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TAM Receptors Affect Adult Brain Neurogenesis by Negative Regulation of Microglial Cell Activation

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TAM tyrosine kinases play multiple functional roles, including regulation of the target genes important in homeostatic regulation of cytokine receptors or TLR-mediated signal transduction pathways. In this study, we show that TAM receptors affect adult hippocampal neurogenesis and loss of TAM receptors impairs hippocampal neurogenesis, largely attributed to exaggerated inflammatory responses by microglia characterized by increased MAPK and NF-κB activation and elevated production of proinflammatory cytokines that are detrimental to neuron stem cell proliferation and neuronal differentiation. Injection of LPS causes even more severe inhibition of BrdU incorporation in the Tyro3/−/−Axl/−/−Mertk/−/− triple-knockout (TKO) brains, consistent with the LPS-elicited enhanced expression of proinflammatory mediators, for example, IL-1β, IL-6, TNF-α, and inducible NO synthase, and this effect is antagonized by coinjection of the anti-inflammatory drug indomethacin in wild-type but not TKO brains. Conditioned medium from TKO microglia cultures inhibits neuron stem cell proliferation and neuronal differentiation. IL-6 knockout in Axl/−/−Mertk/−/− double-knockout mice overcomes the inflammatory inhibition of neurogenesis, suggesting that IL-6 is a major downstream neurotoxic mediator under homeostatic regulation by TAM receptors in microglia. Additionally, autonomous trophic function of the TAM receptors on the proliferating neuronal progenitors may also promote progenitor differentiation into immature neurons. The Journal of Immunology, 2013, 191: 6165–6177.

Microglial cells, a diverse set of innate immune cells distributed throughout the entire CNS, actively scan the CNS microenvironment (1) and provide trophic or maintenance support for normal neuron activity (2). Serving as a major immunosurveillance cell type in the CNS (3), microglia express all necessary receptors and molecules for recognition of invading microbes, pathogenic stimuli, proinflammatory cytokines, and cellular debris (spent or damaged neuronal organelles). When activated, they are able to mount rapid innate immune responses with increased production of proinflammatory cytokines and chemokines not only in response to systemic infection, but also to brain injury and chronic degenerative diseases (4–9). However, chronic inflammation and uncontrolled activation of microglia are detrimental to neuronal functions and neurogenesis (10).

Microglial cells express TLRs, which can be activated by endogenous and exogenous ligands (11–13). Activation of TLRs causes rapid activation of microglial cells and initiates multiple downstream signaling pathways, with the most common being the Erk1/2 and p38 MAPK pathway and the IκB kinase (IKK)/NF-κB signal transduction pathway, which lead, respectively, to activation of AP-1 or NF-κB and their subsequent nuclear binding to AP-1 and κB binding sites on the promoters of multiple proinflammatory genes (14–18). LPS from Gram-negative bacteria binds specifically to TLR4 on microglia and triggers intracellular signaling through the MAPK or IKK/NF-κB pathway, leading to rapid transcriptional activation of innate immune-responsive genes, including those coding for IL-1β, IL-6, and TNF-α. Although microglia are vital in immune surveillance and in defending the CNS from foreign or local danger, unregulated and prolonged activation of brain-resident microglia is detrimental to normal brain function and neuronal survival. There is evidence that systemic or local chronic inflammation in the CNS is detrimental not only to normal neural function (19), but also to the neurogenesis and differentiation of neuronal stem cells (NSCs) into immature neurons (10, 20–24). LPS-elicited microglial inflammation induces the release of proinflammatory cytokines affecting NSC proliferation in vitro and inhibiting hippocampal neurogenesis and neuronal differentiation, and these negative effects are antagonized by immunosuppressive drugs (21–28).

Interestingly, microglia may have evolved to keep the brain immune response in close check. To avoid exaggerated immune responses to infection or pathogenic changes, innate immune cells, including microglia, have developed several regulatory mechanisms to terminate their own innate immune responses. The best studied mechanisms for termination of proinflammatory cytokine gene expression include 1) the rapid cytoplasmic re-expression of

Abbreviations used in this article: DC, dendritic cell; DCX, doublecortin; DG, dentate gyrus; DKO, double-knockout; GFAP, glial fibrillary acidic protein; IKK, IκB kinase; iNOS, inducible NO synthase; NSC, neural stem cell; poly(IC), polyinosinic-polycytidylic acid; qPCR, quantitative PCR; SGZ, subgranular zone; SOCS, suppressor of cytokine signaling; SVZ, subventricular zone; TAM, Tyro3, Axl, and Mertk; TKO, triple-knockout; WT, wild-type.

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IskB that inhibits NF-κB transcriptional activity, 2) the quick gain of phosphatases causing dephosphorylation of MAPKs, and 3) the efficient suppression and termination of multiple cytokine receptor signaling by newly synthesized suppressor of cytokine signaling (SOCS) protein (15) or transcriptional repressors for proinflammatory cytokine genes (29).

In a search for upstream modulators that inhibit cytokine receptor signaling, the Tyr3, Axl, and Merk (TAM) receptor tyrosine kinases, which are expressed on dendritic cells (DCs) and macrophages, were found to function as important immunomodulators (15, 30–33). This family of receptors on innate immune cells plays a pivotal inhibitory regulatory role by limiting prolonged and unrestricted signaling initially triggered by cytokines or pathogen-associated molecular patterns receptors by inhibition of NF-κB signaling and upregulation of SOCS and Twist proteins, which terminate cytokine signaling or block the binding of NF-κB to its target gene promoters (15, 29, 34). In TAM triple-knockout (TKO) mice, the loss of TAM receptors on DCs and macrophages (35–37) or other nonprofessional phagocytes (32, 38) leads to defective phagocytosis and overproduction of proinflammatory cytokines, resulting in chronic inflammation and systemic autoimmune disorders (15, 31). Development of autoimmunity in these mutant mice causes autoantibody deposition in, and autoreactive lymphocyte infiltration into, a variety of tissues, including the brain (30).

