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RAPID COMMUNICATION

CD40 Negatively Regulates ATP-TLR4-Activated Inflammasome in Microglia

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Abstract During acute brain injury and/or sterile inflammation, release of danger-associated molecular patterns (DAMPs) activates pattern recognition receptors (PRRs). Microglial toll-like receptor (TLR)-4 activated by DAMPs potentiates neuroinflammation through inflammasome-induced IL-1β and pathogenic Th17 polarization which critically influences brain injury. TLR4 activation accompanies increased CD40, a cognate costimulatory molecule, involved in microglia-mediated immune responses in the brain. During brain injury, excessive release of extracellular ATP (DAMPs) is involved in promoting the damage. However, the regulatory role of CD40 in microglia during ATP-TLR4-mediated inflammasome activation has never been explored. We report that CD40, in the absence of ATP, synergizes TLR4-induced proinflammatory cytokines but not IL-1β, suggesting that the response is independent of inflammasome. The presence of ATP during TLR4 activation leads to NLRP3 inflammasome activation and caspase-1-mediated IL-1β secretion which was inhibited during CD40 activation, accompanied with inhibition of ERK1/2 and reactive oxygen species (ROS), and elevation in p38 MAPK phosphorylation. Experiments using selective inhibitors prove indispensability of ERK 1/2 and ROS for inflammasome activation. The ATP-TLR4-primed macrophages polarize the immune response toward pathogenic Th17 cells, whereas CD40 activation mediates Th1 response. Exogenous supplementation of IFN-γ (a Th1 cytokine and CD40 inducer) results in decreased IL-1β, suggesting possible feedback loop mechanism of inflammasome inhibition, whereby IFN-γ-mediated increase in CD40 expression and activation suppress neurotoxic inflammasome activation required for Th17 response. Collectively, the findings indicate that CD40 is a novel negative regulator of ATP-TLR4-mediated inflammasome activation in microglia, thus providing a checkpoint to regulate excessive inflammasome activation and Th17 response during DAMP-mediated brain injury.

Keywords CD40 · TLR4 · Inflammasome · ATP/DAMPs · Microglia · T cells

Introduction

Sterile inflammation following acute brain injury such as hemorrhage, ischemic stroke, or trauma is detrimental and largely exacerbates progression of neurodegeneration (Rubartelli 2014). Thus, like in other organs, inflammation can switch from a protective to a destructive role in the brain. Early inflammation induces microglia-mediated protection, while persistent and aberrant inflammation results in destructive response acting as a major contributor for incidences of neurodegeneration (Denes et al. 2010; Trotta et al. 2014). During acute brain injury, danger-associated molecular patterns (DAMPs), such as serum amyloid A (SAA), high-mobility group box (HMGB)-1, heat shock protein (HSP)-70, and ATP, are secreted. These DAMPs are major contributors in accelerating microglia-mediated inflammation and neurotoxicity through TLR4

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signaling (Denes et al. 2010; Trotta et al. 2014; Rubartelli 2014).

Activated microglia rapidly produce interleukin (IL)-1β, a master regulator of neuroinflammation, in response to injury/infection through inflammasome activation (Allan 2005; Vezzani and Granata 2005). Increased IL-1β in the brain has been shown to exacerbate excitotoxic, traumatic, and ischemic insults in vitro and in vivo (Walsh et al. 2014; Allan 2005). In this context, Savage and coworkers show that DAMPs such as ATP enhance the production of inflammatory mediators like IL-6, CXCL1, and proteases by glial cells but the expression of IL-1β remained unaltered. Notably, they also show that SAA provides priming stimulus accelerating increased levels of IL-1β similar to that induced by lipopolysaccharide (LPS) (Savage et al. 2012). Since SAA and other DAMPs activate microglia-expressed TLR4, we have explored ATP-LPS for similar functions.

Increased TLR4 and CD40 levels have been reported during neuroinflammation by independent studies (Gaikwad and Agrawal-Rajput 2015; Rosenberger et al. 2015; Calingasan et al. 2002; Tan et al. 1999). CD40 and its cognate ligand CD40L mediate a broad variety of innate and adaptive immune responses. CD40, through reciprocal regulation of p38-MAPK and ERK1/2, mediates the production of differential T-cell priming cytokines (Mathur et al. 2004). CD40 and TLR4 activation follows common signaling pathway (Chandel et al. 2014). TLRs and CD40 modulate each other's expression in macrophages, but the regulatory role of CD40 during TLR4 activation has never been explored in microglia. Studies have shown increased T-lymphocyte infiltration in patients with ischemic stroke and traumatic brain injury (Brait et al. 2010). Importantly, IL-1β is reported to strikingly enhance detrimental T-cell response with enhanced IL-17 in various neurological diseases (Ben-Sasson et al. 2013; Walsh et al. 2014).

