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TLR2 Expression in Astrocytes Is Induced by TNF-α- and NF-κB-Dependent Pathways[1]

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Astrocytes participate in CNS innate immune responses as evident by their ability to produce a wide array of inflammatory mediators upon exposure to diverse stimuli. Although we have established that astrocytes use TLR2 to signal inflammatory mediator production in response to *Staphylococcus aureus*, a common etiological agent of CNS infections, the signal transduction pathways triggered by this pathogen and how TLR2 expression is regulated remain undefined. Three disparate inhibitors that block distinct steps in the NF-κB pathway, namely SC-514, BAY 11-7082, and caffeic acid phenethyl ester, attenuated NO, TNF-α, and CXCL2 release from *S. aureus*-activated astrocytes. Among these proinflammatory mediators, autocrine/paracrine TNF-α was pivotal for augmenting TLR2 expression, since receptor levels were not elevated in astrocytes isolated from TNF-α knockout mice upon bacterial exposure. Since TLR2 is critical for signaling astrocytic cytokine production in response to *S. aureus*, we evaluated the effect of TNF-α loss on proinflammatory mediator release. Interestingly, among the molecules assayed, only NO production was significantly attenuated in TNF-α knockout astrocytes compared with wild-type cells. Similar results were obtained following LPS treatment, suggesting that TNF-α is an important regulator of astrocytic TLR2 expression and NO release in response to diverse microbial stimuli. In addition, NF-κB inhibitors attenuated TNF-α-induced TLR2 expression in astrocytes. Overall, this study suggests that two important anti-bacterial effector molecules, TLR2 and NO, are regulated, in part, by NF-κB-dependent autocrine/paracrine effects of TNF-α in astrocytes. *The Journal of Immunology,* 2008, 181: 3841–3849.

Brain abscess is a serious infectious disease arising from parencymal seeding of the CNS with pyogenic bacteria such as *Staphylococcus aureus* (1, 2). Among the numerous host responses elicited during brain abscess development, astrocyte activation is a hallmark feature of infection (3–5). Reactive astrocytes are localized along the abscess periphery and the immediate activation of these cells likely plays an important role in initiating the host anti-bacterial immune response before the influx of professional phagocytes from the peripheral circulation (6).

TLR are a family of pattern recognition receptors that allow for the recognition of pathogen-associated molecular patterns, motifs conserved across broad subclasses of bacteria, viruses, or fungi (7, 8). To date, 13 TLRs have been identified, with TLR2 playing a pivotal role in recognizing structural components associated with Gram-positive bacteria such as *S. aureus* (7, 8). Recently, we and others have shown that astrocytes express TLR2, with receptor levels increasing upon exposure to various pathogen-associated molecular patterns including LPS, peptidoglycan, and *S. aureus* (9–13). In addition, TLR2-deficient astrocytes demonstrated impaired immune responses against *S. aureus* (9). Despite these observations, the signaling cascades regulating TLR2 expression and subsequent proinflammatory mediator production in activated astrocytes have not yet been defined.

In response to *S. aureus*, astrocytes produce a wide array of proinflammatory mediators including NO, TNF-α, IL-1β, CXCL2 (MIP-2), and CCL2 (MCP-1) (9, 10). Among these molecules, TNF-α plays an important role in the generation of a protective anti-bacterial immune response during brain abscess development as well as regulating blood-brain barrier permeability (14–17). Since TNF-α is a major product of *S. aureus*-activated astrocytes and these cells also express TNF-α receptors (18), it is possible that TNF-α released upon bacterial exposure may feedback in autocrine/paracrine manner to further modulate astrocyte anti-bacterial responses. However, the functional importance of TNF-α in influencing downstream effector responses of astrocytes following bacterial stimulation has not yet been described.

Ligand binding of TNF-α to TNF-α receptor 1 (TNFR1) leads to the activation of several signal transduction cascades including NF-κB (19, 20). NF-κB is a key transcription factor for regulating TLR expression as well as proinflammatory mediator production in several immune cell types (21–23). Upon ligation with TNF-α, TNFR1 trimerizes with adjacent TNFR1s leading to the recruitment of several proteins to its cytoplasmic domain including receptor interacting protein, TNFR1-associated death domain protein, and TRAF2, ultimately culminating in NF-κB activation (19, 20). In unstimulated cells, NF-κB resides in the cytoplasm in an inactive form associated with IκBs. In response to cytokines and bacterial products, the inhibitor of κB kinase (IKK)[3] complex is

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[3] Abbreviations used in this paper: IKK, inhibitor of κB kinase; CAPE, caffeic acid phenethyl ester; WT, wild type; iNOS, inducible NO synthase; KO, knockout; ROS, reactive oxygen species.