The negative effects of TAM receptors on DCs and macrophages on the innate immune response prompted us to investigate their functional roles on microglia and NSCs and the impact on adult hippocampal neurogenesis. In this study, we explored how TAM receptors regulate microglia activation and examined their effect on adult neurogenesis. We showed that microglia lacking TAM receptors were hyperactivated and produced increased amounts of proinflammatory cytokines, especially in response to activation by LPS, polyinosinic-polycytidylic acid [poly(I:C)], or CpG. This hyperreactivity of mutant microglia was shown to play a major role in the impaired hippocampal neurogenesis observed in vivo. Conditioned medium from TKO microglia cultures was found to be neurotoxic for cultivated wild-type (WT) NSCs and, of the neurotoxic substrates in conditioned medium that might be detrimental to neurogenesis, IL-6 was found to be a major player, because addition of neutralizing anti–IL-6 Ab to the conditioned medium or knockout of the IL-6 gene in TAM TKO mice restored hippocampal neurogenesis to a level comparable to that in WT controls. Additionally, loss of TAM receptors significantly impaired the differentiation process that converted neuronal progenitors to immature neurons, and this effect was not inhibited by immunosuppressive drug. This is consistent with observation that knockout of the TAM receptor ligand Gas6 decreases Bm21 incorporation into the proliferating neuronal progenitors in the brain subventricular zone (SVZ) (39). We conclude that TAM receptors play a dual role in either modulating microglia innate immune responses by damping down production of proinflammatory factors detrimental to neuronal function and NSC renewal or by providing trophic support for neuronal differentiation.

**Materials and Methods**

**Animals**

The Tyr3<sup>−/−</sup> Axl<sup>−/−</sup> Merk<sup>−/−</sup> TKO and Axl<sup>−/−</sup> Merk<sup>−/−</sup> double-knockout (DKO) mice have been described previously (40, 41). CSTBL6, Il6<sup>−/−</sup>, and Tnfα1<sup>−/−</sup> Tnfα2<sup>−/−</sup> (Tnfα1<sup>−/−</sup> Tnfα2<sup>−/−</sup>) DKO mice were purchased from The Jackson Laboratory. Il6<sup>−/−</sup> mice or Tnfα1<sup>−/−</sup> Tnfα2<sup>−/−</sup> DKO mice were bred with TKO mice to generate Il6<sup>−/−</sup> Tnfα1<sup>−/−</sup> Tnfα2<sup>−/−</sup> Tyr3<sup>−/−</sup> Axl<sup>−/−</sup> Merk<sup>−/−</sup> and Tnfα1<sup>−/−</sup> Tnfα2<sup>−/−</sup> Tyr3<sup>−/−</sup> Axl<sup>−/−</sup> Merk<sup>−/−</sup> compound mice. All animals were housed in a pathogen-free facility and were handled according to the regulations of the Institutional Animal Care and Use Committee.

**Reagents and Materials**

The NSC culture supplements B-27 (100×) and N-2 (50×) were purchased from Invitrogen (San Diego, CA). Recombinant mouse Gas6 was purchased from R&D Systems (Minneapolis, MN). LPS (Escherichia coli serotype 055:B5) and indomethacin were purchased from Sigma-Aldrich. The Abs used for Western blotting were rabbit polyclonal anti–mouse p38 or anti-mouse pErk and mouse polyclonal anti–phospho-p38 and anti-mouse Erk42/44 (all from Santa Cruz Biotechnology, Santa Cruz, CA), HRP-conjugated donkey or sheep polyclonal anti-goat/rabbit/mouse IgG (Amersham Biosciences, Piscataway, NJ), goat anti-mouse Axl, Tyr3 or Merk (Santa Cruz Biotechnology), and monoclonal mouse anti-mouse β-actin (Sigma-Aldrich). The Abs used for immunocytochemistry were monoclonal mouse anti-mouse β-tubulin III (anti-TU-1; StemCell Technologies, Vancouver, BC, Canada), anti-mouse Neun (EMD Millipore, Billerica, MA), anti-mouse glial fibrillary acidic protein (GFAP; EMD Millipore), anti-mouse doublecortin (DCX; Santa Cruz Biotechnology), and anti-mouse Iba-1 (Wako Chemicals, Richmond, VA).

**RNA isolation, cDNA synthesis, and real-time quantitative PCR**

Total RNA was extracted from cultured NSCs, microglia, or the hippocampus using TRIzol reagent (Invitrogen) for tissues or RNasey kits (Qiagen) for cultured cells. Total RNA (1 μg) from each sample was treated with DNase I to remove traces of genomic DNA, then reverse transcribed into first-strand cDNA using qScript cDNA SuperMix kits (Quanta BioSciences, Gaithersburg, MD) for real-time quantitative PCR (qPCR) analysis. A sample of 50 ng cDNA was used to quantify gene expression by qPCR using a SYBR Green–based PCR reaction mixture on an MX3005p system (Agilent Technologies, Santa Clara, CA).

**Isolation and primary culture of NSCs**

Newborn mice at postnatal days 0 or 1 were used for isolation of hippocampal stem cells. After trypsinization, the single-cell suspension at a concentration of 1 × 10<sup>5</sup> cells/ml was cultured in complete NSC medium (DMEM/F12, 1× B-27, 1× N-2, 20 ng/ml basic fibroblast factor, 20 ng/ml epidermal growth factor, 1× penicillin/streptomycin [Invitrogen]). The proliferating neuronal stem cells formed NSC spheres in 4 d.

**Isolation and primary culture of brain microglia and astrocytes**

A forebrain single-cell suspension was prepared as described above, and then 3 × 10<sup>5</sup> viable cells in 3 ml microglia culture medium (DMEM/F12 medium [Invitrogen] containing 10% FBS, 2 mM GlutaMAX, 1× penicillin/streptomycin) were plated into each 3.5-cm cell culture dish and cultured for 20 d, with the culture medium being changed every 4–5 d. After reaching confluence, the cells were washed with PBS and trypsinized with 0.06% trypsin at 37°C for 40 min, and then the trypsin was quenched with DMEM containing 10% FBS and the detached astrocytes in the culture medium were saved, transferred to a new tube, and triturated into single cells before plating in a new six-well plate. The cells still attached to the culture dishes were mainly microglia and were further cultured for 1 more day before being used for functional studies.

