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Sneaky Entry of IFNγ Through Arsenic-Induced Leaky Blood–Brain Barrier Reduces CD200 Expression by Microglial pro-Inflammatory Cytokine

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Abstract

Recent studies showed that neuronal surface protein CD200 plays a key role in the regulation of neuroinflammation. Previously, we showed that arsenic (0.38 mg/kg body weight) exposure induces microglial activation and consequently IL-6/TNF-α secretion. This result indicated the possibility of alteration in the expression of CD200. Therefore, the present study was focused on checking arsenic-induced alteration in CD200 expression and revealing the underlying mechanism. Male BALB/c mice were exposed to arsenic (vehicle, 0.038 and 0.38 mg/kg body weight) for 60 days, and the expression level of CD200 was found to be decreased which was rescued by minocycline (33 mg/kg body weight) co-administration. Higher CD68 staining, increased level of IL-6/TNF-α, as well as higher level of IFNγ, were observed in in vivo arsenic-exposed groups. Interestingly, in vitro arsenic exposure could not increase IL-6/TNF-α level in the culture supernatant, whereas, supplementation of IFNγ could mimic the in vivo results. However, arsenic could not induce IFNγ production from brain endothelial cells, microglia, and astrocytes, thereby suggesting the entry of IFNγ through the impaired blood–brain barrier. Evans blue fluorescence in the brain confirms altered blood–brain barrier permeability although no changes were observed in the expression level of tight junction proteins (claudin-5 and occludin). Finally, intracerebral injection of anti-IFNγ neutralizing antibody in arsenic-exposed brain reduced microglia activation (IL-6 and TNF-α and CD68 expression) and subsequently rescued CD200 level. Taken together, the study showed that arsenic-mediated compromised blood–brain barrier is a major driving force to induce microglial IL-6 and TNF-α production through serum IFNγ leading to CD200 downregulation.

Keywords Microglia · Cytokine · Neuron · CD200 · IFNγ · Arsenic · Blood–brain barrier

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Introduction

Neuroinflammation is a tightly controlled phenomenon and kept in check by different inhibitory signals which otherwise can lead to different disease state in the central nervous system. Contact-dependent cell-cell interaction between microglia and neurons through CD200R and CD200 is one of the major inhibitory signals [1]. Chronic neuroinflammatory signals downregulate CD200 expression and thereby sensitize the brain towards neurodegenerative diseases. CD200 expression was found to be decreased in the patients with pediatric epilepsy, Parkinson's, as well as Alzheimer's disease [2–4]. Experimental $CD200^{-/-}$ mouse showed compromised blood–brain barrier (BBB) leading to IFNγ entry into the brain [5] and showed enhanced susceptibility to encephalomyelitis [6]. In contrary, CD200 fusion protein (CD200Fc) shown to reduce the neuroinflammation [7] as well as inhibit the

induction of experimental autoimmune uveoretinitis (EAU) [3]. CD200 expression was observed to be increased both in vitro and in vivo in mouse neurons in the presence of anti-inflammatory cytokine IL-4 [8].

Endogenous factors like Aβ decrease CD200 expression [9]. Apart from its role in neuroinflammation, promoters of both the human and mouse CD200 gene have a binding site for tumor suppressor protein p53 thereby showing its involvement in apoptosis [10]. The transcription factor C/EBPβ regulates the constitutive expression of CD200 by binding to its promoter [11]. Along with endogenous factors, various exogenous factors like bacterial cell wall component, LPS, as well as various environmental toxicants can alter the expression of CD200. Among various environmental chemicals, arsenic is globally infamous for its toxicity in every sphere of life [12, 13]. Arsenic is known to cross blood–brain barrier (BBB) and affect neuronal health in exposed animal [14]. The study indicates that arsenic may affect the integrity of BBB and alter the permeability. Our group has reported that environmentally relevant arsenic dose (0.38 ppm) induces pro-inflammatory cytokines like IL-6 and TNF-α in ex vivo microglia [15]. Therefore, there is high chance that arsenic would affect the expression of CD200 in the brain. There are reports showing compromised cell-cell interaction result in the modest release of inflammatory cytokine from microglia causing cognitive impairment. On the other hand, lower IQ, decreased learning and memory response, as well as altered cognitive response were observed among individuals in arsenic-affected areas [16]. Therefore, environmental stress-mediated dysfunction of neuron-microglial interaction impelled us to explore the mechanism of arsenic-induced alteration in CD200 expression.

In the present study, we tried to find out the role of arsenic in altering the expression of CD200 in the brain. Expression of CD200 was checked in the brain sample of 60-day-exposed mice with or without minocycline treatment as well as in primary cortical neurons following in vitro arsenic exposure. Microglial activation status was checked by immunostaining the activation marker CD68 and cytokine (IL-6, TNF-α, and IFNγ) secretion by primary microglia isolated from experimental mice using multiplex cytokine detection kit. Level of IFNγ was also measured in ex vivo microglia from an adult mouse, neonatal microglia, astrocyte, and endothelial cell culture supernatant as well as in serum. The expression level of blood–brain barrier proteins, i.e., occludin and claudin-5 were checked by western blot analysis. The integrity of the blood–brain barrier was checked by Evans blue dye permeability assay. Finally, the involvement of IFNγ in the arsenic-mediated regulation of CD200 expression was confirmed by injecting anti-IFNγ antibody into the brain by stereotaxy. Thus, in the present study, we unraveled that arsenic exposure compromised the BBB permeability through which IFNγ comes into the brain and induced microglial activation, which in turn leads to downregulation of CD200.