The study aims to answer three key questions: (i) whether CD40 regulates TLR4-mediated inflammation, (ii) whether CD40 governs ATP-TLR4-mediated inflammasome activation, and (iii) whether CD40 governs ATP-TLR4-induced T-cell responses? The study aims to provide insights into the molecular mechanism for CD40-mediated inflammasome activation and T-cell polarization in the absence or presence of ATP. Understanding the regulatory mechanisms of CD40 during ATP-TLR4-induced inflammation can provide feasible strategies during brain injury.

Materials and Methods

Reagents SB202190 (p38 MAPK inhibitor), PD184352 (ERK1/2 inhibitor), N-Acetyl cysteine (NAC, ROS scavenger), *E. coli* LPS (serotype O55:B5), and adenosine 5′-triphosphate disodium salt (ATP) were obtained from Sigma-Aldrich, St. Louis, Missouri. Anti-CD40 monoclonal antibody clone 3/23 (CD40 agonistic antibody) was obtained from BD Biosciences, San Diego, CA. Anti-caspase-1 antibody was sourced from Santa Cruz Biotechnology, Dallas, Texas. Anti-NLRP3 antibody was obtained from IMGENEX, SanDiego, CA. Anti-TLR4, anti-phospho-p38, anti-p38, anti-phospho-ERK 1/2, anti-ERK 1/2, and anti-β-actin antibodies were from Life Technologies, Carlsbad, CA. All the cytokine ELISA kits, recombinant IFN-γ, and $CD4^+$ T-cell enrichment kit were from eBioscience, San Diego, CA. All the other reagents were obtained from Life Technologies, Carlsbad, CA, unless otherwise mentioned.

Cell Culture

Murine BV2 microglia (kind gift from Prof. Anirban Basu, NBRC, India) were maintained at 37 °C in DMEM supplemented with heat-inactivated 10 % FBS and 1× penicillin–streptomycin as described previously (Kaushik et al. 2012).

C57BL/6 mice were used for the study and the animal studies were approved by the Institutional Animal Ethics Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) at the B.V. Patel PERD Centre, Ahmedabad, Gujarat, India. Bone marrow macrophages (BM-MΦ) were derived from low-density marrow cells isolated from femurs and suspended (1×10^6 cells/ml) in RPMI 1640 medium with heat-inactivated 10 % FBS in the presence of 20 ng/ml M-CSF for 5 days and the adherent cells (>95 % $CD11b^+$) were used. For isolation of T cells, RBC-depleted splenocytes were purified using MagniSort-$CD4^+$ T-cell enrichment kit (eBioscience, San Diego, CA) as per manufacturer's instructions.

TLR4 and CD40 Stimulation in Microglia or BM-MΦ

CD40 signaling was triggered in BV2 microglia or BM-MΦ using agonistic CD40 antibody (clone 3/23) (3 μg/ml) following TLR4 stimulation by LPS (1 μg/ml) and activated with or without ATP (500 μM/1 mM). Gene and protein expression for respective molecules and their stimulation time are mentioned in the respective figure legends.

Semi-Quantitative RT-PCR

Total RNA was extracted using TRIZOL (Life Technologies, USA) as per manufacturer's guidelines. RNA concentration was measured using Biophotometer D30

(Eppendorf, Hamburg, Germany). Total RNA (1 μg from each sample) was used for cDNA synthesis using the thermoscript RT-PCR system (Thermo Fisher Scientific, Waltham, MA). The cDNA for indicated genes were amplified using gene-specific primers (Supplementary Table 1) under the following conditions: 95 °C for 5 min, 95 °C for 30 s, annealing at 55–60 °C for 45 s, and 72 °C for 1 min for a total of 25–28 cycles followed by a final extension at 72 °C for 10 min. Each sample was amplified for β-actin to ensure equal cDNA input.

Western Blot

Expression and phosphorylation of the proteins were assayed by Western blot. Briefly, total cell lysates were prepared in lysis buffer (20 mM Tris HCl, 137 mM NaCl, 1 % (v/v) Triton X-100, 10 % glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor cocktail) for 20 min. Supernatants were collected by centrifugation at 13,000×*g* for 20 min at 4 °C, followed by protein quantification using the Bradford assay (Bio-Rad, Hercules, CA). Subsequently, 50 μg cell lysates from each sample were resolved on SDS-PAGE, and electroblotted onto PVDF membranes (Bio-Rad, Hercules, CA). After being blocked for 2 h with 5 % (w/v) BSA in TBST buffer (10 mM Tris, 150 mM NaCl and 0.5 % Tween-20), the membranes were probed overnight at 4 °C with specific primary antibodies: rabbit anti-TLR4, rabbit anti-caspase-1, rabbit anti-NLRP3, mouse anti-phospho-p38, rabbit anti-phospho-ERK1/2, rabbit anti-P38, rabbit anti-ERK1/2, and mouse anti-β-actin at 1:1000 dilution. After three washes in TBST, membranes were incubated with HRP-conjugated antibody for 1 h at room temperature. The protein bands were visualized using ECL kit (Bio-Rad, Hercules, CA) and relative fold change was analyzed using ImageJ software (NIH, Bethesda, USA).

