR4 levels in the null mice rapidly increasing within 90 min, whereas levels increased more slowly in the WT, peaking at 3–6 h.

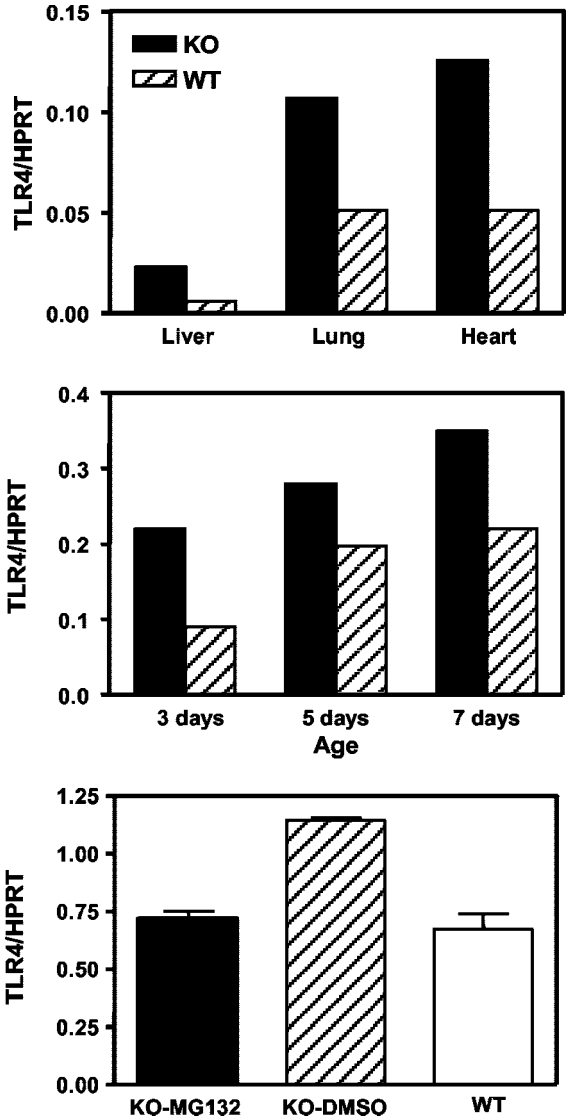

FIGURE 4. TLR4 expression is increased in TGF-β1 null mice. *A,* Total RNA from multiple tissues of 21-day-old TGF-β1 null mice and littermates was reverse transcribed and amplified by PCR using TLR4- and HPRT-specific primers. PCR products were separated by gel electrophoresis and stained with ethidium bromide. Data represent densitometric values for TLR4-specific bands that have been normalized to HPRT. *B,* Total RNA from heart tissue from TGF-β1 null and WT littermates was analyzed by RT-PCR for TLR4 and HPRT. *C,* Total RNA from heart tissue from MG132- or DMSO-treated TGF-β1 null and WT mice was analyzed by RT-PCR for TLR4 and HPRT.

Increased NO production in mice lacking TGF-β1 or a functional TGF-β signaling pathway

Because of the rapid and striking induction of iNOS in the TGF-β1 null mice in response to LPS, we next looked for evidence of NO in the circulation, characteristic of inflammatory disease (8). Plasma levels of NO in the LPS-injected mice were measured by a NO analyzer. In TGF-β1-deficient mice, NO levels were profoundly elevated (60-fold as compared with WT) within 3 h following LPS challenge (Fig. 6*A*). Consistent with cytokine involvement in endotoxic shock (29), levels of TNF-α and IL-1β proteins were also increased in the plasma of LPS-treated null mice (20-fold and 5-fold, respectively) as compared with LPS-treated WT littermates (data not shown). To determine whether this increased response to LPS was unique to the TGF-β1 null mice, we performed parallel studies in mice lacking the TGF-β transcription factor Smad3. In similar fashion, Smad3 null mice also were hyperresponsive to endotoxin, although to a lesser degree and somewhat delayed as compared with TGF-β1 null mice (Fig. 6*B*). Thus, the absence of TGF-β1 or a functional TGF-β signaling pathway contributes to a hyperresponsiveness to LPS. Furthermore, Smad3 null mice also express elevated levels of TLR4 mRNA (Fig. 6*C*) similar to those of TGF-β1 null mice, linking the LPS hyperresponse to TLR4 overexpression.