Materials and Methods

Reagents and Antibodies

Sodium arsenite (SA) ($NaAsO_2$), Percoll, cell culture medium (DMEM/F12), papain, sodium dodecyl sulphate (SDS), acrylamide, bis-acrylamide, bromophenol blue, Tween 20, N-ethylmaleimide, o-phthalaldehyde, EDTA, protease inhibitor cocktail, 10× Western blocking buffer, methanol (LC grade), minocycline, Evans Blue, Cytosine arabinoside, and Poly L Lysine were obtained from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Cell Clone Inc. PVDF membrane, Occludin antibody, claudin-5 antibody, and chemiluminescence substrates were obtained from Merck-Millipore (USA). CD200 antibody was purchased from Santacruz biotechnology. CD68 antibody is obtained from Abcam. IFNγ neutralizing antibody, N-2 supplement, B27 supplement, B5 supplement, Glutamax, neurobasal medium, and Alexa Fluor 594 anti-rabbit were obtained from Thermo Scientific. Recombinant IFNγ was purchased from BD Biosciences. Tissue-freezing medium was obtained from Leica.

Animals and Treatment

Six to eight-week old male BALB/c mice (20–25 g) were procured from the CSIR-Indian Institute of Toxicology Research (CSIR-IITR) animal facility. All the protocols for this study were approved by the Institutional Animal Ethics Committee of CSIR-IITR, Lucknow, India, and all experiments have been carried out in accordance with the guidelines laid down by the committee for control and supervision of experiments on animals (CPCSEA), Ministry of Environment and Forests (Government of India), New Delhi, India. Mice were housed at 25 °C with food and water supplied ad libitum. Three sets of animal experiments were performed, and animals were divided randomly by investigator who is blind about treatment groups; in the first set, animals were divided into three groups: control, 0.038 ppm, and 0.38 ppm arsenic treatment group, in the second set, animals were divided into three groups: control, 0.38 ppm arsenic, and 0.38 ppm arsenic + minocycline (33 mg/kg) [17], and in the third set, animals were divided into three groups: vehicle control (sham control), 0.38 ppm arsenic, and 0.38 ppm arsenic + anti-IFNγ neutralizing antibody. Sodium arsenite solution was gavage fed daily in the arsenic treatment groups. In the second set, minocycline was given for the last 2 weeks every alternate day till sacrifice, and in the third set, 3.5 μg, the anti-IFNγ neutralizing antibody was intracerebrally injected in arsenic-treated group 6 days before sacrifice. The control group received water.

Isolation of Primary Microglia (Neonatal and Adult) and Culture

Neonatal microglia was isolated from post natal day 0–3 pups according to the protocol described in Saura et al. [18]. Briefly, brain cortices were isolated and digested to form a cell suspension. 0.4×10^6 cells/well were seeded in 12 well plates in DMEM/F12 medium with 10% FBS, and the medium was replaced every third to fourth day till cells reached full confluency. Then, mild trypsinization with DMEM/F12:Trypsin solution in 1:1 ratio gives pure microglial cells. Sixty thousand cells in 500-μl medium were given treatments according to experimental requirements. Adult microglia isolation was performed as established in our lab [15]. Briefly, brain homogenate is centrifuged at 500×*g* for 20 min at 20 °C on 30% isotonic percoll gradient, and 50,000 microglial cells were cultured in DMEM/F12 supplemented with 2% FBS and N-2 supplement for 18 h, and the medium was collected for cytokine estimation.

Primary Neuron Culture

Neuronal cultures were obtained with some modifications of the protocol described by Pardo et al. [19]. Briefly, brain cortices were isolated from mice embryonic day 18 fetuses and kept in cold hanks buffer followed by meninges removal. After enzymatic digestion of cortices by 0.25% trypsin for 5 min at 37 °C, resulting cell suspension were centrifuged at 1000 rpm for 5 min. Finally, cells were counted, and 0.5×10^6 cells were plated in poly-L-lysine-coated 12 well culture plates in neurobasal medium (containing 1% penicillin-streptomycin, 1% glutamax) with 20% horse serum for 3 h. After that, medium was replaced with fresh serum-free neurobasal medium with 2% B27 supplement and replaced half every third day in culture. Sodium arsenite treatment was given on the seventh day in vitro.

Microglia-Neuron Co-culture

Mix glial culture plates were prepared as discussed above and mildly trypsinized to remove astrocytes. Resulting attached microglia were incubated with 0.25% trypsin for 10 min at 37 °C. Trypsinized cells were centrifuged at 1500 rpm for 10 min, and the cell pellet was suspended in the neurobasal medium. After cell counting, microglia is plated on neuronal cultures at a ratio of 0.5:1 [1]. Therefore, 50-μl medium is containing microglial cells; 50,000 cells were seeded on 7 days in vitro 0.1×10^6 neurons and kept for 24 h at 37 °C in 5% CO_2. Treatments were given 24 h after addition of microglia on neuronal cultures.

Astrocyte and bEND.3 Cell Culture

Briefly, cerebral cortices of 1-day-old neonatal mice pups were dissected, and meninges were removed. Cortices were dissociated with 0.05% trypsin/EDTA at 37 °C for 15 min into a cell suspension. After centrifugation, the pellet obtained was suspended in complete DMEM/F12 media, and cells were seeded on poly-l-lysine-coated culture flasks and grown until confluent in a humidified CO_2 incubator at 37 °C with 5% CO_2. At the third day, cells were supplemented with G5 growth supplement. The confluent cells were rinsed with PBS and then detached with 0.05% trypsin/EDTA, and 30,000 cells were plated in 100 μl serum-free DMEM/F12. Sodium arsenite treatment was given 12–16 h after seeding, and culture medium was collected 48 h after treatment. bEND.3 cell line (CRL-2299™) was cultured in DMEM/F12 with 10% FBS and 1% pen-strep. Treatment was given in 30,000 cells similarly as in astrocyte culture.

Preparation of Cell Lysate and Western Blot Analysis

Following treatment, cells were rinsed with cold PBS, scrapped, and centrifuged at 4 °C. Cell pellet was resuspended in cell lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, protease inhibitor cocktail). Cells were freeze-thawed thrice in lysis buffer and centrifuged at 12000*g* and supernatant was collected. Protein concentration in the supernatant was quantitated by Bradford reagent [20]. Twenty to fifty micrograms of the protein of each sample was run on 7–10% SDS-PAGE, transferred to PVDF membrane, and probed with primary antibodies of CD200 (1:3000), occludin (1:3000), and claudin-5 (1:3000) in TBS overnight at 4 °C. After washing thrice for 15 min each, anti-rat and anti-rabbit secondary antibodies (1:10,000) for respective primary antibodies were added for 2–3 h followed by washing with TBST. Then visualization of protein was done by chemiluminescence substrate followed by bright field image capturing of protein marker in a gel documentation system (G-box H-16, Syngene). The same blot was stripped using stripping buffer (Sigma) and re-probed with β-actin primary antibody followed by incubation with secondary antibody and visualized under gel documentation system. Finally, densitometric analysis of the developed protein bands was done by Image-J software.