To assess cell purity, microglial cultures were immunostained with anti-Iba-1 Ab, showing that >98% were Iba-1<sup>+</sup>, whereas astrocyte cultures were labeled with anti-GFAP Ab and 85% were found to be GFAP<sup>+</sup>.

**Preparation of microglia- or astrocyte-conditioned medium and treatment of NSCs**

Microglia or astrocytes at 80–90% confluence were activated by addition of LPS (0.1 μg/ml, serotype 055:B5; Sigma-Aldrich) for 6 h and washed three times with PBS to remove all traces of LPS, and then fresh medium was added and culture continued for another 24 h before collection of conditioned medium. Microglia-conditioned medium (25%) was added to NSC cultures.

**Unbiased stereological estimation and statistical analysis of granular cells in the granular layer of the hippocampal dentate gyrus**

To estimate the volume and number of the granular cell layer in hippocampus, the number of granule cells was determined in every sixth section in a series of 40-μm coronal sections using the unbiased stereology method described above. Granule cells were identified by blue nuclear staining with 4′,6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR; 0.5 ng/ml in TBS for 15 min) and, in most cases, immunostained with anti-Neun Ab (1:100; EMD Millipore), and any that lay across the top focal plane or the exclusion boundaries of the unbiased sampling frame were excluded from
counting. The granule cell layer reference volume was determined by the traced granule cell areas for each section multiplied by the distance between the sections sampled, and then the mean granule cell number per disector volume was multiplied by the reference volume to estimate the total granule cell number.

Injection of LPS, indomethacin, and BrdU

To induce acute inflammation, a single i.p. injection of LPS (1 mg/kg body weight) was given and BrdU (100 mg/kg body weight) or PBS as a control was injected i.p. 30 h later and daily for the following 6 d, and then the mice were sacrificed 24 h after the last injection. In the groups treated with the anti-inflammatory drug indomethacin, the first injection of indomethacin (2.5 mg/kg body weight) was given 18 h before the LPS injection, and then the same dose was injected twice daily for 7 consequent days. Twenty-four hours after the last injection of BrdU or PBS, the mice were processed for BrdU immunohistochemistry. For measurement of LPS-induced proinflammatory cytokines in hippocampus, a single i.v. injection of LPS (1 mg/kg body weight) was given at 8 h prior to RNA isolation from the hippocampal tissues.

Immunocytochemistry

Free-floating brain sections or differentiated cells on chamber slides were incubated overnight at 4°C with Abs against BrdU, β-tubulin III (anti-TUJ-1, 1:200; StemCell Technologies), NeuN or GFAP (1:200; EMD Millipore), DCX (1:100; Santa Cruz Biotechnology), or Iba-1 (1:200; Wako Chemicals) and analyzed on a Carl Zeiss Axio Imager M2 cell imaging microscope equipped with Apatome and AxioCam systems (Carl Zeiss, Jena, Germany).

Statistical analysis

Data were analyzed using ANOVA, followed by a Student t test for independent means where appropriate to determine which results showed a significant difference. All experiments were performed at least three times in triplicate. Data are expressed as the means ± SD.

Results

Adult brain neurogenesis is reduced in the hippocampus of the Tyro3+/−/Axl+/−/Merk−/− TKO brain

Because all three TAM family receptors are expressed in brain, especially in the hippocampus (42, 43), this prompted us to investigate whether neurogenesis in the mutant adult brain was defective. To assess NSC proliferation, we first labeled proliferating NSCs with BrdU, a thymidine analog that can be inserted into newly synthetic genomic DNA, on days 1–7, and then anti-BrdU immunohistochemistry was performed on brain sections prepared on day 8. BrdU reactivity in the subgranular layer of the dentate gyrus (DG) represents proliferating NSCs, and the TKO brain section clearly showed reduced BrdU labeling (Fig. 1B) compared with the WT control (Fig. 1A), with an ~50% reduction in the TKO brains (Fig. 1C, **p < 0.05). To assess neuronal differentiation of the surviving TKO NSCs, we next studied immature neurons in the subgranular region by immunostaining hippocampal sections for the immature neuron marker DCX, a cytoskeleton-associated protein expressed transiently during adult neurogenesis, and we found that the number of DCX+/BrdU+ double-positive immature neurons in TKO brains showed an even more dramatic decrease than did the BrdU+ cells when compared to the WT controls.
with the WT controls (Fig. 1A–C, Supplemental Fig. 1), with an average 60% reduction in the TKO brains (Fig. 1C, **p < 0.01).

Decreased NSC proliferation and immature neuron generation in the TKO hippocampal DG led to a slightly reduced total volume (Fig. 1D, p = 0.203, n = 5) and cell population (Fig. 1E, p = 0.024, n = 5) in the DG granular layer as studied by unbiased stereological quantification. Reduced hippocampal neurogenesis was also noticed in the Axl⁻/⁻ Mertk⁻/⁻ DKO mice, but not in all three single gene knockout mice (Supplemental Fig. 2).

Adult NSCs express the transcriptional factors Sox2, c-myc, and KLF4 (44, 45). Comparison of the relative expression of these three transcriptional factors in the adult hippocampus by real-time qPCR showed that mRNA levels of both Sox2 and c-myc were significantly reduced in 8-wk-old TKO mice (Fig. 1F, p < 0.05), whereas the KLF4 mRNA showed slight reduction (Fig. 1F, p = 0.073). This suggests a reduction in the cell population expressing these factors.