Mice lacking a functional TGF-β signaling pathway are more sensitive to endotoxemia

Having demonstrated the association between accentuated LPS responses and TLR4 expression, we next compared the consequences of endotoxemia in the TGF-β1 null and WT mice. Mice were injected with LPS and monitored for survival (Fig. 7). Quite dramatically, 50% of the TGF-β1 null mice died within 12 h after LPS challenge and 90% by 24 h. In similar fashion, 50% of Smad3 null mice died within 48 h of LPS injection. In contrast, 100% of the WT littermates survived this dose of LPS although a subset of mice displayed limited symptoms of endotoxemia (weakness, lethargy, ruffled hair), but recovered completely. To confirm the role of TLR4 in the LPS hypersensitivity, TGF-β1 null mice were bred onto a C3H/HeJ (LPS-resistant, mutated TLR4) background. When the TGF-β1 null:C3H/HeJ mice were injected with the dose of LPS which was lethal in the TGF-β1 null mice, 100% of the mutant mice survived the 6-day observation period (Fig. 7), consistent with an essential role for the TLR4 molecule in LPS hypersensitivity in the TGF-β1 null mice and the absence of other contributing elements from the TGF-β1 genotype. Importantly, in the absence of a LPS challenge, the TGF-β1 null:C3H/HeJ mice exhibited an extended life span (31.4 ± 2.0 days, $n = 28$, vs 22.7 ± 2.1, $n = 14$, $p = 0.016$), supporting a role for TLR4 in the pathophysiology of the TGF-β1 null mice.

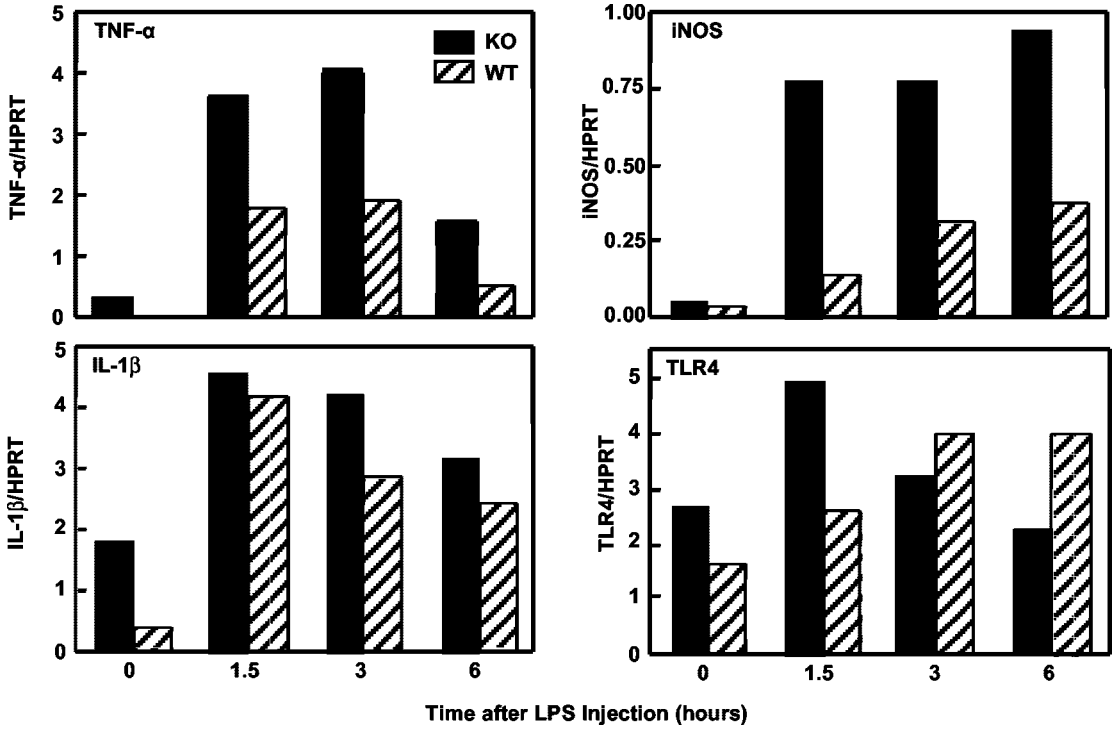

FIGURE 5. TGF-β1 null mice are hyperresponsive to endotoxin challenge. TGF-β1 null mice and littermates were injected i.p. with LPS and total RNA from lung tissue was analyzed by RT-PCR for inflammatory mediators and TLR4. Densitometric values for ethidium-stained TLR4 products were normalized to HPRT. Data are representative of three experiments.