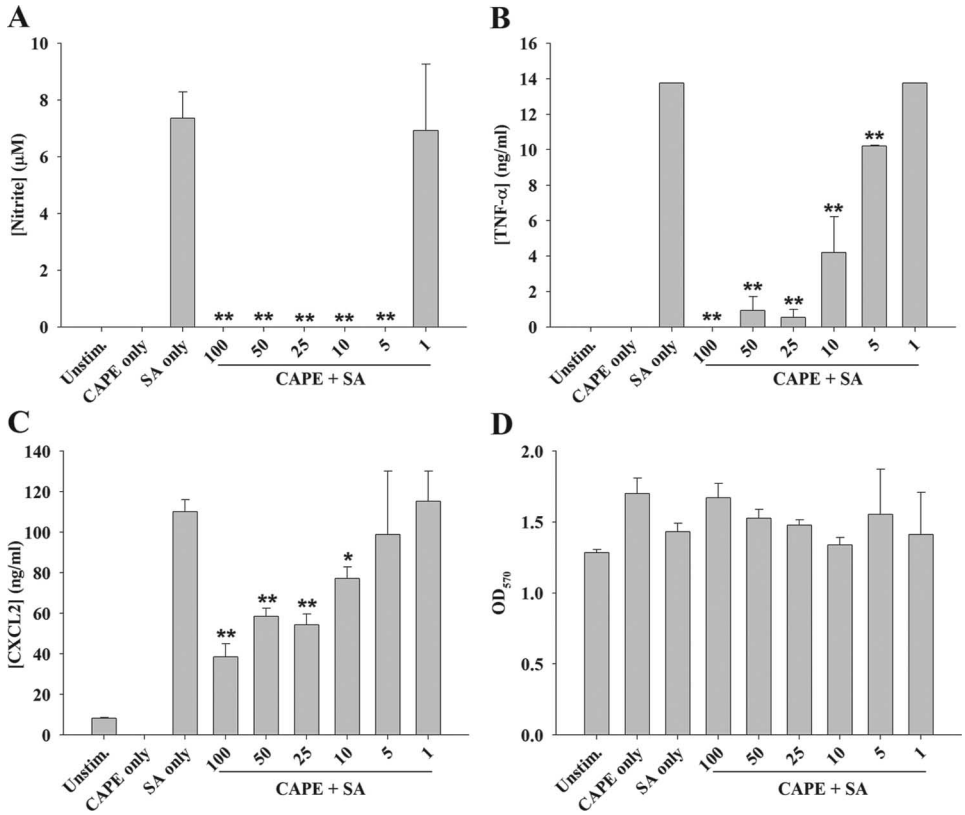

FIGURE 1. The broad spectrum NF-κB inhibitor CAPE attenuates proinflammatory mediator production by *S. aureus*-stimulated astrocytes. Primary astrocytes were seeded in 96-well plates at 1×10^5 cells per well and incubated overnight. The following day, cells were pretreated for 1 h with the indicated concentrations of CAPE (1–100 μM) followed by stimulation with 10^7 CFU of heat-inactivated *S. aureus* (SA). Cell-free supernatants were collected at 24 h following bacterial exposure and analyzed for nitrite (*A*), TNF-α (*B*), and CXCL2 (*C*) production. Astrocyte viability was assessed using a standard MTT assay and the raw OD_{570} absorbance values are provided (*D*). Results are reported as the mean ± SD of three independent wells for each experimental treatment. Significant differences between *S. aureus*-stimulated astrocytes vs *S. aureus* plus CAPE are denoted with asterisks (*, $p < 0.05$; **, $p < 0.001$). Results are representative of four independent experiments.

activated, leading to the phosphorylation, ubiquitination, and subsequent degradation of IκBα (24–26). The liberated NF-κB complex then translocates to the nucleus and initiates the transcriptional activation of target genes. Although it is presumed due to the ability of *S. aureus* to trigger TLR2 activation (27), the relative importance of NF-κB signaling in astrocyte responses to *S. aureus* has not yet been demonstrated. In this study, we report that NF-κB is pivotal for regulating astrocyte activation in response

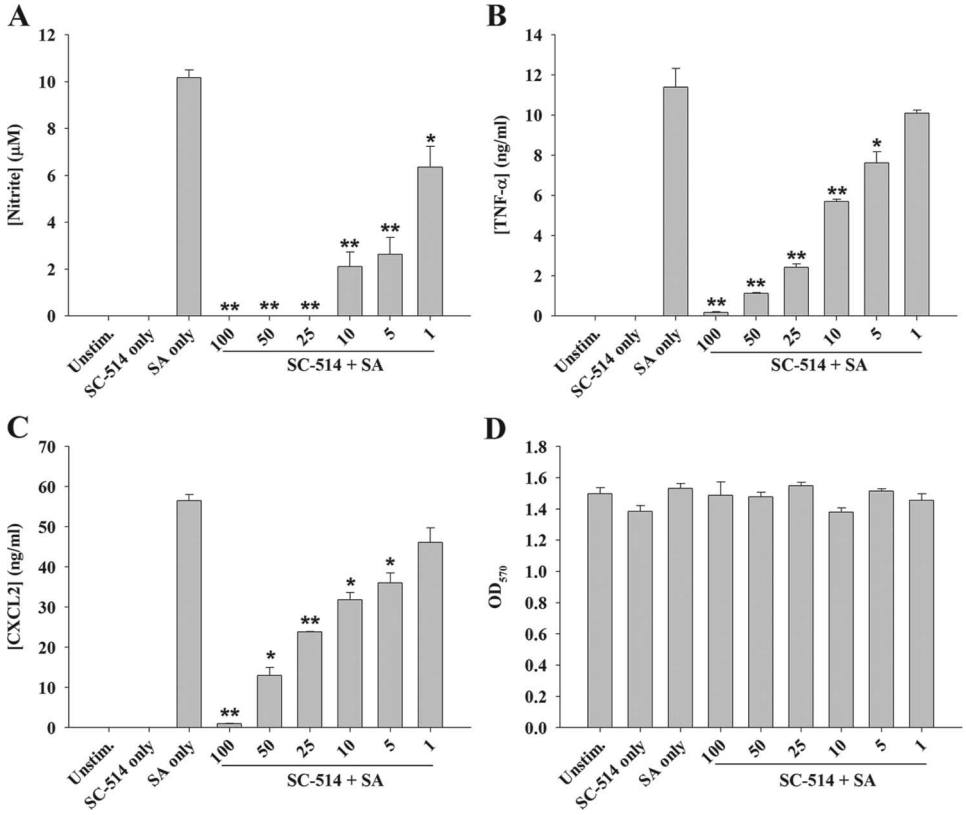

FIGURE 2. The IKK inhibitor SC-514 attenuates astrocytic proinflammatory mediator release in response to *S. aureus*. Primary astrocytes were seeded in 96-well plates at 1×10^5 cells per well and incubated overnight. The following day, cells were pretreated for 1 h with the indicated concentrations of SC-514 (1–100 μM) followed by stimulation with 10^7 CFU of heat-inactivated *S. aureus* (SA). Cell-free supernatants were collected at 24 h following bacterial exposure and analyzed for nitrite (*A*), TNF-α (*B*), and CXCL2 (*C*) production. Astrocyte viability was assessed using a standard MTT assay and the raw OD_{570} absorbance values are provided (*D*). Results are reported as the mean ± SD of three independent wells for each experimental treatment. Significant differences between *S. aureus*-stimulated astrocytes vs *S. aureus* plus SC-514 are denoted with asterisks (*, $p < 0.05$; **, $p < 0.001$). Results are representative of two independent experiments.