Detection of Cytokine Level in the Culture Supernatant

Cytokine levels in cell culture supernatant, serum, and brain lysate were measured using Milliplex mouse cytokine assay kit (Millipore) following the manufacturer's instructions. In brief, the magnetic assay plate was washed with 200 μL of

wash buffer followed by addition of 25 μL of undiluted sample along with a 25-μL mixture of antibody-coated magnetic beads for IL-6 and TNF-α into the well of a magnetic assay plate (96 well plate) and kept on a plate shaker at 25 °C for 2–3 h at 240 rpm. Following incubation, the magnetic plate was washed twice with wash buffer by using a hand-held magnet. Biotinylated detection antibody (25 μL) was added to each well and incubated for 1 h at room temperature followed by incubation with 25 μL of streptavidin−phycoerythrin for 30 min at room temperature. The plate was finally washed twice with wash buffer and run on the Bio-Plex MAGPIX multiplex reader (Bio-Rad). The infrared (IR) fluorescence associated with different antibody-coated magnetic beads was read to quantitate respective cytokines from a standard curve of that particular cytokine.

Evans Blue Permeability Assay

Blood–brain barrier permeability test was performed using Evans blue dye as described in Gasche et al. [21]. Briefly, 3% Evans blue in saline was injected though the tail vein 4 h before sacrifice. For quantification of Evans blue fluorescence in the brain, tissue was isolated, immediately weighed, and mixed with 500 μl formamide. After homogenization, the lysate was kept at 55 °C overnight followed by centrifugation at 10,000×*g* for 30 min, and the absorbance of the collected supernatant is measured at 620 nm in a microplate reader (Fluostar Omega, BMG Labtech).

Cryosectioning, Fluorescence Microscopy, and Infra-Red Fluorescent Imaging

Anesthetized mice were perfused by chilled PBS followed by 4% paraformaldehyde (PFA). The brain was removed and kept overnight in 4% PFA. For cryosectioning, the brain was washed with PBS and then kept in 15 and 30% sucrose solution for 1 day each. Coronal sections of the brain were cut using a cryotome (Thermo scientific) and kept in PBS. Brain cryosections were observed under a fluorescence microscope as well as in an in vivo imaging system (IVIS spectrum), and images were taken.

Immunohistochemistry

Coronal sections of the brain are washed with PBS followed by addition of blocking buffer (2% Horse serum and 0.1% Triton X-100 in PBS) for 1 h. After blocking, sections were washed with PBST (phosphate buffer saline with 0.1% Tween 20) followed by overnight incubation with the CD68 primary antibody (1:500 in blocking buffer). Sections were washed with PBST five times 10 min each and incubated with appropriate secondary antibodies for 2 h at RT. Nuclei were stained with DAPI (1 μg/ml) for 2 min and washed, and sections were mounted on glass slides using vectashield mounting medium (Vector lab). Slides were observed under Nikon Eclipse 90i microscope (Nikon, Kawasaki, Japan).

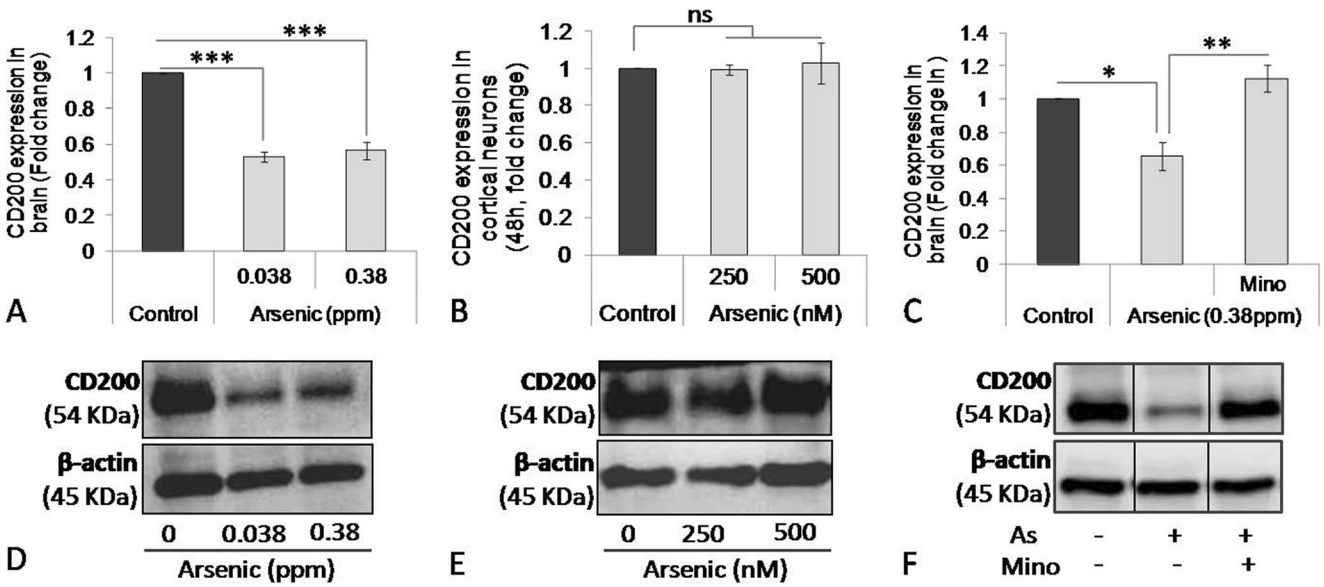

Fig. 1 Effect of arsenic on CD200 expression in the brain. **a** In vivo arsenic exposure decreases CD200 expression in the brain ($n = 4$ each group), but (**b**) in vitro arsenic exposure does not alter CD200 expression ($n = 3$). **c** Minocycline treatment reverses arsenic-induced reduced expression of CD200 ($n = 3$ each group). **d**–**f** are respective representative western blot of data presented in **a**, **b**, and **c**. Densitometric analysis was performed using image J software. *p* denotes level of significance in comparison to control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; *ns* non significant. Vertical bars in between lanes in **f** depicts that lanes were cropped and joined together