Discussion

Mice deficient in TGF-β1 develop inflammatory lesions in multiple organs accompanied by overexpression of inflammatory cytokines and NO and activation of the transcription factor NF-κB. Although the underlying instigatory mechanism of the inflammatory pathology is not known, the ability to modulate the inflammatory sequelae by blocking recruitment and migration (30, 31), activation (8, 32), and, as demonstrated here, signal transduction, by targeting either NF-κB activation or TLR4 expression, highlights the complexity of the inflammatory phenotype of the TGF-β1 null mouse. However, in the absence of any identifiable pathologic agent, the initiator of these events remains a mystery.

Recent studies have defined Toll receptors as critical in host defense for the early detection of microorganisms, and activation of the receptors leads to rapid induction of antimicrobial proteins for the containment and elimination of the pathogen (15). As vital as Toll receptors are to innate immune responses, hyperactivation of the receptors can also have adverse effects such as septic shock (18, 33), apoptosis (34), and bone destruction (35). Key to maintaining a balance between protective host defense and immunopathology is the expression of anti-inflammatory cytokines, one of the most potent being TGF-β. Our data suggest that in the TGF-β1 null mice, without the suppressive power of TGF-β1, abnormal activation of NF-κB-dependent signaling pathways through the Toll receptors, namely TLR4, leads to a debilitating and ultimately fatal inflammatory syndrome. Preventing NF-κB activation through the use of proteasome and IκBα phosphorylation inhibitors to block IκB degradation (22, 26) is effective in reducing TLR4 and inflammatory cytokine and mediator gene expression as well as circulating NO and TNF-α levels and extending the life span of the TGF-β1 null mice, thus highlighting the essential role of this signaling pathway in the pathologic inflammatory syndrome. However, the ability to completely cure the mice may be limited due to the route of administration of the inhibitor or multiple lethal factors contributing to the pathology (8, 36).

Although it remains unclear whether the elevation in TLR4 is a primary or secondary consequence of the lack of TGF-β1, our data suggest a participatory role for these receptors in the unrestrained inflammation in the TGF-β1 null mice. Furthermore, elevations in TLR4 mRNA were observed as early as 3 days after birth, before evidence of inflammatory cell infiltration, suggesting that the receptors may serve as an early target for activation. Whether early exposure to enteric bacteria in the normal flora of the gut or other environmental Ags in the TGF-β1 null mice predisposes them to aberrant activation through the TLR4 receptors and initiates the cascade of events leading to the rampant inflammatory syndrome is not known. TGF-β1 null mice on a 129 $\times$ CF_1 background develop considerable gastric inflammation which is not reduced under germfree conditions, although death is delayed in these mice (37) and it remains unclear whether our pathogen-free TGF-β1 null mice on a 129 $\times$ C57BL/6 background which develop late-stage colitis with little gastric inflammation would survive longer under germfree conditions. It is noteworthy that TLR4 mRNA and protein expression is up-regulated in other inflammatory conditions, including intestinal macrophages from inflamed mucosa of Crohn's disease and ulcerative colitis patients and, in response to LPS, the isolated macrophages expressed increased levels of intracellular IL-1 protein (38). Since TGF-β1 is a potent negative regulator of mucosal inflammation (39), the overexpression of Smad7, an inhibitory transcription factor of TGF-β signaling, in colonic mucosal tissues from patients with active Crohn's disease and ulcerative colitits (40) may inhibit its activity. Treatment of lamina propria mononuclear cells with specific antisense oligonucleotides to inhibit Smad7 restored TGF-β signaling and inhibition of cytokine production. In similar fashion, our own data demonstrate that abrogation of TGF-β signaling either by deletion of