Measurement of ROS Production

The intracellular ROS production was examined using Cell-Rox Deep Red as per manufacturer's instruction (ThermoFisher Scientific, Waltham, MA). Briefly, after incubated the cells were washed with PBS followed by Cell-Rox Deep Red staining (10 μM) for 30 min and ROS was quantified in multimode plate reader at 644/655 nm (SpectraMax, Molecular Devices, Sunnyvale, CA) and plotted as fold change of the mean fluorescence intensity. In parallel experiment, following Cell-Rox Deep Red staining (10 μM) for 30 min intracellular ROS was analyzed under fluorescence microscope (Olympus, Tokyo, Japan).

BM-MΦ–CD4$^+$ T-Cell Coculture

To study microglia-mediated T-cell response, we used BM-MΦ–CD4$^+$ T-cell coculture assay, as an alternative to microglia–T-cell coculture. Bone marrow-derived macrophages from C57BL/6 mice were prepared as described above and treated with agonist anti-CD40 Ab (3 μg/ml) for 2 h followed by LPS (1 μg/ml) stimulation for 24 h and activated with ATP (500 μM). CD4$^+$ T cells were cocultured with BM-MΦ for another 48 h at 1:10::APC:T-cell ratio. Secretory IFN-γ and IL-17 were assayed by ELISA.

ELISA

Cytokines in the cell-free culture supernatants were detected using ELISA for the indicated cytokines as per manufacturer's guideline (eBioscience, San Diego, CA).

Statistical Analysis

The statistical significance of differences between groups was determined by one-way ANOVA followed by Tukey's post hoc multiple comparison test. The data are expressed as mean ± SEM from three independent experiments. A statistical p value less than 0.05 ($p \leq 0.05$) was considered significant.

Results

CD40 Synergizes TLR4-Mediated Inflammation in Microglia

Antigen-presenting cells, including microglia, present antigen to CD4$^+$ T cells and CD40 is an important costimulatory molecule in this regard. The effect of CD40 and TLR4 activation on microglia-mediated inflammation was investigated. CD40 stimulation significantly enhanced LPS-induced TLR4 and CD40 expression (Fig. 1a, b, supplement figure S1A, S1B, and S1C). Further, CD40 activation synergized TLR4-mediated microglial activation as evidenced by increased production of proinflammatory cytokines like IL-12, IL-6, and TNF-α accompanying decreased IL-10 secretion (Fig. 1c). CD40 alone or in combination with LPS did not induce IL-1β secretion (data not shown). It was observed that independent stimulation of TLR4 and CD40 induced p38 and ERK 1/2 phosphorylation. Interestingly, CD40 in the presence of TLR4 stimulation significantly enhanced ERK-1/2 phosphorylation, while p38 phosphorylation was slightly inhibited (Fig. 1d, supplement figure S1D and S1E). The data