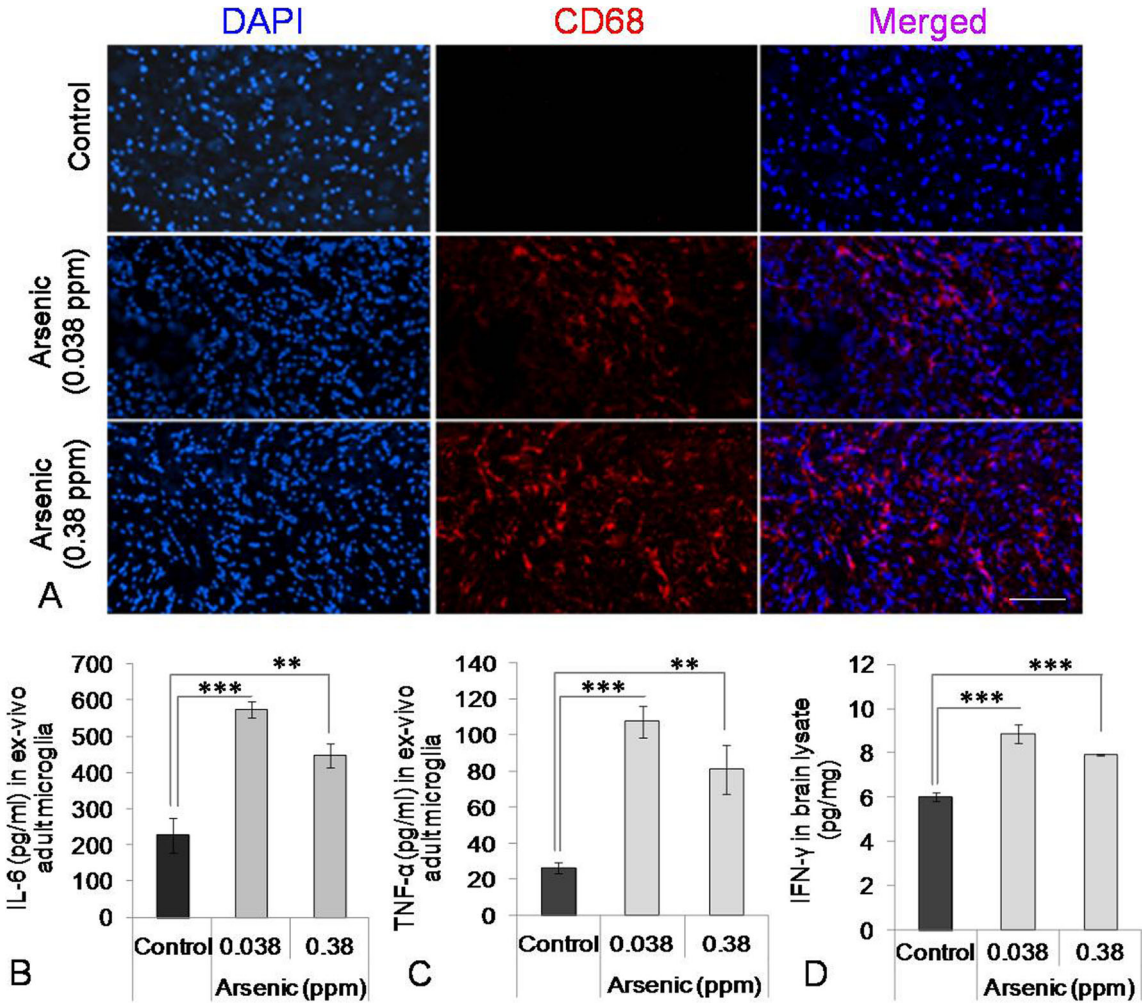

Fig. 2 Effect of in vivo arsenic exposure on the microglial activation and cytokine secretion. **a** In vivo arsenic exposure for 2 months activated microglia as determined by increased expression of CD68 in brain sections ($n = 3$ each group). In vivo arsenic exposure also increased the level of **b** IL-6 and **c** TNF-α in ex vivo microglia culture supernatant ($n = 3$ each group) as well as **d** IFNγ in brain lysate ($n = 4$ each group). p denotes level of significance in comparison to control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; *ns* non significant. The scale bar represents 50 μm

Anti-IFNγ Neutralizing Antibody Treatment Using the Stereotaxic Technique

To confirm the IFNγ-mediated activation of microglia and secretion of IL-6 and TNF-α cytokines, we performed intracerebral injection of recombinant anti-IFNγ neutralizing antibody using the stereotaxic technique. Briefly, mice were anesthetized with intraperitoneal injection of ketamine and xylazine (60 and 20 mg/kg body weight, respectively). The mice were placed in the stereotaxic frames (Stoelting Co), skull exposed, and disinfected with betadine. An incision was made into the scalp, and a 0.5-mm burr hole was drilled at 3 mm caudal and 2 mm horizontal from bregma on medial and lateral sides [22]. At a depth of 1.6 mm from dorsal to ventral, recombinant anti-IFNγ neutralizing antibody (1.75 μg in 1.75 μl saline in each hemisphere) was injected slowly (1 μl/min) with a 10-μl Hamilton syringe on the 54th day of arsenic exposure and dissected on the sixth day following injection [23]. The vehicle control mice for these groups were treated with sterile PBS that followed the same procedure. Microglia was isolated and cultured for 18 h ex vivo. Levels of IL-6 and TNF-α were measured in the culture supernatant using multiplex cytokine detection kit.