**TAM receptors are expressed in both microglia and astrocytes**

TAM receptors are expressed in macrophages and DCs (30, 34, 35, 40, 46), in a few nonprofessional phagocytes including the Sertoli cells in testis (38, 40) and retinal pigment epithelium cells in eye (32), and in the CNS (42, 43). As the absence of TAM receptors, macrophages and DCs produce increased levels of proinflammatory cytokines (34). To determine whether brain glial cells express all three members of TAM receptors that might negatively regulate brain glial cell cytokine production responsible for inhibition of neurogenesis in TKO mice, we first examined the expression profile of TYRO3, AXL, and MERTK proteins in primary cultured brain microglia and astrocytes by Western blotting. Although all three receptors were expressed in both microglia and astrocytes, the expression of each receptor varied significantly. WT microglia showed a dominant Mertk signal and weak expression of both Tyro3 and Axl, whereas WT astrocytes showed equally high expression of Tyro3 and Axl and weak Mertk expression (Fig. 2, left panel). Both cell types from TKO brains were negative for all three proteins. WT mouse tests, a tissue known to express all three receptors (40), was used as the positive control, and tests from Axl⁻/⁻ Mertk⁻/⁻ DKO mice and Tyro3⁻/⁻ Mertk⁻/⁻ DKO mice were used as negative controls (Fig. 2, right panel). Whether such relative expression profile of the three receptors in the microglia and astrocytes that had been cultured for >3 wk represents the in vivo expression profile is currently not clear.

**Primary microglia from TKO brains are hyperreactive to activation of several different TLRs**

Systemic or local chronic inflammation inhibits adult hippocampal neurogenesis (21, 24, 47). Microglia are brain-resident immunoresponsive cells with the similar developmental origin as peripheral macrophages. Given that TAM TKO macrophages show hyperreactivity to LPS stimulation (15, 33), TAM receptor–deficient microglia in the CNS might also show an unrestricted innate immune response and produce increased amounts of proinflammatory cytokines detrimental to hippocampal neurogenesis. To test this hypothesis, we isolated and purified primary microglia from both WT and TKO brains (Supplemental Fig. 3) and examined proinflammatory cytokine expression by primary cultured microglia stimulated with LPS, poly(I:C), or CpG and found that these components of infectious pathogens induced a rapid immune response by both WT and TKO microglia, with peak expression of mRNAs coding for the proinflammatory cytokines IL-1β, IL-6, and inducible NO synthase (iNOS) within 4–8 h (Fig. 3A). Remarkably, at the peak, the TKO cells produced more than twice the amount of these factors as did WT cells. This enhanced production of proinflammatory cytokines by TKO microglia was found not only in response to LPS, which acts through TLR4, but also to poly(I:C) and CpG; activating, respectively, TLR3 and TLR9 (48). After 24 h of stimulation, levels of mRNA for these cytokines were reduced to basal levels in both the WT and TKO groups, whereas, as shown in Fig. 3B, IL-6 release into the culture medium increased continuously during the 24-h stimulation period. Additionally, TKO microglia produced more TNF-α than did WT controls in response to LPS treatment (Fig. 3C). These data demonstrate that TAM receptors play a negative role in the TLR-initiated signal transduction pathways.

**LPS stimulation leads to increased activation of the MAPK and IKK/NF-κB signal transduction pathways in TKO microglia and macrophages**

Given that TKO microglia showed enhanced expression of proinflammatory mediators in response to TLR activation, we asked what signal transduction pathways were responsible. It is well established that TLR activation triggers multiple downstream signaling pathways, including the Erk1/2 and p38 MAPK pathway and the IKK/NF-κB pathway (14–17). Microglia express TLRs and all components of the IKK/NF-κB signaling pathway and produce proinflammatory mediators on activation (8, 49). To examine the negative functional role of TAM receptors on microglia in response to TLR activation, we activated TLR4 on primary cultured microglia with LPS and found that LPS stimulation caused rapid activation of both Erk1/2 and p38 in TKO cells compared with WT microglia (Fig. 4A). p38 has been suggested to be a major MAPK for regulating proinflammatory cytokine production by microglia (17). Indeed, we observed early activation of p38 in the 0 time sample (Fig. 4A, p-p38 row at 0 h) and rapid induction of phosphorylation within the first hour of LPS stimulation in the TKO microglia (Fig. 4A, top row). This suggests that negative regulation of TLR signaling by TAM receptors is conserved in all myeloid-derived cell lineages and that loss of TAM receptors on microglia is responsible for the overproduction of proinflammatory cytokines provoked by LPS stimulation.

We also studied NF-κB activation by LPS in TKO macrophages. Activation of the NF-κB pathway after LPS stimulation can be assessed by IKK phosphorylation and IκB degradation (16, 50). LPS stimulation caused rapid phosphorylation of IKK1/2 and...
are shown as means ± SD for five wells per group in a single experiment and are representative of those in three experiments. *p < 0.05, **p < 0.01.

FIGURE 3. Enhanced expression of IL-1β, IL-6, and iNOS by TKO microglia. (A) Real-time qPCR quantification of cytokine production. Microglial cells were isolated from newborn mouse brains and cultured in vitro for 21 d before separation from the cocultured astrocytes by low concentration of trypsin digestion. After 24 h recovery, the microglia in culture dishes were stimulated by LPS (100 ng/ml), poly(I:C) (30 μg/ml), and CpG-oligo-deoxynucleotide 1668 (1 μM; InvivoGen, San Diego, CA), accordingly, for indicated time points. The total RNA was extracted by TRizol and reverse transcribed using a qScript cDNA SuperMix kit. Real-time qPCR was performed to measure the relative mRNA levels of IL-1β, IL-6, and iNOS genes in the WT (○) and TKO (●) microglia. The mRNA expression levels relative to β-actin mRNA levels are shown as means ± SD; n = 3. **p < 0.05. (B) ELISA measurement of IL-6 by the LPS-, poly(I:C)-, and CpG-stimulated microglia or of TNF-α expression by the LPS-stimulated microglia. The microglial cells were prepared and treated as described above. At each time point, the culture medium was collected and subjected to either IL-6 or TNF-α measurement using specific ELISA kits (eBioscience, San Diego, CA), and the assays were performed following the manufacturer’s instruction. The concentration of IL-6 (B) or TNF-α (C) concentration in the WT (○) or the TKO (●) microglia medium was plotted against stimulation times. Data are shown as means ± SD for five wells per group in a single experiment and are representative of those in three experiments. *p < 0.05, **p < 0.01.

degradation of IκB in both cell types, but the mutant cells displayed earlier IKK phosphorylation (15 min in TKO versus 30 min in WT) and enhanced, prolonged IκB degradation (Fig. 4B), suggesting that macrophages lacking TAM receptors respond to TLR activation with increased NF-κB activation and thus downstream target gene expression.