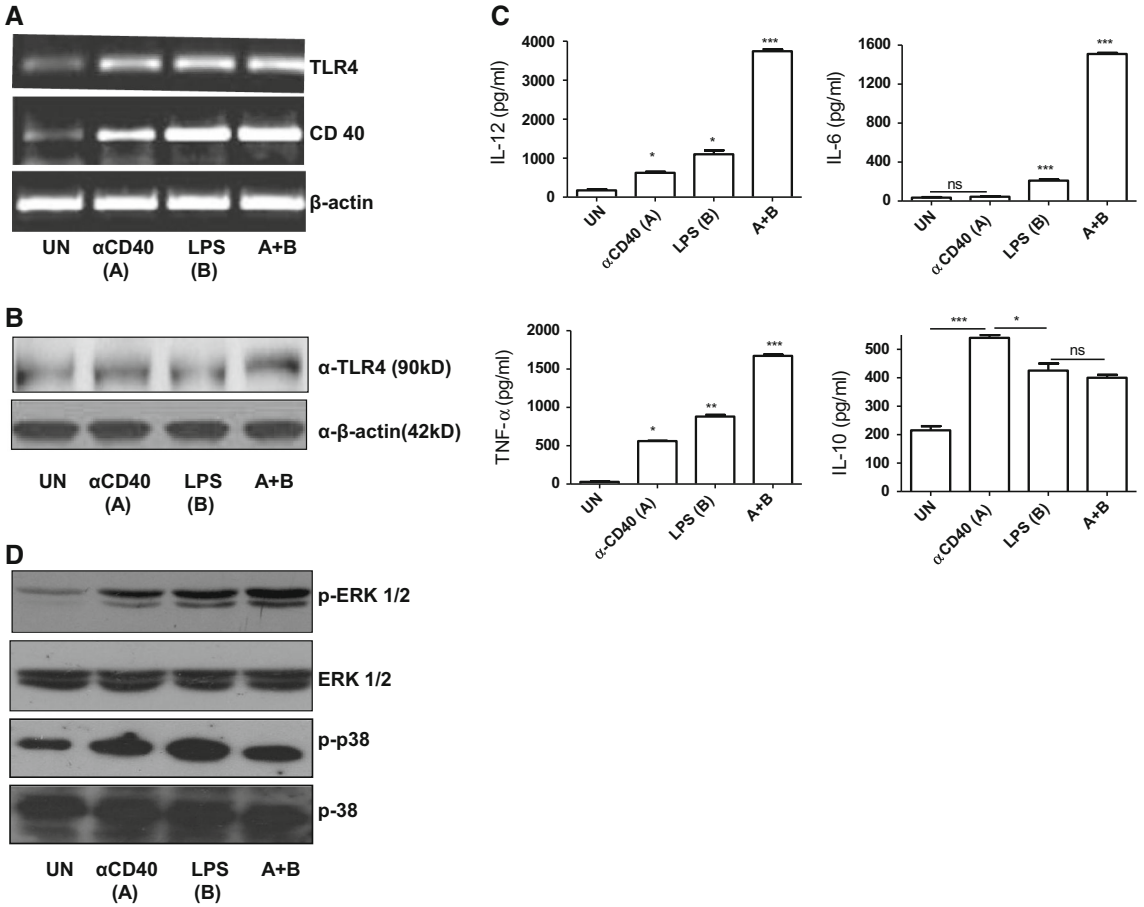

Fig. 1 CD40 synergistically enhances TLR4-mediated inflammation in microglia. **a** BV2 microglial cells were treated with agonistic anti-CD40 Ab for 2 h in the absence or presence of LPS for 6 h. CD40 and TLR4 mRNA levels were assayed by semi-quantitative RT-PCR. β-actin was used as an endogenous control. **b** BV2 microglial cells were treated with agonistic anti-CD40 Ab for 2 h in the absence or presence of LPS for 24 h. TLR4 protein expression was detected by Western blotting and **c** IL-6, IL-12 (p70), TNF-α, and IL-10 secretion was measured by ELISA. **d** BV2 cells were pretreated with agonistic anti-CD40 Ab for 30 min followed by treatment with or without LPS for 30 min. Total and phosphorylated protein levels of p38 and ERK were assayed by Western blotting ($*p < 0.005$; $**p < 0.001$; $***p < 0.0001$ as compared to untreated control)

suggest that CD40 synergistically enhanced TLR4-mediated microglial activation, MAPK phosphorylation, and subsequent inflammation.

CD40 Negatively Regulates ATP-TLR4-Induced Inflammasome Activation

Acute brain injury accompanies increased release of extracellular ATP, which provides danger signal for inflammasome activation and promotes brain injury (Beamer et al. 2015). Using LPS-ATP as priming-activating stimuli, the effect of CD40 on inflammasome activation was examined. We observed that ATP induces NLRP3 inflammasome activation during TLR4 and/or CD40 activation. Unlike the synergy observed with TLR4 activation, CD40 stimulation does not potentiate ATP-TLR4-mediated priming of NLRP3 inflammasome (Fig. 2a). ATP alone fails to induce caspase-1 cleavage; similarly, TLR4 activation in the absence of ATP cannot form active caspase-1. The presence of ATP facilitated the release of IL-1β during TLR4 or CD40 activation. CD40 inhibited ATP-TLR4-induced caspase-1 cleavage (Fig. 2b). Consequently, in the absence of ATP microglia fail to release IL-1β during TLR4 and/or CD40 activation. However, ATP-TLR4-induced inflammasome activation follows the release of IL-1β. Interestingly, CD40 activation resulted in a remarkable inhibition of IL-1β secretion during ATP-TLR4 activation (Fig. 2c). In contrast, ATP-TLR4-induced IL-12 was enhanced, while IL-6 remained unaltered (Supplement figure S2A and S2B). The data suggest that CD40 negatively regulates ATP-TLR4-induced NLRP3 inflammasome activation and secretion of IL-1β by microglial cells.