Statistical Analysis

Results were expressed as mean ± SEM. Statistical significance was determined by unpaired Student's t test for two groups and one-way ANOVA for more than two groups, followed by Newman-Keuls post-hoc analysis by Graph-Pad prism (GraphPad Software, San Diego, CA). $p < 0.05$ is considered as statistically significant.

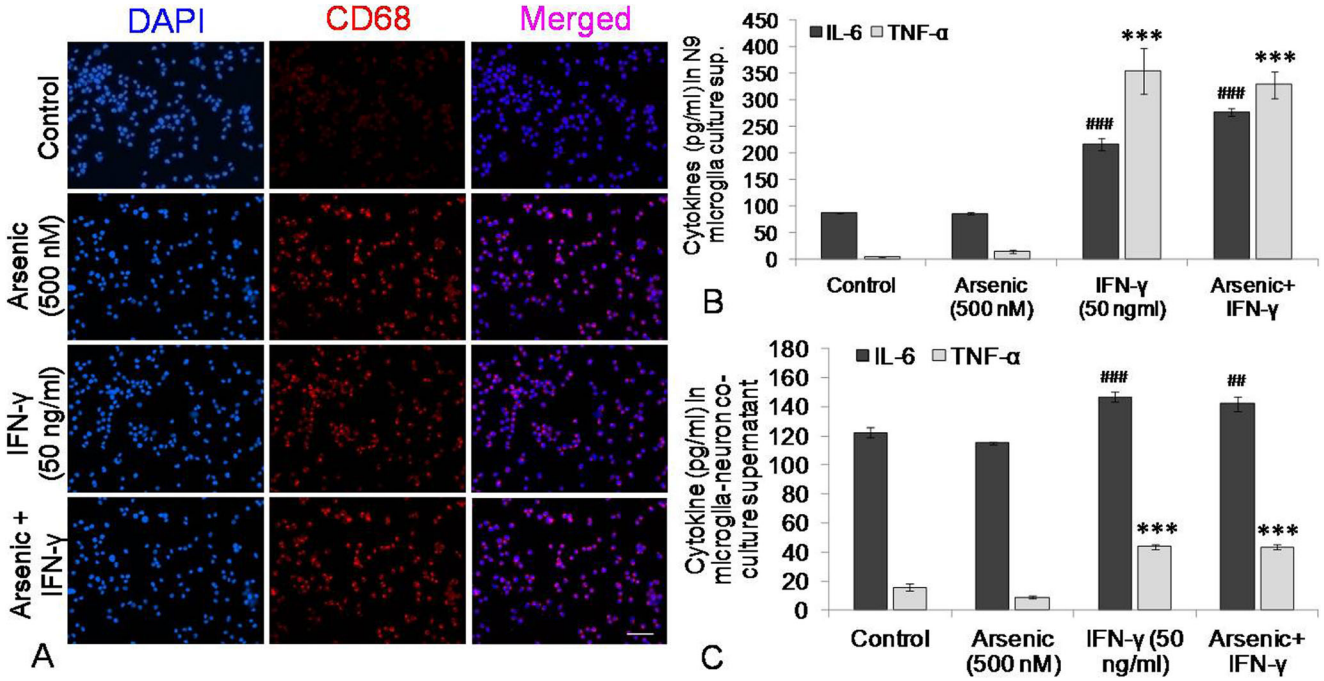

Fig. 3 Effect of in vitro arsenic exposure and the role of IFNγ on the microglial activation and cytokine secretion. **a** Expression of CD68 increased following arsenic as well as IFNγ treatment. **b** In vitro arsenic exposure does not alter the IL-6 and TNF-α level in culture supernatant of microglia cell line (N9) although IFNγ treatment increased the level of both the cytokines significantly ($n = 3$). **c** IFNγ treatment increased the level of IL-6 and TNF-α in the culture supernatant of primary microglia-neuron co-culture whereas arsenic does not alter the cytokine level ($n = 3$). p denotes level of significance in comparison to control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; *ns* non significant. The scale bar represents 10 μm

Results

In Vivo Arsenic Exposure Downregulates the Expression of CD200 but Not In Vitro

Expression of CD200 following in vivo arsenic exposure Six- to eight-week-old BALB/c mice were exposed to arsenic (0.038 and 0.38 ppm) for 2 months on a daily basis. Following the completion of the arsenic exposure, animals were sacrificed, and the expression of CD200 was checked in the brain lysate by western blot analysis. It was observed that arsenic exposure significantly suppressed the expression of CD200 around 50% compared to control group (Fig. 1a, d).

Expression of CD200 following in-vitro arsenic exposure Effect of in vitro arsenic exposure on the expression of CD200 was also checked. Cortical neurons were isolated from embryonic day 18 fetus and cultured in vitro. Following 6 days in culture, cortical neurons were exposed to a non-toxic dose of arsenic (250 and 500 nM) for 48 h, and CD200 expression was checked. Unlike in vivo exposure, arsenic exposure did not alter the expression of CD200 in-vitro (Fig. 1b, e).

Effect of In-Vivo Minocycline Administration on the Arsenic-Induced Altered Expression of CD200 By the expression pattern of CD200 following in vivo and in vitro arsenic exposure, we hypothesize that, decreased CD200 expression is mediated through microglia-induced inflammation in the brain under the influence of arsenic exposure. To test this hypothesis, we have administered minocycline (33 mg/kg intraperitoneally) every alternate day in the last 14 days from sacrifice along with arsenic exposure. Interestingly, minocycline was found to rescue the expression level of CD200 to the level of the control group (Fig. 1c, f).

In Vivo Arsenic Exposure Activates Microglia and Increases the Level of IL-6, TNF-α, and IFNγ in the Brain

Effect of In Vivo Arsenic Exposure on Microglial Activation Status Microglial activation status was checked by immunostaining the microglia-specific activation marker, CD68 on brain sections from 2-month arsenic-exposed mice as well as control. It was very clear from the CD68 expression in brain sections that arsenic (0.038 and 0.38 ppm) increased the CD68 expression thereby the activation (Fig. 2a).