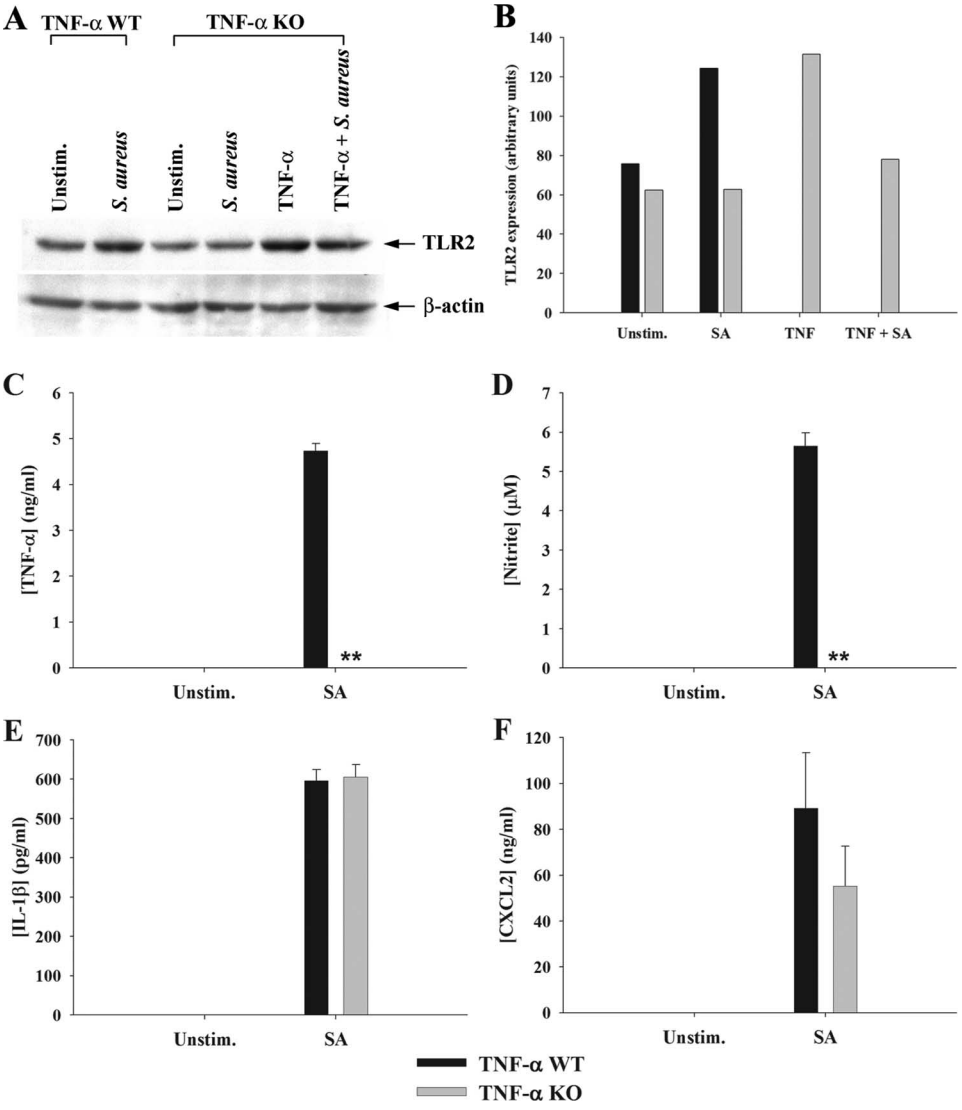

FIGURE 3. Astrocytes produce TNF-α in response to *S. aureus* that functions in an autocrine/paracrine manner to augment TLR2 and NO expression. Primary astrocytes isolated from TNF-α WT or KO mice were stimulated with heat-inactivated *S. aureus* (SA) ± 100 ng/ml of recombinant mouse TNF-α for 24 h, whereupon whole cell extracts were prepared and analyzed for TLR2 expression by Western blotting (*A* and *B*). Results are presented as the raw gel data (*A*) and quantitative analysis of TLR2 expression by densitometry (*B*). For quantitation in *B*, the pixel intensity of each TLR2 band was normalized to the amount of actin to verify uniformity in gel loading. In addition, cell-free supernatants were collected at 24 h following bacterial exposure and analyzed for TNF-α (*C*), nitrite (*D*), IL-1β (*E*), and CXCL2 (*F*) production. Results are reported as the mean ± SD of three independent wells for each experimental treatment (*C–F*). Significant differences between TNF-α WT and KO astrocytes stimulated with *S. aureus* are denoted with asterisks (**, $p < 0.001$). Results are representative of two (*A* and *B*) or six (*C–F*) independent experiments.

to *S. aureus* since three discrete inhibitors of the NF-κB signaling pathway attenuated proinflammatory mediator production. In addition, *S. aureus*-induced TLR2 expression was found to be mediated via NF-κB as well as autocrine/paracrine effects of TNF-α. Similar effects on astrocytic TLR2 levels were observed in response to the TLR4 ligand LPS. Interestingly, the results also identify autocrine/paracrine TNF-α as a pivotal regulator of inducible NO synthase (iNOS) induction in response to diverse microbial stimuli. Collectively, these findings suggest that TNF-α plays an important role in amplifying the immune response of astrocytes upon bacterial exposure, in part, by augmenting TLR2 expression.

Materials and Methods

Mouse strains

TNF-α knockout (KO) mice on a C57BL/6 background were obtained from The Jackson Laboratory. C57BL/6 mice were used as a source of wild-type (WT) astrocytes and were purchased from Harlan Sprague Dawley. The animal use protocol was approved by the University of Arkansas for Medical Sciences Animal Care and Use Committee and is in accord with National Institutes of Health guidelines for the use of rodents.

Primary astrocyte cell culture and reagents

Primary astrocytes were derived from C57BL/6 WT or TNF-α KO mice (1–4 days of age) as previously described (9, 10). One concern when working with primary astrocytes relates to the issue of cell purity (28). The summation of several lines of evidence indicates that the results obtained in the current study can be attributed to astrocytes. First, we subjected our mixed glial cultures to a shaking protocol for a minimum of three occurrences, which progressively diminished the number of contaminating microglia over time. Second, upon transitioning mixed glial cultures for astrocyte experiments, the culture medium was supplemented with 0.1 mM L-LME, a microglial cytotoxic agent that has been used extensively as a method for microglial depletion (9, 10, 28, 29). Astrocytes were cultured in L-LME-containing medium for at least 2 wk before use in experiments and were not used until the third passage in culture (approximately days 35–42 in vitro). Continued passage of astrocytes also facilitated the elimination of residual microglia that remained firmly attached to the tissue culture flask surface following trypsinization to recover astrocytes. Similar approaches have been cited by other laboratories (30). A final observation to confirm the relative purity of astrocyte cultures prepared in this manner is provided by our extensive experience working with *S. aureus* and its cell wall product peptidoglycan in glia. Our studies have reproducibly demonstrated that these Gram-positive microbial stimuli are poor inducers of iNOS expression and subsequent NO production in primary microglia, whereas they are potent activators of iNOS and NO in astrocytes (9, 31, 32). The purity of astrocyte cultures used in these studies as determined by glial fibrillary acidic protein immunoreactivity and the absence of CD11b staining was routinely ≥95%.