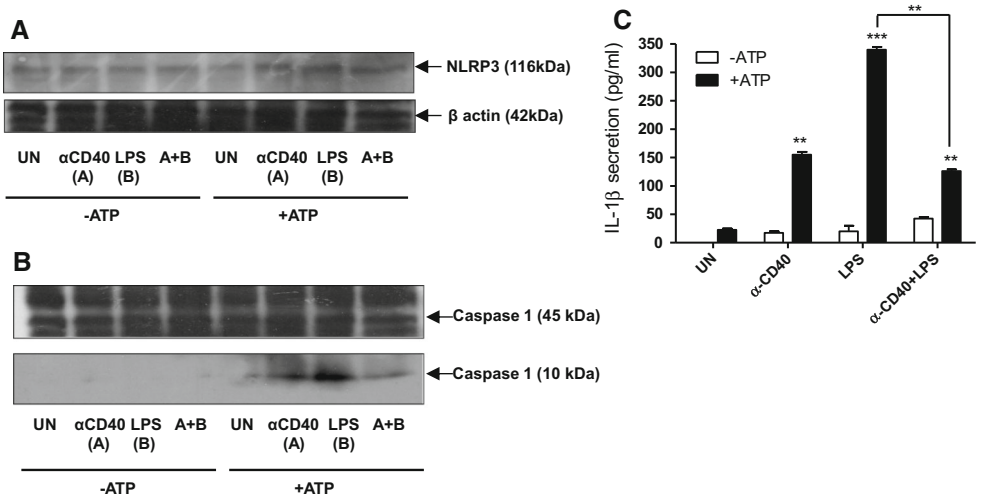

Fig. 2 CD40 inhibits ATP-TLR4-induced inflammasome activation. BV2 cells were treated with agonistic anti-CD40 Ab for 2 h followed by LPS stimulation for 24 h later with or without ATP (1 mM) for 45 min before termination of experiments. **a** NLRP3 protein levels were assayed by Western blotting; **b** caspase-1 activation was assayed by Western blotting, and **c** secreted IL-1β was measured by ELISA (**$p < 0.001$; ***$p < 0.0001$)

CD40 Inhibits ATP-TLR4-Induced Inflammasome Through Decreased Phosphorylation of ERK1/2 and ROS Inhibition

We investigated the molecular mechanisms of inflammasome inhibition during CD40 activation. Interestingly, we observed that CD40 activation significantly inhibited ATP-TLR4-induced phosphorylation of ERK1/2 (Fig. 3a) accompanied with a parallel increase in p38-MAPK phosphorylation (Fig. 3b). Using selective inhibitor of ERK 1/2 and p38, we demonstrate that inhibition of ERK 1/2 abrogated ATP-TLR4-induced secretion of IL-1β, while p38 inhibition had no significant effect on IL-1β secretion (Fig. 3c). ATP-TLR4 activation significantly triggered intracellular ROS levels, while CD40 activation significantly attenuated the ATP-TLR4-induced ROS production (Fig. 3d and supplement figure S3). ROS inhibition by ROS scavenger, NAC, abrogated IL-1β secretion confirming its potential role in inflammasome activation (supplement figure S4A, S4B, and S4C). The data suggest that CD40 negatively regulates ATP-TLR4-induced inflammasome activation through inhibition of ERK 1/2 phosphorylation and ROS generation.

Th17/Th1 Polarization During ATP-TLR4 Activation is Governed by CD40

Infiltration of inflammatory T cells is a well-described mechanism during sterile brain injury (Brait et al. 2010). Inflammasome activation and the release of IL-1β are known to initiate Th17 polarization leading to exacerbated neuronal injury (Brait et al. 2010). We explored the role of CD40 in ATP-TLR4-mediated T-cell response. Results show that inflammasome mediated the release of IL-17 from T cells during ATP-TLR4 activation. Following CD40 activation, there was a significant decrease in IL-17, while there was a significant increase in the release of IFN-γ (Fig. 4a, b). The increase in IFN-γ during CD40 activation intrigued us to further explore the role of IFN-γ in governing IL-1β secretion. We observed that exogenous recombinant IFN-γ increased CD40 expression (data not shown) and limits IL-1β secretion (Fig. 4c) suggesting its importance in regulating inflammasome activation. Taken together, these results suggest that CD40 mediates IFN-γ secretion which negatively regulates inflammasome.

Discussion

DAMP-induced sterile inflammation plays a critical role in acute brain injury (Savage et al. 2012). DAMPs like SAA, HSPs, HMGB1, and many others are well-known ligands for TLR4 (Trotta et al. 2014), and contribute to neurodegeneration (Rosenberger et al. 2015). LPS stimulation is known to induce CD40 gene expression through the activation of NF-κB and STAT-1α in macrophages and microglia (Qin et al. 2005). Further, CD40 is reported to contribute to inflammatory responses and brain infarction followed by leukocyte recruitment (Ritter et al. 2000). Microglial activation and neuroinflammation are important characteristics in the pathogenesis and progression of neurodegenerative diseases. Lipopolysaccharide (LPS)-treated mice are important and widely used animal models to study neuroinflammation and neurodegeneration (Catorce and Gevorkian 2015). Studies have shown that systemic injection of LPS markedly induces microglial activation, aberrant neuroinflammation, and oxidative stress in mouse brain. Increased IL-1β mRNA levels were found in the mouse brain following systemic LPS treatment. Although LPS treatment did not significantly alter