Effect of In Vivo Arsenic Exposure on Microglial Cytokine Level Following 60-day arsenic exposure, microglia were isolated and cultured for 18 h (ex vivo microglia), and level of IL-

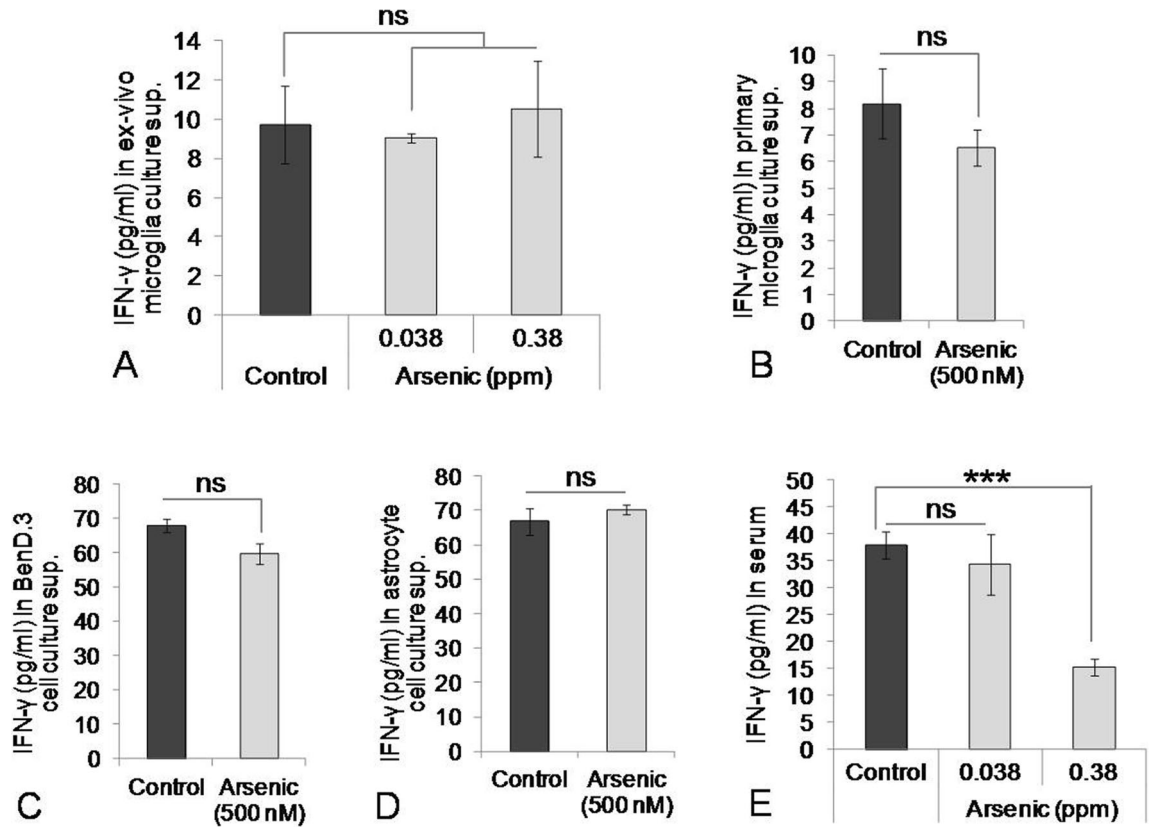

Fig. 4 Source of IFNγ in the brain of arsenic-exposed mouse. Level of IFNγ was checked in culture supernatant of different cell types to find out the exact source of IFNγ. **a** Ex vivo microglia ($n = 3$ each group). **b** Primary microglia. **c** Primary astrocyte. **d** bEND.3 cells. **e** Mouse serum (n = at least 4 each group). p denotes level of significance in comparison to control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; *ns*: non significant

6 as well as TNF-α was measured in the culture supernatant. IL-6 level increased significantly at 0.038 and 0.38 ppm arsenic to 550 and 420 pg/ml respectively with respect to control 200 pg/ml (Fig. 2b). Whereas, level of TNF-α increased to 100 and 80 pg/ml at 0.038 and 0.38 ppm arsenic respectively, compared to around 26 pg/ml in the control group (Fig. 2c).

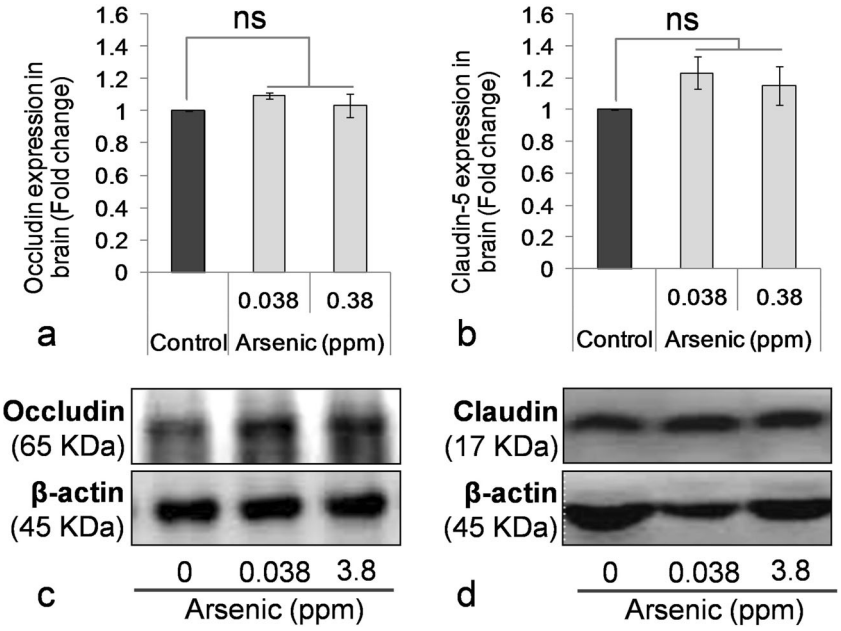

Fig. 5 Effect of arsenic on the expression of blood–brain (BBB) barrier proteins. Changes in the expression level of **a** occludin, and **b** claudin-5. Representative western blot of **c** occludin and **d** claudin-5 (n = at least 4 each group). Densitometric analysis was performed using image J software. p denotes level of significance in comparison to control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; *ns* non significant