The results shown above indicate that activation of TLRs in TKO microglia or macrophages resulted in high and prolonged activation of MAPK and NF-κB signaling, which leads to elevated proinflammatory cytokine production, suggesting that TAM signaling may inhibit TLR-induced proinflammatory cytokine production. Alternatively, we also examined whether activation of TAM receptors on WT microglia by their ligand Gas6 would inhibit innate immune responses. We pretreated cultured WT microglia with Gas6 for 2 h, then with LPS and Gas6 for 6 h, and quantified levels of mRNAs for inflammatory factors by real-time qPCR. As shown in Fig. 4C, the LPS-induced expression of IL-1β, IL-6, and iNOS mRNAs was inhibited by Gas6 pretreatment, further confirming that TAM receptors play a negative regulatory role on those cells.

Conditioned medium from LPS-treated TKO microglia inhibits NSC proliferation and neuronal differentiation in vitro and this effect is inhibited by anti–IL-6 Ab

Because mounting evidence showed that TKO microglia produced increased levels of proinflammatory cytokines, especially after exposure to different TLR cognate ligands, we examined whether conditioned medium from LPS-stimulated TKO microglia cultures was neurotoxic for cultured NSCs. To generate conditioned medium, primary cultures of microglia were subjected to LPS stimulation for 6 h, followed by extensive wash-off of residual LPS, and then the cells were transferred to normal medium for 24 h, after which the conditioned medium was collected and purified through a 0.2-μm filter and added to NSC cultures for 48 h, and then proliferating NSCs were labeled with BrdU (shown in Fig. 5A). Although conditioned medium from LPS-stimulated WT cells had an inhibitory effect on NSC proliferation (Fig. 5B, WT-by-LPS), the conditioned medium from LPS-treated TKO microglia led to a more dramatic reduction in BrdU incorporation into either WT or TKO proliferating NSCs (Fig. 5B, TKO-by-LPS), indicating that TKO microglial cells released higher levels of neurotoxic mediators after LPS stimulation. Notably, TKO NSCs were more vulnerable to the neurotoxicity of LPS-treated conditioned medium than were WT NSCs (Fig. 5B). Furthermore, LPS-stimulated TKO cell–conditioned medium caused more extensive NSC death than did LPS-stimulated WT cell–conditioned medium (Fig. 5C). Interestingly, close examination of both NSC genotypes clearly showed that TKO NSCs were more committed to programmed cell death than were WT cells.

We next studied the effect of TKO-conditioned medium on NSC differentiation into β-tubulin III⁺ neurons. TKO microglia–conditioned medium in the absence of LPS preactivation had a significant inhibitory effect on WT NSC differentiation into β-tubulin III⁺ neurons (Fig. 5D, open bars, TKO-by-PBS versus WT-by-PBS), and this effect was as high as that using conditioned medium from LPS-stimulated WT microglia (Fig. 5D, open bars, TKO-by-PBS versus WT-by-LPS). Interestingly, differentiation of TKO NSCs into β-tubulin III⁺ neurons was significantly reduced compared to the WT cells, and LPS pretreatment had a negligible inhibitory effect (Fig. 5D, open bars), suggesting that TAM
Astrocytes, similar to microglia, produce higher levels of proinflammatory cytokines deleterious to adult neurogenesis

Astrocytes have been postulated to exaggerate the neurotoxic effect of microglial inflammation by amplifying the release of inflammatory neurotoxic mediators (53). Given that astrocytes express TLRs and IL-1β receptors mediating the innate immune response (9, 49, 53–57) and express all three TAM receptor members (shown in Fig. 2), we postulated that the paracrine action of proinflammatory cytokines on TKO astrocytes might also be unchecked in the absence of negative regulation by TAMs. We therefore investigated inflammatory mediator production by cultured astrocytes. Consistent with the effect seen on microglia (Fig. 3B), LPS-elicited IL-6 production by TKO astrocytes was significantly higher than that seen with WT cells (Fig. 6A, 6C), because astrocytes show high expression of IL-1β receptor (53), it was possible that the enhanced production of IL-1β by TKO microglia might stimulate astrocytes to generate more inflammatory cytokines. This was indeed found to be the case, as, similar to LPS, IL-1β stimulation triggered increased expression of mRNAs for IL-6 and IL-1β itself (Fig. 6B) and increased release of IL-6 into the culture medium (Fig. 6D). These data suggest that mutant astrocytes and microglia work together to impair hippocampal neurogenesis.

LPS, mimicking pathogen infection, inhibits NSC proliferation in vivo

Neuroinflammation inhibits adult NSC replication and differentiation (24, 58). Because we previously showed that TAM receptors played a negative role on microglia in the innate immune response and that naïve TKO mice exhibited reduced NSC proliferation and differentiation into immature neurons, we then examined whether systemic administration of LPS induced increased proinflammatory cytokine production, and, if so, whether this contributed to inhibition of neurogenesis. LPS is a potent inducer of microglia activation and proinflammatory cytokine release in the CNS (8, 59, 60). A single i.v. injection of LPS (1 mg/kg) resulted in an increase in mRNA levels for the proinflammatory cytokines IL-1β, IL-6, and TNF-α in 8 h, and this effect was greater in the TKO hippocampus (Fig. 7A). LPS pretreatment for 4 h prior to the initial BrdU injection significantly inhibited BrdU incorporation into proliferating NSCs in WT brains, in which the percentage of the BrdU+ NSCs was reduced to a level similar to that seen in the untreated TKO brains (Fig. 7B, compare PBS in the TKO group and LPS in the WT group). Notably, LPS treatment further reduced BrdU incorporation into TKO brains (Fig. 7B, PBS versus LPS groups). LPS treatment also inhibited differentiation into BrdU+/DCX− immature neuronal progenies in the WT brains (Fig. 7C, PBS versus LPS in the WT groups); however, this differentiation was still higher than in the naive TKO brain (Fig. 7C, TKO in PBS versus WT in LPS groups). Interestingly, examination of BrdU+/DCX− double-positive immature neurons in the TKO brain revealed that LPS treatment did not cause a further decrease in the number of immature neurons (Fig. 7C, solid bars in the PBS and LPS groups). The percentage of immature neurons in the BrdU+ subgranular zone (SGZ) cell population was significantly lower than that seen in WT brains (Fig. 7C), even when compared with LPS-stimulated WT brains, suggesting that TAM receptors play an intrinsic functional role in supporting neurogenesis and that TAM receptor knockout renders NSCs more susceptible to toxic mediators in conditioned medium.