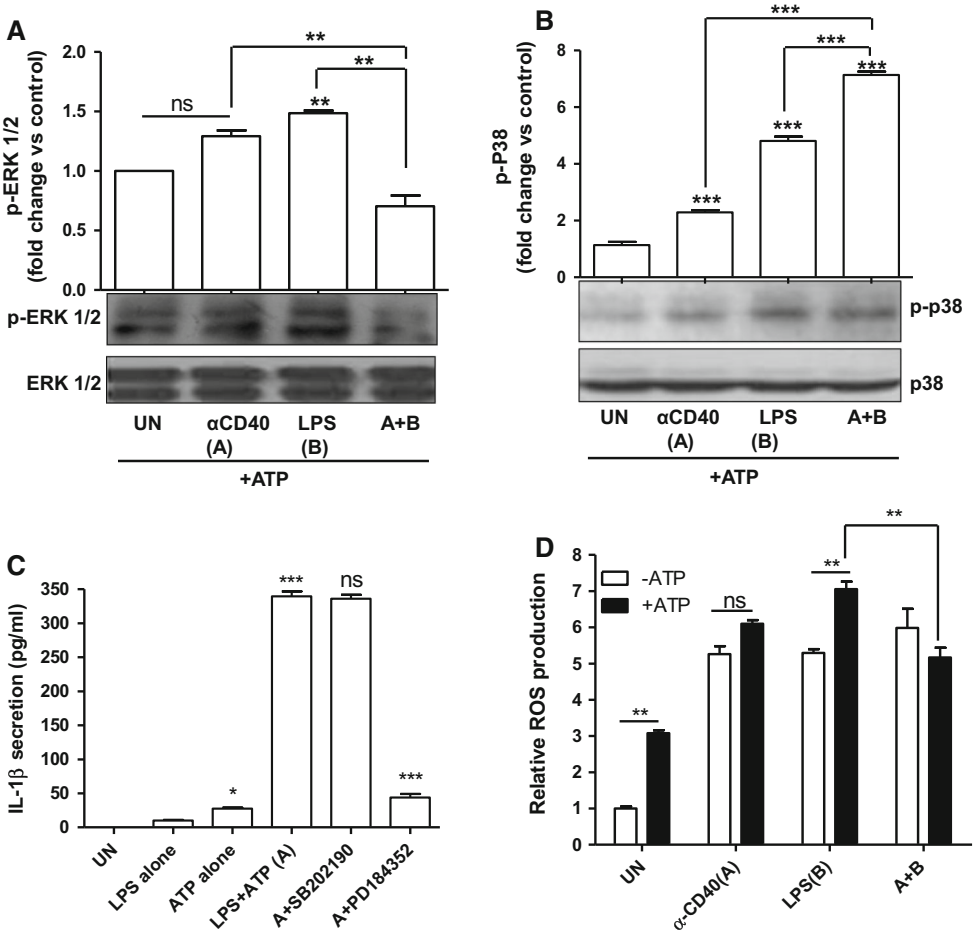

Fig. 3 CD40 inhibits NLRP3 inflammasome through inhibition of ERK 1/2 and ROS production**. a** and **b** BV2 cells were pretreated with agonistic anti-CD40 Ab for 30 min, followed by LPS stimulation later ATP was added for 30 min. Total and phosphorylated protein levels of p38 and ERK were assayed by Western blotting and relative fold change was analyzed by ImageJ software. **c** BV2 cells were pretreated with ERK 1/2 and p38 MAPK inhibitors for 1 h followed by LPS stimulation for 24 h and activated by ATP for 45 min before termination of experiments. Secreted IL-1β was measured by ELISA. **d** BV2 cells were pretreated with agonistic anti-CD40 Ab for 2 h and later with LPS for 24 h followed by ATP treatment for 45 min. ROS production was detected using Cell-Rox deep red reagent and ROS was measured using SpectraMax multimode plate reader at 644/655 nm, (**$p < 0.001$; ***$p < 0.0001$)

ATP levels, the ATP levels correlated with the levels of IL-1β and TLR4 in the LPS-treated mice (Noh et al. 2014). Furthermore, inhibition of TLR4 signaling has been reported to ameliorate neuroinflammation and neurodegeneration (Gaikwad and Agrawal-Rajput 2015; Tang et al. 2007; Kilic et al. 2008).

The study aimed to correlate the function of CD40 and its contribution during ATP-TLR4-mediated signaling and subsequent inflammasome activation. Our study demonstrated for the first time that (i) CD40 synergistically enhanced TLR4-mediated microglial activation and inflammation in the absence of DAMP (ATP); (ii) conversely, in the presence of ATP CD40 activation inhibits ATP-TLR4-mediated caspase-1 cleavage and IL-1β secretion in microglia; (iii) CD40 reverts reciprocally regulated ERK 1/2 and p38 phosphorylation to inhibit ATP-TLR4-induced inflammasome; and (iv) CD40 activation dampens inflammasome-IL1β-dependent Th17-mediated response through elevated IFN-γ.