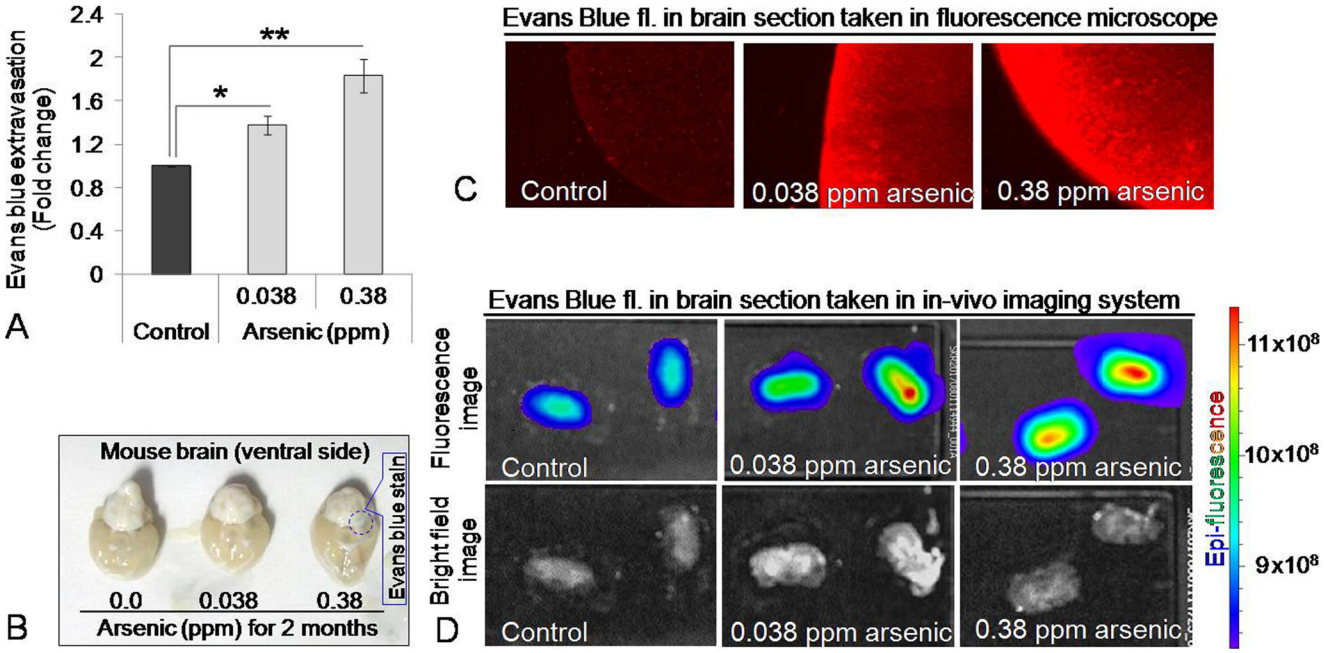

Fig. 6 Effect of arsenic on blood–brain barrier (BBB). Evans blue permeability was performed to check whether the integrity of BBB becomes compromised. **a** Evans blue fluorescence in brain lysate ($n = 4$ each group). **b** Image of whole brain following Evans blue injection. **c** Image of brain sections captured in fluorescence microscope.(**d** Image of brain sections captured in in vivo imaging system. p denotes level of significance in comparison to control; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; *ns* non significant

Effect of In Vivo Arsenic Exposure on IFNγ Level in the Brain We have also observed that there is a significant increase in interferon-γ levels in brain lysate of 2-month exposed samples. In 0.038 and 0.38 ppm arsenic-exposed group, IFNγ levels are 9 and 8 pg/mg protein respectively as compared to control 6 pg/mg protein (Fig. 2d).

In Vitro Arsenic Exposure Activates Microglia but Needs IFNγ Help for Cytokine Production

Effect of In Vitro Arsenic and IFNγ Exposure on Microglial Activation Status We have checked the levels of microglial activation marker, CD68, following arsenic and IFNγ alone as well as co-exposure. It was observed that arsenic as well as IFNγ both are capable of activating microglia alone and in combination (Fig. 3a).

Effect of In Vitro Arsenic and IFNγ Exposure on Microglial Cytokine Level To identify the role of IFNγ in inducing microglial inflammation, microglia monoculture (N9 microglia) and microglia-neuron co-culture were exposed to arsenic and IFNγ alone as well as in combination. It was observed that arsenic could not stimulate cytokine secretion in either of monoculture and co-culture although increased the expression of microglial activation marker, CD68. Levels of IL-6 detected in the culture supernatant of microglia monoculture were around 86 pg/ml in the control group, 85 pg/ml in the arsenic group, 235 pg/ml in the IFNγ group, and 286 pg/ml in the arsenic-IFNγ co-exposed group. In the case of microglia-neuron co-culture supernatant, the IL-6 levels were around 120, 115, 145, and 140 pg/ml respectively in the control, arsenic, IFNγ, and in an arsenic-IFNγ co-exposed group. A similar pattern of cytokine level was observed for TNF-α. The level of TNF-α detected in microglial monoculture supernatant was around 5 pg/ml in the control group, 21 pg/ml in the arsenic group, 312 pg/ml in the IFNγ group, and 297 pg/ml in the arsenic-IFNγ co-exposed group. In co-culture supernatant, a similar trend of TNF-α was 18 pg/ml in the control, 12 pg/ml in the arsenic group, 53 pg/ml in IFNγ group, and 53 pg/ml in arsenic and IFNγ co-exposed group. Altogether, these results showed that although arsenic activates microglia but cannot induce cytokine production. Induction of cytokine production needs IFNγ stimulation (Fig. 3b, c).