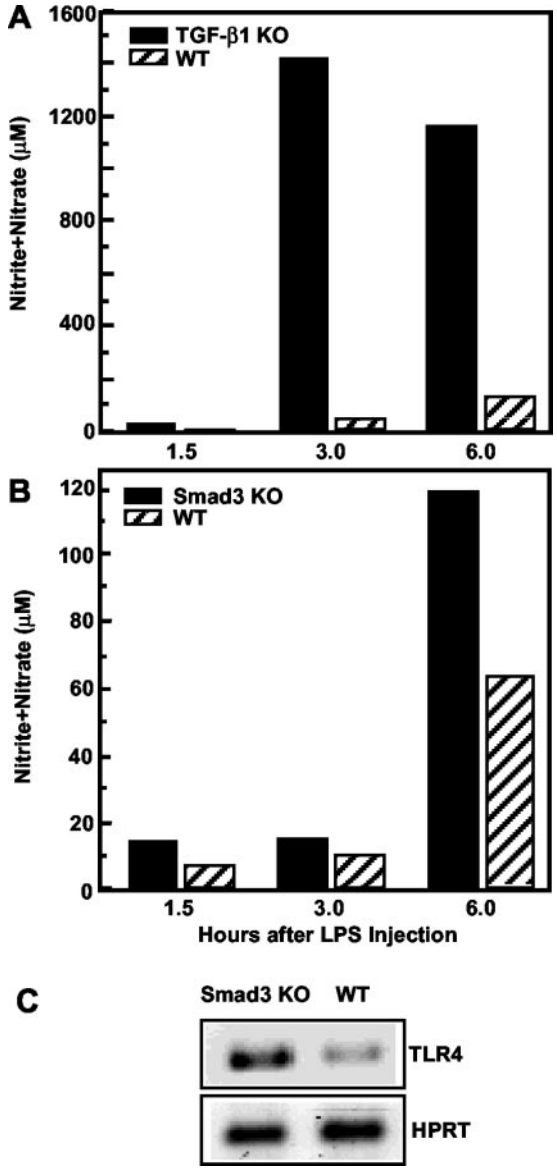

FIGURE 6. Increased NO production in mice lacking TGF-β1 or a functional TGF-β signaling pathway. Plasma levels of nitrite plus nitrate in LPS-injected TGF-β1 null mice (*A*) and Smad3 null mice (*B*) and WT littermates were measured using a NO analyzer, and data are expressed as mean ± SEM. Total RNA from Smad3 null and WT mice was analyzed by RT-PCR for TLR4 and HPRT (*C*).

TGF-β1 or Smad3, is associated with TLR4 elevation, increased inflammation, and aberrant cytokine and NO production, giving further credence to cross-talk between the TGF-β and TLR4 signaling pathways.

The functional importance of Toll receptors is highlighted by the association of LPS hyporesponsiveness and susceptibility to Gram-negative infections with disabling mutations in the TLR4 gene, demonstrated by a single point mutation in C3H/HeJ mice (18) and a deletion in C57BL/10ScCr mice (19). Similar associations have been made to TLR4 polymorphisms in humans (41) and links to chronic inflammatory diseases have been suggested (33, 42). Whereas TLR4 is advantageous and essential for innate immunity against Gram-negative pathogens, persistent activation of this innate pathway may magnify release of proinflammatory mediators, resulting in hypotension, multiorgan failure, and ultimately death, typical of TGF-β1-deficient mice. Importantly, we demonstrate in the TGF-β1 null:C3H/HeJ mice that TLR4 expression is essential in the LPS hypersensitivity of the TGF-β1 null mouse. The beneficial aspects of abrogating TLR4 signaling in the TGF-β1 null mice (i.e., increased life span) underscores the potential for targeting TLR4 in the treatment of human chronic inflammatory diseases as well as to counteract exaggerated inflammatory responses during infection (43). The efficacy of such an approach would have to be balanced between the protective host-microbe response and pathological immune response. Key to the regulation of this balance is TGF-β.

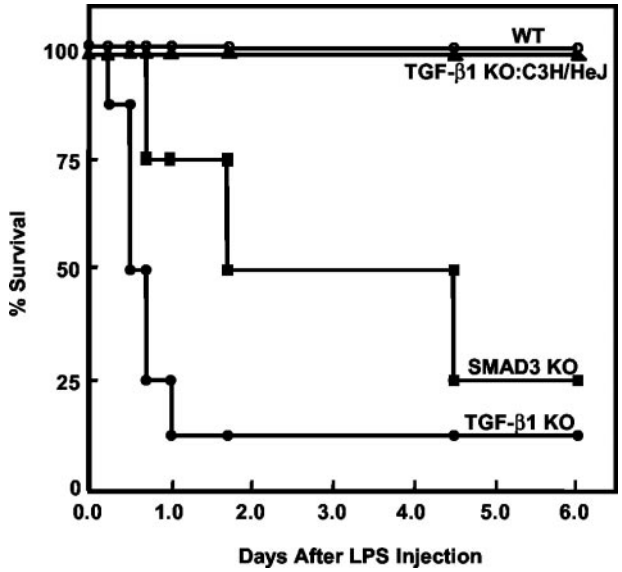

FIGURE 7. Mice lacking a functional TGF-β signaling pathway are more sensitive to endotoxemia. TGF-β1 null ($n = 8$), Smad3 null ($n = 4$), TGF-β1null:C3H/HeJ ($n = 6$), and WT ($n = 9$) mice were injected with LPS and monitored for survival.

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