The role of CD40–CD40L during CNS homeostasis and pathology is controversial. Studies have shown that the presence of functional CD40–CD40L maintains neuronal homeostasis and CD40-deficient mice remarkably show neurodegeneration and brain abnormalities, while intact CD40–CD40L dyad reinforces neuroprotection (Tan et al. 2002; Burwinkel et al. 2004). Constitutive expression of CD40 on microglia and the presence of its cognate ligand CD154 (CD40L) on astrocytes, endothelial cells, and vascular smooth muscle cells (Abdel-Haq et al. 1999; Tan et al. 1999) speculate important role of CD40–CD40L dyad. Controversially, CD40–CD40L interactions have also been implicated in various inflammatory disorders. Their expressions are elevated during ischemic stroke (Michels et al. 2015). CD40-mediated immune and inflammatory responses can play detrimental roles in neurodegeneration (Michels et al. 2015; Ke et al. 2005).

We aimed to explore CD40 activation-regulated microglial TLR4-mediated neuroinflammation. We report that CD40 stimulation can enhance TLR4 expression. Additionally, CD40 stimulation synergizes TLR4 to escalate IL-12 production, a cytokine promoting Th1 response and IFN-γ release. The synergy is also accompanied by elevated IL-6 and TNF-α along with inhibited IL-10 production. CD40 and TLR4 share common signaling

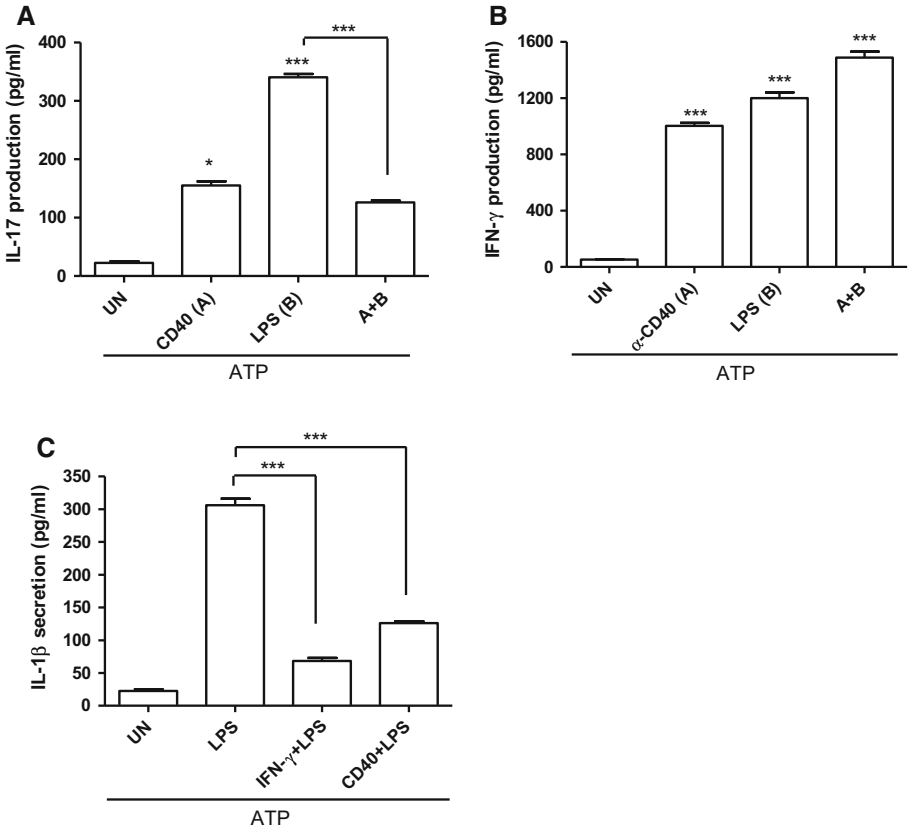

Fig. 4 CD40 inhibits inflammasome–IL-β–Th17 pathway through enhanced IFN-γ production. BM-MΦ were pretreated with agonistic anti-CD40 Ab for 2 h followed by stimulation of LPS for 24 h and activated with ATP (500 μM), $CD4^+$ T cells were cocultured for 48 h, and secreted cytokines IL-17 (**a**) and IFN-γ (**b**) were measured by ELISA. BM-MΦ were pretreated with r-IFN-γ (100U) for 2 h followed by sequential treatment with or without agonistic anti-CD40 Ab (2 h), LPS (24 h), and ATP (1 mM) for 45 min. Secreted IL-1β was measured using ELISA (**c**) (*$p < 0.005$; ***$p < 0.0001$)

intermediates such as ERK 1/2 and p38 MAPK (Mathur et al. 2004; Liu et al. 2012). ERK 1/2 and p38 MAPK activation play pivotal roles during CNS pathologies (Cruz and Cruz 2007). Our findings suggest that CD40 activation enhanced LPS-mediated ERK 1/2 phosphorylation with mild inhibition of p38 phosphorylation. However, the synergy did not induce IL-1β production suggesting the requirement of activation signal like DAMPs for inflammasome-mediated IL-1β secretion. Our data suggest that CD40 positively regulates TLR4-mediated inflammation and their crosstalk may provide Th1-mediated protective response during early/or and mild CNS pathologies. The Th1 cytokine, IFN-γ, has been reported to exert neuroprotective effect that prevents the lethal injury of motor neurons following infection with Theiler's murine encephalomyelitis virus (Rodriguez et al. 2003).