Reagents

Recombinant mouse TNF-α was purchased from BD Biosciences in a low endotoxin/no azide form. Caffeic acid phenethyl ester (CAPE), a nonspecific inhibitor of the NF-κB pathway (33), and BAY 11-7082 and SC-514,

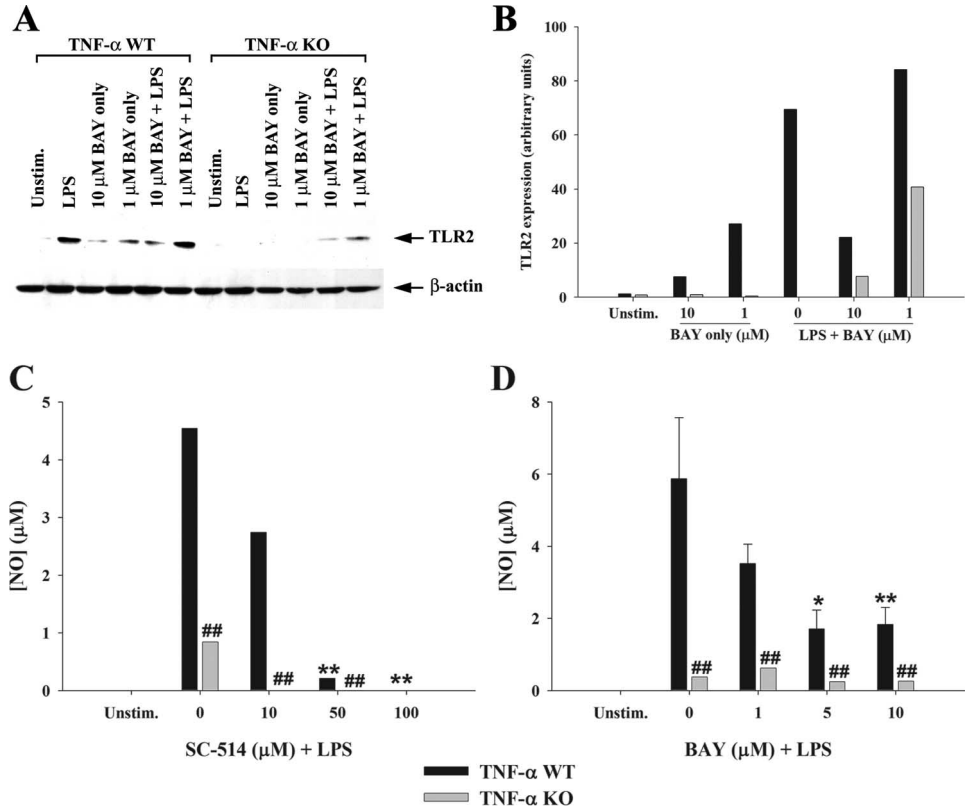

FIGURE 4. Autocrine/paracrine TNF-α and NF-κB-dependent signaling augment TLR2 and NO expression in response to LPS stimulation. Primary astrocytes isolated from TNF-α WT or KO mice were pretreated with the indicated concentrations of the IKK or IkBα inhibitors SC-514 or BAY 11-7082, respectively, for 1 h followed by LPS (100 ng/ml). Whole cell extracts were prepared 24 h following LPS stimulation and analyzed for TLR2 expression by Western blotting (*A* and *B*). Results are presented as the raw gel data (*A*) and quantitative analysis of TLR2 expression by densitometry (*B*). For quantitation in *B*, the pixel intensity of each TLR2 band was normalized to the amount of actin to verify uniformity in gel loading. In addition, cell-conditioned supernatants were collected 24 h following LPS exposure, whereupon nitrite levels were determined (*C* and *D*). Significant differences between WT astrocytes treated with LPS only vs LPS plus NF-κB inhibitors are denoted with asterisks (*, $p < 0.05$; **, $p < 0.001$), whereas alterations between TNF-α WT vs KO astrocytes are indicated by hatched signs (##, $p < 0.001$). Results are representative of two independent experiments.

potent inhibitors of IκB-α phosphorylation and IKK-2, respectively (34–36), were purchased from Calbiochem. Heat-inactivated *S. aureus* (strain RN6390; kindly provided by Dr. Ambrose Cheung, Dartmouth Medical School, Hanover, NH) was prepared as previously described (37) and *E. coli* O11:B1 LPS was obtained from List Biological Laboratories. All non-LPS reagents and culture media were verified to have endotoxin levels < 0.03 EU/ml as determined by *Limulus* amebocyte lysate assay (Associates of Cape Cod).

Nitrite assay

Nitrite, a stable end product of resulting from the reaction of NO with molecular oxygen, was used to quantitate NO levels in astrocyte-conditioned supernatants as previously described (10).

Cell viability assays

The effects of NF-κB inhibitors on astrocyte cell viability were evaluated using a standard MTT assay as previously described (9).

ELISA

Quantitation of cytokine and chemokine levels in astrocyte-conditioned medium was performed using standard sandwich ELISA kits according to the manufacturer's instructions (OptEIA mouse IL-1β and TNF-α; BD Biosciences; and DuoSet mouse CXCL2; R&D Systems).

Protein extraction and Western blotting

Protein extracts were prepared from primary astrocytes as previously described (38) and quantified using a standard protein assay (bicinchoninic acid protein assay reagent, BCA; Bio-Rad). TLR2 and iNOS expression was evaluated by Western blot using goat anti-mouse TLR2 (R&D Systems) or rabbit anti-mouse iNOS (Santa Cruz Biotechnology) polyclonal Abs as previously described (38). For quantitation, nonsaturated autoradiographs were scanned, and the pixel intensity for each band was determined using the Image/J program (NIH Image) and normalized to the amount of actin. Results are expressed in arbitrary units as the ratio of TLR2 or iNOS to actin.