In an effort to search for neurotoxic mediators, we focused on one well-characterized proinflammatory cytokine, IL-6, which could be detrimental to NSC renewal and survival (51, 52). ELISA quantification clearly showed increased release of IL-6 by LPS-activated TKO microglia compared with LPS-activated WT cells (Fig. 5E). To examine whether the toxicity of TKO microglia-conditioned medium could be attributed to the enhanced production of IL-6, we prepared conditioned media from LPS-treated IL6−/− TKO and IL6−/− microglia culture and compared their effects on WT NSC proliferation with those of the WT- and TKO-conditioned media. Knockout of IL-6 in the TKO microglia indeed alleviated the neurotoxicity of the conditioned medium from TKO microglia (Fig. 5F). This result shows that the enhanced release of IL-6 from LPS-activated TKO microglia was responsible for the enhanced neuronal toxicity and suggests that this factor is a major candidate for the downregulation of hippocampal neurogenesis in TKO mice.

**FIGURE 4.** Activation of TLR induces enhanced activation of MAPK and NF-κB signal transduction pathways. (A) Western blotting analysis of p38 and Erk activation in the LPS-stimulated microglia. Microglia isolation and purification follow the procedure described in Materials and Methods. After 48 h recovery from mild trypsin digestion, both WT and TKO microglia were stimulated with 100 ng/ml LPS for the indicated time points. By the end of activation, the equal numbers of cells were directly lysed into cold 1× NuPAGE LDS sample buffer (Invitrogen) and subjected to SDS-PAGE electrophoresis, transferred onto nitrocellular membrane, and subsequently immunoblotted for phospho-p38, total p38, phospho-ERK, total ERK, and β-actin. (B) Western blot analysis of IKK phosphorylation and IκB degradation in the purified peritoned macrophage protein lysate. Western blots were probed with anti-IKK1/2 pSer, anti-IKK2, and anti-IκB Abs. (C) TAM receptor ligand Gas6 inhibits proinflammatory cytokine production by microglia. The WT microglia were pretreated with 50 nM GAS6 for 2 h and then stimulated with 100 ng/ml LPS for 6 h. The RNA isolation, cDNA synthesis, and real-time qPCR measurement of IL-1β, IL-6, and iNOS gene expression are described in Materials and Methods and in the legend to Fig. 3.
intrinsic role in NSC differentiation and that loss of these receptors dramatically hampers differentiation into immature neurons in the hippocampus.

To confirm that inflammation elicited by LPS stimulation was responsible for reduced NSC proliferation, we treated the mice with the anti-inflammatory drug indomethacin prior to, and during, LPS stimulation and BrdU labeling and found that administration of indomethacin to LPS-stimulated mice was able to antagonize the inhibitory effects of LPS on hippocampal neurogenesis in WT mice, but had no effect in TKO mice (Fig. 7B, LPS versus LPS/indomethacin groups). This observation suggests that inflammation affects neurogenesis in adult brains, but TAM knockout creates a harsher environment antagonizing the immunosuppressive effect of indomethacin. Interestingly, when comparing the number of BrdU+/DCX+ immature neurons in the PBS-treated TKO mice and those in the LPS-, LPS/indomethacin-, or indomethacin-treated TKO brains, LPS, LPS/indomethacin, or indomethacin alone did not change the number of the BrdU+/DCX+ immature neurons, suggesting that LPS-triggered inflammation or an anti-inflammatory drug does not contribute much to the decreased generation of BrdU+/DCX+ immature neurons seen in the PBS-treated TKO mice (Fig. 7C). However, whether those observations

FIGURE 5. The TKO microglia–conditioned medium inhibits NSC proliferation, neuronal differentiation, and promotes NSC death. (A) Schematic diagram shows how the conditioned medium is prepared. After mild trypsinization, both WT and TKO microglia were cultured for 48 h prior to 6 h of LPS (100 ng/ml) stimulation, after which the residual LPS was intensively washed off with sterile PBS and replaced with fresh medium (DMEM/F12 containing 10% FBS and antibiotics) for preparation of microglia-conditioned medium (MG-cMD). The conditioned media prepared from the LPS-stimulated TKO or WT microglial cells are labeled as TKO-by-LPS or WT-by-LPS, respectively. The control conditioned medium was prepared from the cells treated with same volume of PBS without LPS, and those MG-cMD are named either TKO-by-PBS or WT-by-PBS. The conditioned medium was collected 24 h later and passed through a 0.2-µm filter, and the resulting medium, served as MG-cMD, was subsequently mixed with 10% differentiation supplements (Invitrogen) and used in the following NSC culture for another 48 h. (B) Proliferating NSCs were labeled by addition of 10 µM BrdU into the culture medium in the last 12 h. The percentage of BrdU+ cells in the total DAPI-stained cells were counted and calculated. (C) Apoptotic cells after 48 h of conditioned medium treatment were identified by TUNEL assay (Roche) following the manufacturer’s instructions. The TUNEL+ cells in the DAPI-stained cells were calculated and plotted. (D) After the cells were cultured in the conditioned medium for 48 h, the medium was removed and the cells on the chamber slides were washed and fixed with 4% paraformaldehyde for 20 min at room temperature before immunostaining with anti–β-tubulin Ab. The β-tubulin+ cells in total DAPI-stained cells were counted and the percentage was calculated. (E) The IL-6 level in the conditioned medium was measured by ELISA. The cells were stimulated with LPS for 6 h and replaced with fresh medium without LPS, and the cell culture media used for ELISA assays were collected from the media either by the end of 6 h of LPS stimulation (LPS-6 h, chase 0 h) or by 24 h after chase with fresh medium (chase for 24 h after 6 h of LPS treatment) fed with fresh medium for 24 h. The IL-6 was measured by an ELISA kit (eBioscience) following the manufacturer’s instructions. (F) The MG-cMD was collected from primary cultured microglial cells prepared from the genotypes of WT, TKO, Il6−/−Tyro3−/−Axl−/−Mertk−/− (Il6−/− TKO), and Il6−/− mice and was used to treat the WT NSCs for 48 h. The percentages of β-tubulin+ and BrdU+ cells were calculated and the fold changes over the WT-conditioned medium was plotted. The open and filled bars in (B)–(D) represent data obtained from the WT or TKO NSC culture, respectively. Means ± SD are shown. *p < 0.05, n ≥ 3; **p < 0.01.
suggest an alternative Cox-independent mechanism accounting for the increased vulnerability of TAM TKO mice or an endogenous regulatory role that the TAM receptors play in the hippocampal neuronal stem cells remains to be further elucidated.