Inflammasome activation is known to play a key role during acute brain injury (Schroder et al. 2010; Walsh et al. 2014). Inhibition of inflammasome protects neuronal cells against ischemic stroke (Fann et al. 2013), suggesting that inhibition of inflammasome may provide potential clinical benefit. The key events of inflammasome activation include the priming and activation signals that promote caspase-1 cleavage, which facilitates the release of IL-1β, a crucial mediator of the acute neurodegeneration (Walsh et al. 2014; Allan 2005). However, the regulation of NLRP3 inflammasome in the CNS is poorly understood. DAMPs trigger inflammasome activation and consequent secretion of IL-1β (Savage et al. 2012; Rubartelli 2014; Trotta et al. 2014). As observed, CD40 and TLR4 activation is not sufficient for inflammasome activation. It may require danger signal like ATP to potentiate caspase-1 activation required for IL-1β secretion. We used ATP-TLR4 stimulation as an alternative approach to study DAMP-induced TLR4-mediated inflammasome activation in microglial cells (Rubartelli 2014; Kaushik et al. 2012). The presence of ATP during TLR4 activation resulted in NLRP3 activation followed by caspase-1 cleavage and release of IL-1β. Surprisingly, CD40 activation dampened ATP-TLR4-induced caspase-1 activation and the release of IL-1β, suggesting it as a novel negative regulator of inflammasome activation in microglia. CD40 activation triggers a series of signaling events including the reciprocal regulation of ERK 1/2 and p38 MAPK (Mathur et al. 2004). Studies have shown that ERK signaling is indispensible for LPS-mediated inflammasome priming (Ghonime et al. 2014), while increased p38 phosphorylation induces autophagy (Wu and Cederbaum 2013), which accompanies inflammasome inhibition (Shi et al. 2012; Chang et al. 2015). However, the role of CD40 in this context has never

been explored. We demonstrate that CD40 activation inhibits ATP-TLR4-induced inflammasome activation through negative regulation of ERK 1/2 accompanied with increased p38 MAPK phosphorylation. Inhibition in the levels of intracellular ROS was also observed which is a consequent event following inhibition of ERK 1/2 and it is essential for activation of NLRP3 inflammasome (Zhou et al. 2010; Liu et al. 2012; Ghonime et al. 2014).

Following innate activation, T lymphocytes infiltrate in the brain within few hours after ischemic stroke, contributing to neuropathology (Brait et al. 2010). IL-1β plays a pivotal role in differentiation of Th17 cells, which contributes significantly to pathophysiology of inflammation and acute brain injury (Asgari et al. 2013; Chung et al. 2009; Li et al. 2005). However, regulation of inflammasome–IL-1β–Th17 in CNS is poorly known. We report that CD40 activation inhibited IL-1β-dependent IL-17 production and enhanced IFN-γ production, suggesting that CD40 promotes Th1 response and dampens pathogenic Th17 response. Our data are supported by a report showing that CD40–CD40L interaction dampens innate immune responses through inhibition of inflammasomes (Guarda et al. 2009). In addition, we demonstrate that CD40 mediates IFN-γ release that negatively regulates IL-1β secretion. IFN-γ is known to increase STAT-1α-mediated CD40 expression in microglia (Nguyen and Benveniste 2000) citing a possible reason for CD40 activation. Our data are also supported by the finding that IFN-γ decreased IL-1β and subsequently IL-17 (Masters et al. 2010), suggesting the role of CD40 in limiting IL-1β-mediated inflammation. Notably, CD40 activation and IFN-γ-producing $CD4^+$ T cells have been shown to provide neuroprotection through regulation of microglial response to injury (Tan et al. 2002; Beers et al. 2008). The CD40-mediated feedback loop mechanism thus regulates NLRP3 inflammasome through Th1 activation, providing a checkpoint to limit excessive inflammasome activation, while the inflammasome independent inflammatory response (IL-6 and IL-12) remains intact to ensure protective immunity.

In summary, we demonstrate the dual role of CD40 in regulating TLR4-mediated inflammation in microglia through inflammasome-dependent and independent pathways. The absence of danger signal during TLR4 activation results in synergy between TLR4 and CD40 to induce protective response. In the presence of danger signal (ATP) during TLR4 activation, CD40 provides a checkpoint to limit deleterious immune outcome marked by a decrease in pathogenic IL-1β–IL-17, while inflammasome independent inflammatory response remains intact to extend optimum immunity. However, the findings need to be validated in the in vivo model systems.

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Compliance with Ethical Standards

Conflict of Interest The authors report no conflicts of interest.

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