Statistics

Significant differences between experimental groups were determined using the Student's *t* test at the 95% confidence interval using Sigma-Aldrich.

Results

The NF-κB pathway plays a pivotal role in inducing inflammatory mediator production by astrocytes in response to S. aureus

Astrocytes recognize *S. aureus* via TLR2, which results in the downstream production of numerous proinflammatory mediators (9). TLR2 engagement leads to the activation of NF-κB and p38 MAPK signaling pathways (22, 23); however, the relative importance of the former has not yet been demonstrated in astrocytes in response to *S. aureus*. To evaluate the role of NF-κB signaling, astrocytes were treated with CAPE, a broad spectrum NF-κB inhibitor (33). Pretreatment of cells with various concentrations of CAPE for 1 h before *S. aureus* exposure led to a dose-dependent inhibition of NO, TNF-α, and CXCL2 expression (Fig. 1, *A–C*, respectively). Cell viability assays revealed

that CAPE was not cytotoxic at any of the concentrations examined, indicating that the anti-inflammatory effects observed were not due to cell death (Fig. 1*D*).

Since CAPE is a rather nonspecific inhibitor of NF-κB, the importance of this signaling cascade was confirmed with the use of additional, more specific blockers. Similar to what was observed with CAPE, the IKK complex inhibitor SC-514 was also capable of attenuating NO, TNF-α, and CXCL2 expression in a dose-dependent manner (Fig. 2, *A–C*, respectively). Likewise, BAY 11-7082, which affects NF-κB signaling downstream by blocking IκBα phosphorylation, also inhibited proinflammatory mediator release from astrocytes following *S. aureus* exposure (data not shown). Cell viability assays revealed that neither SC-514 nor BAY 11-7082 were cytotoxic at any of the concentrations examined, indicating that the inhibitory actions of these compounds were not attributable to cell death (Fig. 2*D* and data not shown). Collectively, these findings indicate that NF-κB signaling is a primary pathway for inducing a broad range of astrocytic proinflammatory mediators in response to *S. aureus*.

Astrocytes produce TNF-α upon S. aureus *stimulation that acts in an autocrine/paracrine manner to augment TLR2 expression*

Besides inducing proinflammatory mediator release, another consequence of *S. aureus* stimulation in astrocytes is an increase in TLR2 expression (9, 10). Since TNF-α is a major product of *S. aureus*-activated astrocytes, it was plausible that this cytokine may regulate TLR2 expression in an autocrine/paracrine fashion. To investigate this possibility, TLR2 levels were assessed in both TNF-α KO and WT astrocytes in response to *S. aureus* stimulation. A pivotal role for autocrine/paracrine TNF-α in augmenting TLR2 expression was demonstrated by the fact that receptor levels remained unchanged in TNF-α KO astrocytes following bacterial exposure, whereas TLR2 expression was increased in WT cells in response to *S. aureus* (Fig. 3, *A* and *B*). Treatment of TNF-α KO astrocytes with recombinant TNF-α was capable of restoring the increase in TLR2 expression, confirming the pivotal autocrine/paracrine actions of this cytokine in response to bacterial activation (Fig. 3, *A* and *B*). Similar effects were observed following LPS stimulation of astrocytes (Fig. 4, *A* and *B*), suggesting that TNF-α is an important regulator of astrocytic TLR2 expression in response to diverse microbial stimuli. Although a slight increase in TLR2 expression was observed in TNF-α KO astrocytes in response to the combination of LPS plus BAY 11-7082, this was not a consistent finding and is considered to represent biological variation. Although LPS is not a TLR2 ligand, previous studies from other groups have demonstrated that LPS augments TLR2 expression in glia (13, 39–41). The novel finding in this study is that this increase is modulated by the autocrine/paracrine action of TNF-α.

S. aureus*-induced NO production is TNF-α-dependent*

In addition to investigating the role of TNF-α in regulating TLR2 expression, we also examined whether proinflammatory mediator production was affected upon TNF-α loss. As expected, TNF-α KO astrocytes did not produce TNF-α in response to bacterial challenge, confirming the absence of cytokine in KO cells (Fig. 3*C*). Interestingly, *S. aureus*-induced NO production was completely ablated in TNF-α KO astrocytes compared with WT cells (Fig. 3*D*), whereas the expression of numerous other proinflammatory mediators (i.e., IL-1β and CXCL2) was not affected (Fig. 3, *E* and *F*). We also examined iNOS expression in *S. aureus*-stimulated TNF-α WT and KO astrocytes and, similar to our findings with NO release, iNOS was not induced in TNF-α KO cells, whereas TNF-α restoration led to an increase in iNOS expression (Fig. 5). A similar TNF-α- and NF-κB-dependence for NO production was observed in response to LPS stimulation (Fig. 4, *C* and *D*). Collectively, these results suggest that TNF-α produced by astrocytes in response to diverse microbial stimuli functions in an autocrine/paracrine manner to induce subsequent NO production.