Knockout of IL-6 but not the TNF-α receptor in the TAM TKO or AM DKO mice restores neurogenesis

Direct evidence for proinflammatory cytokines being detrimental to adult neurogenesis came from a study showing that transgenic mice overexpressing IL-6 uniquely in astrocytes showed impaired hippocampal neurogenesis (61). To further test the hypothesis that the increase in microglial cell–produced proinflammatory cytokines in TKO mice, especially that of IL-6, was deleterious to hippocampal neurogenesis (61). To further test the hypothesis that the increased vulnerability of TAM TKO mice or an endogenous regulatory role that the TAM receptors play in the hippocampal neuronal stem cells remains to be further elucidated.

Knockout of IL-6 but not the TNF-α receptor in the TAM TKO or AM DKO mice restores neurogenesis

Discussion

Mounting evidence shows that activated microglia produce multiple proinflammatory cytokines that are essential for normal tissue protection from pathogen invasion and tissue damage insults (62); however, uncontrolled activation of the innate immune system and an excessive inflammatory response in the CNS may contribute significantly to the pathogenesis and progression of many neurodegenerative diseases (9, 63, 64) and to impairment of adult neurogenesis (21, 24, 47, 65). The inhibitory function of TAM receptors in innate immunity has received much attention recently (15, 29, 31, 34, 46, 66). This family of receptors plays a regulatory role in DCs and monocyte-derived cells and causes late-stage inhibition of cytokine receptor signaling either by upregulation of SOCS, which are inducible negative regulators of cytokine signaling (15, 34, 67), or by induction of expression of an NF-κB repressor, Twist, which binds to E-box elements in the TNF promoter and inhibits NF-κB–dependent transcription (29). Our present data demonstrated that TAM receptors on microglia also have a suppressive effect on cytokine signaling. Microglia, a brain-resident innate cell type with most of the major characteristics of peripheral macrophage, express all major TLRs (11–13).
and, in our study, activation with LPS, poly(I:C), or CpG triggered proinflammatory cytokine production. Notably, our data showed that pathogen-induced proinflammatory cytokine expression was much higher in TKO microglia than in WT controls, suggesting that TAM receptors indeed function as negative regulators to keep microglia cytokine signaling in check and maintain a homeostatic balance for microglia innate immune responses. Consistent with this observation, TKO microglia exhibited activated MAPK and IKK/NF-κB signaling before treatment and responded to TLR activation with prolonged phosphorylation of MAPKs, especially p38, and increased degradation of IκB, which normally inhibits NF-κB transcription activity. Interestingly, in microglia, p38 has been postulated to be the major MAPK responsible for the production of proinflammatory cytokines upon activation (17, 68, 69). Inhibition of p38 by specific inhibitors or gene deletion impairs proinflammatory cytokine production by microglia in the inflammatory or aging brain (17, 68, 69). The unrestricted activation of p38 and NF-κB signaling in TKO microglia is therefore considered a major signaling pathway for increased proinflammatory cytokine production in the TKO brains.

The unrestrained cytokine signaling and enhanced production of proinflammatory cytokines by TKO microglia inspired us to evaluate the effect of loss-of-function mutation on adult hippocampal neurogenesis. Adult NSCs are restricted to a few regions in the brain, including the subventricular zone and hippocampal SGZ (70–74), and they are characterized by a self-renewal capacity and the potential to differentiate into neurons or glia (75–78). Adult NSCs with differentiation potential provide a rich source for the replacement of cells lost during normal cell turnover and after brain injury (79–82). Interruption of adult neurogenesis leads to impairment of hippocampus-dependent learning and behavior (24, 25, 79, 81, 83–85). Compared to WT controls, TKO brains showed significantly lower BrdU incorporation in dividing NSCs in the hippocampal subgranular layer, and systemic administration of LPS, a potent inducer of proinflammatory cytokine production as a result of its activating TLR4-initiated signaling in microglia and astrocytes, resulted in even more dramatic inhibition of neurogenesis.

To further assess the neurotoxic role of TKO microglia and to test whether the enhanced production of proinflammatory mediators by TKO microglia inhibited NSC proliferation and differentiation, we used an in vitro culture system and exposed WT NSC cultures to conditioned medium collected from TKO microglia cultures and found that TKO microglia released harmful factors that significantly inhibited WT NSC proliferation and neuronal differentiation and caused apoptosis, and that these effects were increased by LPS pretreatment of the cultured microglia, suggesting that unrestricted innate immune responses by the TKO microglia released high level of proinflammatory mediators that were detrimental to the cultured NSCs. Recent studies of the effect of proinflammatory cytokines on neurogenesis have shown that the detrimental or supportive effects of microglia on neurogenesis are determined by their activation status. In response to infection, most commonly mimicked by LPS stimulation in animal models, activated microglia release proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, which have a negative effect on hippocampal neurogenesis and neuronal differentiation (21–24, 27, 28, 86). However, microglia activated with anti-inflammatory cytokines, such as IL-4, IL-15, or low-dose IFN-γ, support neurogenesis and glial differentiation (87–90). Of the proinflammatory factors that suppress neurogenesis, IL-6, TNF-α, and IL-1β have been intensely studied, and IL-6 has been shown to play the major role based on the fact that IL-6–neutralizing Ab inhibits the negative effect of LPS-activated microglia on neurogenesis (24). This inhibitory effect of IL-6
was also demonstrated in IL-6 transgenic mice, in which overexpression of IL-6 uniquely in astrocytes dramatically inhibited neurogenesis (61). We indeed detected a level of IL-6 by ELISA in the conditioned medium or by real-time qPCR from TKO microglia with or without LPS activation. To further test whether increased IL-6 produced by the mutant glial cells was responsible for the neurotoxic effect, we crossed Il6–/– mice with Axl–/–/Mertk–/– DKO mice to eliminate IL-6 expression in DKO mice and found no significant improvement for the impaired neurogenesis seen in untreated or LPS-treated TKO mice, suggesting that the brain local inflammation may play a major role in the impaired neurogenesis seen in the adult TKO brain. There is no strong evidence that a single injection of LPS in vivo can cause permanent neuronal damage and neurodegeneration (92), except in the case of dopaminergic neurons in the substantia nigra (53, 93). However, long-term exposure to infectious pathogens has been shown to have detrimental effects on the nervous system (19, 94–96). This again suggests that TKO brains undergo chronic inflammation that is harmful for adult neurogenesis.

Microglial cells actively patrol the CNS and sense any danger signals. The next question was why TKO microglia were constantly activated. On the one hand, TAM receptors play a trophic supporting role in a variety of cell types, which, in the absence of TAM receptors, undergo apoptosis (30, 40, 46). Additionally, all three TAM receptors have been shown to be expressed in brain blood vessel endothelial cells, and loss of function mutation destroys blood vessel integrity (97, 98), rendering the CNS highly vulnerable to local or peripheral insults (99). Microglia can efficiently sense such risk factors and are constantly activated and produce neurotrophic factors that penetrate the blood–brain barrier and cause strong upregulation of proinflammatory cytokine production by activated microglia (91). Indomethacin, a non-steroidal anti-inflammatory drug, inhibits cyclooxygenase (COX)-mediated PG synthesis and thus inhibits LPS-induced systemic inflammation (24). Notably, indomethacin did not alleviate the impaired neurogenesis seen in untreated or LPS-treated TKO mice, suggesting that the brain local inflammation may play a major role in the impaired neurogenesis seen in the adult TKO brain. This indicates that it is IL-6 but not TNF-α responsible for the impaired neurogenesis in the mutants.

LPS-induced inflammation has been shown to inhibit hippocampal neurogenesis (21, 24). Our results are in agreement with this finding, and furthermore, systemic administration of the anti-inflammatory drug indomethacin to a great extent overcame the LPS-induced inhibition of hippocampal neurogenesis in the WT brain. LPS-induced inhibition of neurogenesis is thought to be caused by primarily peripheral inflammatory mediators that penetrate the brain–blood barrier and cause strong upregulation of proinflammatory cytokines.
prevent prolonged activation of microglia in a similar way to that demonstrated for DCs and macrophages (15, 29, 31). We observed constitutive activation of MAPKs, especially p38 MAPK, and increased IKK/NF-κB signaling in response to LPS stimulation. Unrestricted activation of IKK and downstream NF-κB and sustained secretion of proinflammatory cytokines have also been seen in apoptotic cell–induced inhibition of the inflammatory response by monocyte-derived DCs from Mertk knockout mouse (66). In our study, when stimulated with LPS in vitro, TKO microglia produced increased levels of proinflammatory cytokines, as shown at either the mRNA or protein level, creating a chronic inflammatory environment detrimental to adult brain stem cell renewal and neuronal differentiation.

TAM receptors may also play a role in supporting NSC differentiation into DCX+ immature neuroblasts or immature neurons. Given the NSCs expressing all three receptors (39, 100) and the TAM receptor ligand, Gas6, lowering incorporation of BrdU into the proliferating neuronal progenitor cells located in brain SVZ (39), the defective neurogenesis could also be caused by loss of direct support from TAM signaling on NSC proliferation and differentiation, in addition to the overreactive innate immune response by TKO glial cells discussed above. Detailed comparison of differentiation of WT and TKO NSCs into β-tubulin III+ neurons, presented in Fig. 5D, showed that a significantly lower percentage of immature neurons was produced by TKO NSCs and that LPS-activated microglia-conditioned medium did not result in a further decrease, whereas WT NSCs showed an equal decrease in differentiation into β-tubulin III+ neurons after exposure to the microglia-conditioned medium from either PBS-treated TKO or LPS-treated TKO and WT cells, implying that TAM receptors might play a functional role in NSC proliferation and neuronal differentiation. In vivo LPS treatment not only inhibited NSC proliferation, but also hindered differentiation into BrdU+/DCX+ neuronal progenies in WT brains, but this LPS inhibition of immature neuron differentiation in the WT brain never reached the level seen in naive TKO brain, as shown in Fig. 7C. In contrast, LPS treatment did not cause a further decrease in the number of immature neurons (BrdU+/DCX+) in the TKO brain, indicating that differentiation into immature neurons may be affected by other endogenous factors normally under positive regulation by TAM receptors and/or that TAM knockout had already exerted a maximal deleterious effect on immature neuron production.

In summary, TAM receptors are expressed on microglia. Loss of these receptors results in adult hippocampal neurogenesis being significantly comprised. TAM receptors have a negative regulatory effect on microglia to prevent overproduction of proinflammatory mediators detrimental to NSC proliferation and differentiation. Loss of TAM receptors that the microglia produces enhanced IL-6 and IL-1β, and IL-1β acting as a paracrine factor, in turn, stimulates astrocytes to produce more IL-6. Overproduction of IL-6 by both microglia and astrocytes inhibits NSC proliferation and differentiation, leading to an impaired neurogenesis in adult hippocampus. Additionally, autonomous trophic function of the TAM receptors on the proliferating neuronal progenitors may also promote neuronal differentiation of progenitor into immature neurons.

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Disclosures
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