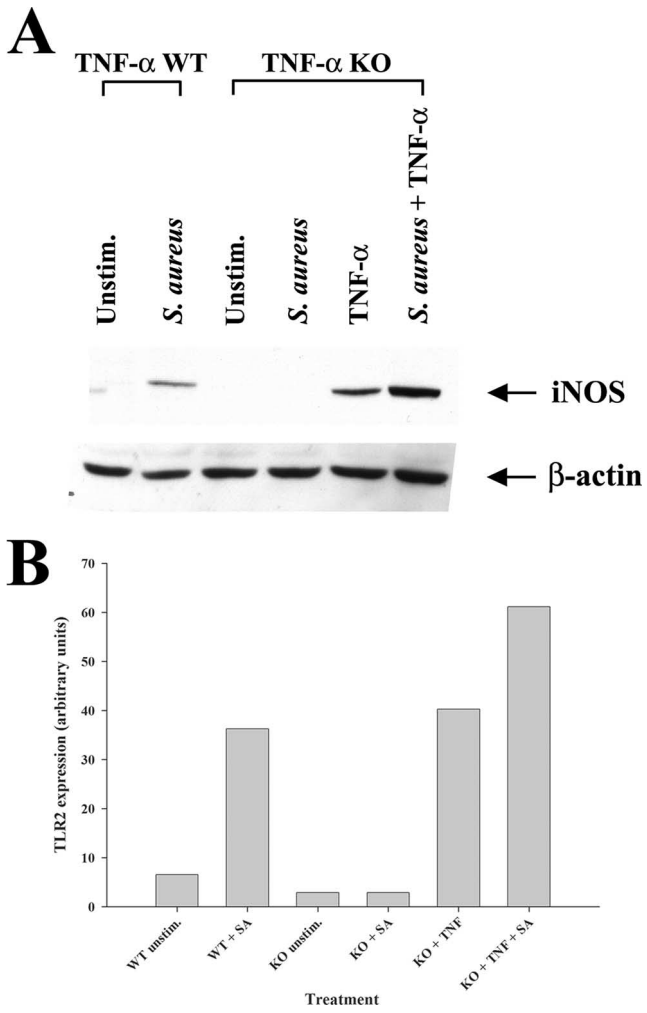

FIGURE 5. iNOS expression is regulated by the autocrine/paracrine action of TNF-α in response to *S. aureus*. Primary astrocytes isolated from TNF-α WT or KO mice were seeded in 6-well plates at 1×10^6 cells per well and incubated overnight. The following day, cells were stimulated with 10^8 CFU of heat-inactivated *S. aureus* ± 100 ng/ml of recombinant mouse TNF-α for 24 h, whereupon whole cell extracts were prepared and analyzed for iNOS expression by Western blotting. Results are presented as the raw gel data (*A*) and quantitative analysis of iNOS expression by densitometry (*B*). For quantitation in *B*, the pixel intensity of each iNOS band was normalized to the amount of actin to verify uniformity in gel loading. Results are expressed in arbitrary units as the ratio of iNOS to actin and are representative of two independent experiments.

Autocrine/paracrine IL-1β is not sufficient for astrocytic NO production in response to microbial stimuli

Previous studies have reported that IL-1β is a major inducer of NO production in astrocytes, whereas TNF-α had relatively little effect (42–45). These findings contradict our results with TNF-α KO astrocytes, where a strict TNF-α-dependence for NO release was demonstrated. However, our studies differ since a microbial stimulus was applied to astrocytes, which elicits a complex milieu of inflammatory mediators that may rely on the autocrine/paracrine action of TNF-α to induce maximal NO release. To evaluate the effects of IL-1β on NO production, we treated TNF-α KO and WT

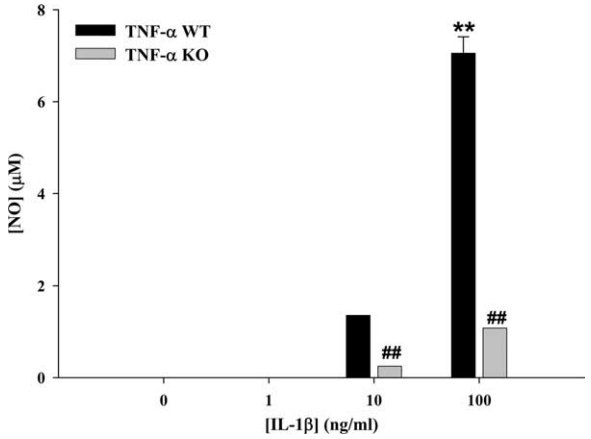

FIGURE 6. High dose IL-1β is capable of inducing NO production in primary astrocytes. TNF-α WT and KO astrocytes were seeded in 96-well plates at 1×10^5 cells per well and incubated overnight. The following day, cells were treated with the indicated concentrations of IL-1β for 24 h, whereupon nitrite levels were determined. Significant differences between unstimulated and IL-1β-treated WT astrocytes are denoted with asterisks (**, $p < 0.001$), whereas alterations between TNF-α WT vs KO astrocytes are indicated by hatched signs (##, $p < 0.001$). Results are representative of two independent experiments.

astrocytes with various concentrations of recombinant mouse IL-1β (i.e., 1–100 ng/ml). The results demonstrated that high doses of IL-1β (i.e., 10 and 100 ng/ml) were capable of inducing NO release from primary astrocytes; however, the lowest concentration of IL-1β tested (i.e., 1 ng/ml) was not sufficient to elicit NO production (Fig. 6). This is an important finding since the amount of IL-1 produced by *S. aureus*-activated astrocytes is significantly less compared with the lowest dose of IL-1β examined (i.e., <1 ng/ml). Interestingly, NO levels generated by high dose IL-1β in TNF-α KO astrocytes were ~7-fold lower compared with WT cells, revealing an important role for TNF-α in regulating NO expression even in response to IL-1β treatment. Collectively, these results indicate that although IL-1β is capable of inducing NO production in primary astrocytes, the level of cytokine required for this response well exceeds that typically produced by astrocytes in response to *S. aureus* stimulation. Therefore, an autocrine/paracrine effect of IL-1β does not appear to influence NO production by primary astrocytes; rather, our data suggests that TNF-α is a major player in dictating NO release.

NF-κB-dependent signaling is pivotal for the TNF-α-induced increase in astrocytic TLR2 expression

In several cell types of the innate immune system including macrophages and microglia, TLR2 expression is driven, in part, via NF-κB (46–48). In addition, TNF-α also triggers the NF-κB signaling pathway (19, 20). However, the involvement of NF-κB in regulating astrocytic TLR2 expression in response to TNF-α has not yet been examined. To determine whether TNF-α-induced TLR2 expression in astrocytes is mediated via a NF-κB signaling pathway, we used the same series of NF-κB pathway inhibitors previously described. As expected, TNF-α exposure augmented TLR2 expression in astrocytes, which was attenuated upon pretreatment with either CAPE, SC-514, or BAY 11-7082 in a dose-dependent manner (Fig. 7). Collectively, these results suggest that

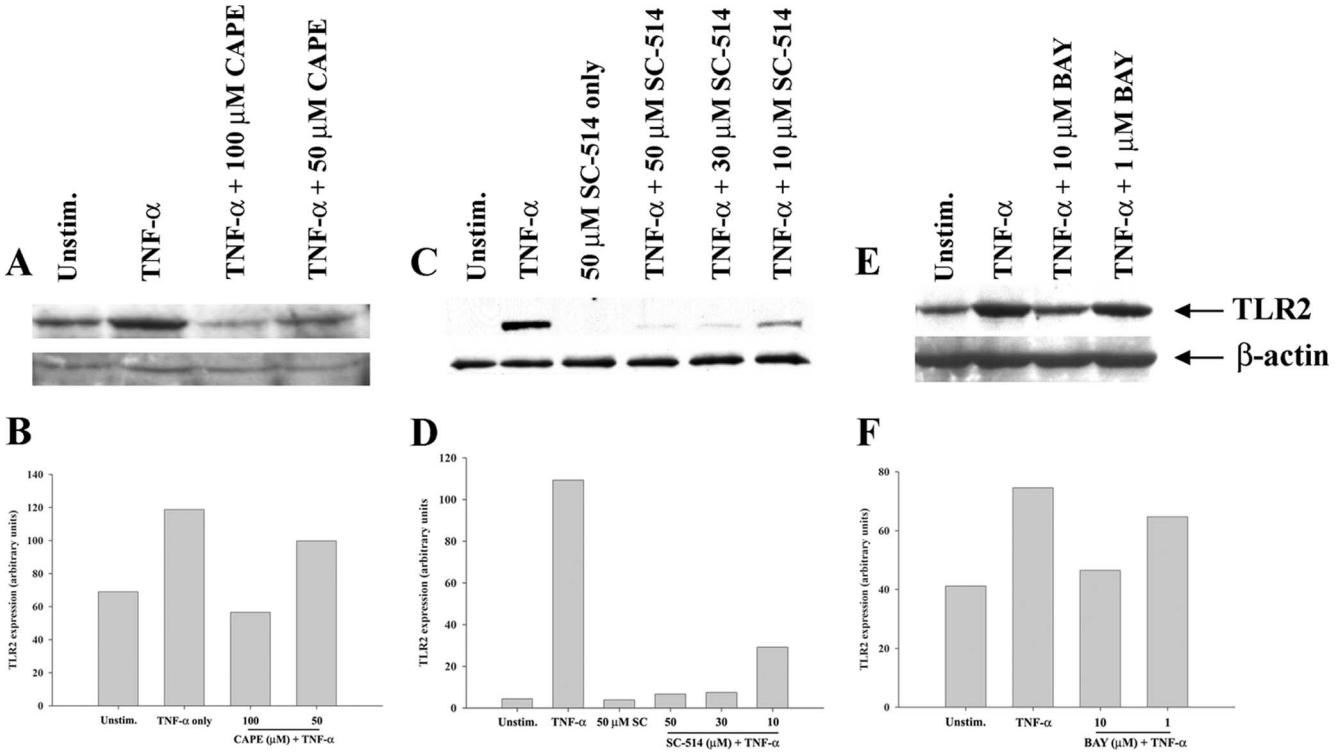

FIGURE 7. Three distinct NF-κB inhibitors attenuate astrocytic TNF-α-induced TLR2 expression. Primary astrocytes were seeded in 6-well plates at 1×10^6 cells per well and incubated overnight. The following day, cells were pretreated with various concentrations of CAPE (*A* and *B*), SC-514 (*C* and *D*), or BAY 11-7082 (*E* and *F*) for 1 h before TNF-α stimulation (100 ng/ml) for 24 h, whereupon whole cell extracts were prepared and analyzed for TLR2 expression by Western blotting. Results are presented as the raw gel data (*A*, *C*, and *E*) and quantitative analysis of TLR2 expression by densitometry (*B*, *D*, and *F*). For quantitation, the pixel intensity of each TLR2 band was normalized to the amount of actin to verify uniformity in gel loading. Results are expressed in arbitrary units as the ratio of TLR2 to actin and are representative of three independent experiments.

NF-κB represents a major signaling cascade responsible for the TNF-α-mediated induction of TLR2 expression in astrocytes.

Discussion

Initially thought to act as mere supporting cells, emerging evidence indicates that astrocytes participate in innate immune responses to injurious or infectious stimuli (49–51). Despite the important role TLR2 plays in *S. aureus* recognition by astrocytes (9), the cell signaling cascades regulating TLR2 expression and subsequent proinflammatory mediator production in activated astrocytes remained to be defined. Based on the known downstream signaling cascades triggered by TLR2 activation (23, 52), NF-κB was envisioned as the most likely pathway involved in regulating *S. aureus*-induced astrocyte activation. Indeed, other studies have reported that the NF-κB pathway is important for eliciting astrocytic proinflammatory mediator production in response to the TLR4 ligand LPS (53–55). To determine whether the NF-κB pathway participates in regulating astrocytic responses to *S. aureus*, we performed studies using three distinct pharmacological inhibitors acting against discrete steps along the NF-κB pathway. The results indicated that NF-κB signals are a significant contributor toward inflammatory mediator release in astrocytes following *S. aureus* exposure.

Subsequent studies revealed that TNF-α is pivotal for regulating *S. aureus*- and LPS-induced TLR2 expression since TNF-α KO astrocytes failed to up-regulate TLR2 upon bacterial exposure. Additional evidence to support this relationship was provided when TLR2 levels were restored upon treatment of TNF-α KO astrocytes with recombinant TNF-α. The effects of TNF-α were observed at concentrations that approximate those detected during brain abscess development (16, 17), although it is difficult to make direct comparisons due to the likelihood that TNF-α concentrations in various tissue microdomains may dramatically differ. To our knowledge, this is the first report to demonstrate the autocrine/paracrine action of any inflammatory mediator on TLR2 expression in astrocytes.

NF-κB is one of the primary signaling cascades triggered by TNF-α binding to its receptor (19, 20). Based on the finding that NF-κB inhibitors attenuated astrocytic inflammatory mediator release and the fact that the mouse TLR2 promoter contains several consensus NF-κB binding motifs (46, 47, 56), we evaluated whether NF-κB influences TLR2 expression in astrocytes following TNF-α exposure. We found that TNF-α-induced TLR2 expression in astrocytes is NF-κB-dependent since all of the NF-κB inhibitors tested blocked the characteristic increase in TLR2 expression observed following cytokine treatment. In addition, NF-κB-dependent signaling was also found to regulate the characteristic increase in astrocytic TLR2 expression observed following both *S. aureus* and LPS stimulation. Although LPS is not a TLR2 ligand, other studies have also demonstrated that LPS augments TLR2 levels (13, 39–41). Increasing TLR2 expression may facilitate more efficient pathogen recognition since TLR2 also triggers cytokine signaling pathways in response to bacterial lipoproteins that are contained within the outer cell walls of both Gram-positive and -negative bacteria. However, this possibility remains speculative. Collectively, these observations are in agreement with previous reports where the NF-κB pathway has been shown to regulate TLR2 expression in various immune cells in response to select stimuli including microglia and macrophages (46–48, 56).

Next we evaluated whether TNF-α loss affected the production of additional immune molecules in astrocytes. Interestingly, we found that TNF-α loss resulted in a selective suppression of NO production in both *S. aureus*- and LPS-stimulated astrocytes. This observation is in agreement with earlier studies where TNF-α has been shown to induce iNOS expression and subsequent NO production. For example, iNOS expression in response to β-amyloid plus IFN-γ stimulation was shown to be mediated, in part, by TNF-α through a TRAF6-, TRAF2-, and NF-κB-inducing kinase-dependent pathway in astrocytes (57). Another recent report has demonstrated that in macrophages, TNF-α is required for iNOS induction in response to IFN-γ via a NF-κB-dependent mechanism (58). However, an important distinction between these reports and our work is the lack of IFN-γ involvement in the current study. In contrast to these reports, others have demonstrated that IL-1β is a major inducer of iNOS in rodent astrocytes, whereas TNF-α is not effective by itself but rather requires the presence of an additional cytokine such as IL-1β or IFN-γ (42–45). Because of these discrepancies, we directly examined whether IL-1β was capable of inducing NO production in TNF-α WT and KO astrocytes. Only high concentrations of IL-1β (i.e., 10–100 ng/ml) were effective at inducing NO release in primary WT astrocytes, which greatly exceeds levels normally produced by *S. aureus*-activated astrocytes (i.e., <1 ng/ml). Collectively, these findings suggest that TNF-α is a major regulator of astrocytic NO production in response to microbial stimuli. The complex interplay between multiple inflammatory mediators in regulating astrocytic NO production is similar to earlier studies by others (30, 45). Specifically, these authors demonstrated that IFN-γ primed astrocytes for maximal TNF-α release in response to LPS stimulation, whereas either stimulus alone was not effective. Likewise, a combination of IFN-γ plus IL-1β was also capable of inducing TNF-α production, revealing synergistic effects of distinct inflammatory mediators (30). This theme of synergy is similar to our findings where endogenous TNF-α is required for NO production in response to microbial stimuli. Surprisingly, we did not observe any consistent differences in the expression of other proinflammatory mediators between TNF-α KO and WT astrocytes. One possibility to explain this finding could stem from the fact that iNOS is a late inducible gene and typically requires priming by alternative mediators such as IFN-γ or IFN-β (30, 44, 58, 59). This is generally not the case for the other inflammatory mediators examined in this study (i.e., IL-1β and CXCL2), which are induced rapidly upon cell activation. However, we cannot exclude the possibility that TNF-α KO astrocytes may produce low levels of NO upon bacterial exposure with extended incubation periods (i.e., 48 h). In addition, IL-1β is unique in that it exists intracellularly in a preformed state and requires processing by caspase-1 for maturation into the biologically active cytokine (60, 61). Therefore, inherent differences in the timing of gene expression following cell activation and processing pathways might be responsible, in part, for the selective influence of TNF-α on NO production. It remains possible that TNF-α impacts alternative inflammatory mediators in astrocytes that were not examined in the current study, an issue that remains to be investigated.

It is interesting that a complete blockade of NO production was observed in TNF-α KO astrocytes following *S. aureus* stimulation since iNOS expression is regulated by several distinct pathways (42). Although we have demonstrated that NF-κB activation is important for regulating iNOS expression, the precise mechanism(s) responsible for this outcome remain to be defined. However, several possibilities may be considered when contemplating the mechanism by which TNF-α dictates iNOS expression in astrocytes. First, other stimuli such as LPS plus IFN-γ induce the production of reactive oxygen species (ROS) that have been reported to positively regulate iNOS expression via NF-κB (44, 62). In this instance, the loss of TNF-α may indirectly affect NO production by interfering with ROS release; however, the ability of *S.*

aureus to induce ROS production has not yet been examined. Second, compounds that increase cAMP have been shown to inhibit LPS- and cytokine-induced NO production in several cell types including astrocytes (59, 63, 64); therefore, it is possible that cAMP levels may be elevated in TNF-α KO astrocytes following *S. aureus* treatment. A similar relationship could occur with regard to PI3K, since elevated PI3K levels have been associated with an increase in iNOS expression (65). In addition, NF-κB activation in response to TNF-α requires PI3K activity (66). Since we did not measure cAMP or PI3K levels in response to bacterial exposure, these possibilities remain highly speculative. Finally, a recent study has shown that TNF-α induces the expression of GTP cyclohydrolase, the rate-limiting enzyme for tetrahydrobiopterin synthesis, an important cofactor for iNOS activity (67). In the absence of TNF-α, this cofactor may not be induced, which could also lead to a reduction in iNOS expression in agreement with our findings.

Previous studies from our laboratory and others have revealed an important role for TNF-α in the host anti-bacterial immune response during brain abscess development (16, 17). By extension, these findings suggest that priming the CNS by TNF-α administration may be a potential method to augment *S. aureus* recognition and inactivation through elevated TLR2 and NO expression. Particularly, heightened NO production may facilitate efficient pathogen clearance via the formation of peroxynitrite (formed by the reaction between NO and hydrogen peroxide). However, a recent study from our laboratory has demonstrated that although TNF-α is capable of increasing TLR2 expression in microglia, this did not translate into enhanced cytokine production or phagocytosis (48), although bactericidal activity was not examined. It remains possible that enhanced TLR2 expression may significantly impact the host immune response during brain abscess development since the treatment paradigm used during our in vitro studies is much less complex compared with what occurs during CNS infections such as brain abscess.

Collectively, these results suggest that *S. aureus*-induced astrocyte activation is mediated via a NF-κB pathway involving TNF-α as an important effector molecule for amplifying TLR2 expression. The identification of such regulatory pathways and their potent inhibitors might provide effective and safe treatment options for diseases, such as brain abscess, where persistent inflammation contributes to disease pathology.

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Disclosures

The authors have no financial conflict of interest.

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