None of the Brain Cells Secrete IFNγ. Therefore, Serum IFNγ Enters the Brain

It has been very much evident from the earlier results of the present study that IFNγ is essential for the initial induction of microglial inflammation which leads to the downregulation of CD200 expression. To find out the source of IFNγ detected in arsenic-exposed brain lysate (Fig. 2d), we first tested the IFNγ levels in 2-month control and arsenic exposed ex vivo microglia culture supernatant and observed no significant alterations in IFNγ levels (Fig. 4a). Furthermore, neonatal microglia, astrocytes, and brain endothelial cell line, bEND.3, all were failed to induce IFNγ following in vitro exposure to 500 nM arsenic (Fig. 4b, c, d). Finally, we have checked the

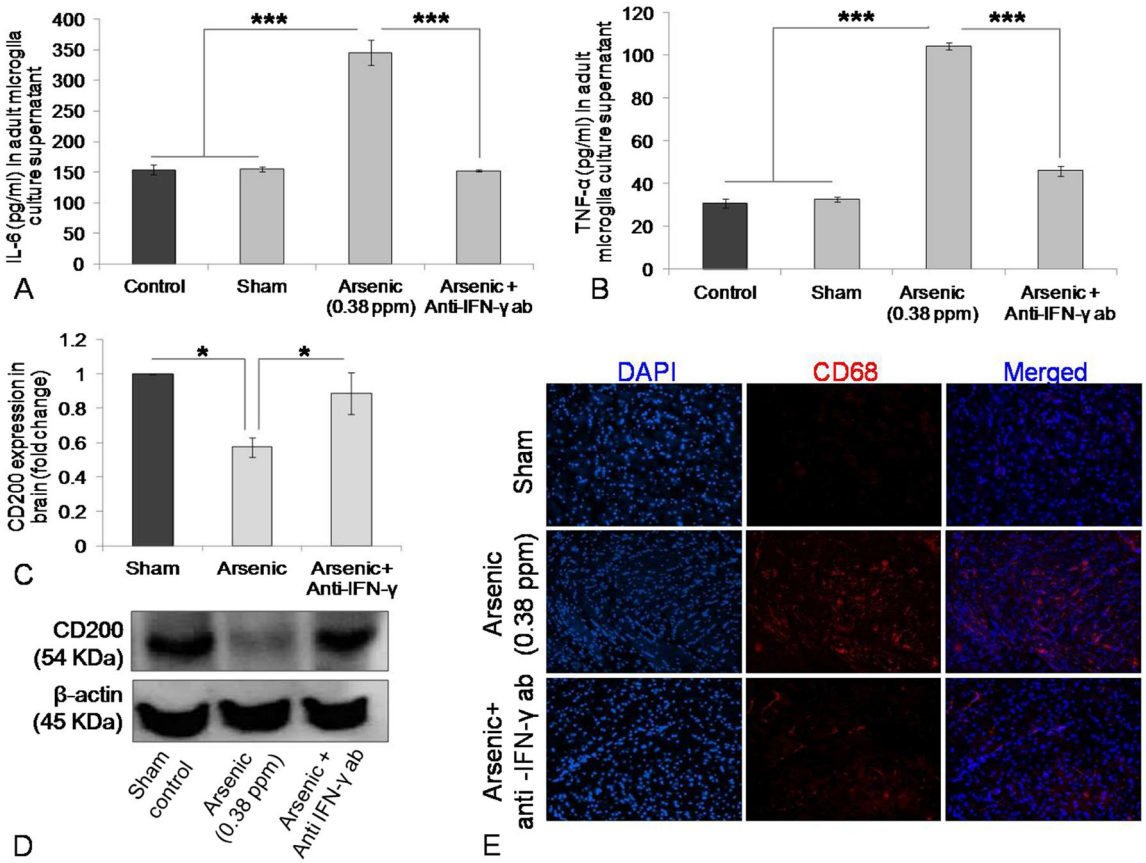

Fig. 7 Effect of in vivo IFNγ neutralization on the induction of IL-6 and TNF-α and level of CD200 expression in arsenic-exposed animals. Anti IFNγ neutralizing antibody (3.5 μg/animal) was injected in the brain directly by stereotaxy 6 days before completion of 2-month arsenic exposure. Primary microglia were isolated and levels of **a** IL-6 and **b** TNF-α were measured in the culture supernatant following 18 h of ex vivo incubation ($n = 3$ each group). The brains were isolated, and expression level of CD200 was checked by western blot analysis ($n =$ at least 3 each group). **c** Densitometric analysis of CD200 expression level. **d** Representative western blot of CD200 in the brain. **e** Representative photomicrographs of expression of microglial activation marker CD68 in brain section. p denotes level of significance in comparison to control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; *ns* non significant. The scale bar represents 50 μm

levels of IFNγ in serum samples of 2-month control and arsenic-exposed mice (0.038 and 0.38 ppm). There is no significant decrease in IFNγ levels in 0.038 ppm arsenic-exposed group with respect to control. Although 0.38 ppm arsenic exposure significantly decreased the level of IFNγ to 15 pg/ml with respect to 35 pg/ml in control group indicating towards immunosuppression but still the levels were believed to be sufficient to activate microglia to secrete IL-6 and TNF-α (Fig. 4e).

In Vivo Arsenic Exposure Renders Blood–Brain Barrier Leaky Without Affecting Blood–Brain Barrier Protein Level

Effect of In Vivo Arsenic Exposure on the Expression of Blood–Brain Barrier Protein Occludin and Claudin-5 We found that none of the brain cells were able to increase the IFNγ compared to control group. On the other hand, a higher level of IFNγ was detected in the blood indicating a possibility of the disrupted blood–brain barrier which might allow the entry of IFNγ from the blood. We have checked the expression level of blood–brain barrier proteins occludin and claudin-5 level in brain lysate following 2-month arsenic exposure. No significant alteration in the expression level was observed for both occludin (Fig. 5a, d) and claudin-5 (Fig. 5b, d).

Effect of In Vivo Arsenic Exposure on the Blood–Brain Barrier Permeability We have further checked if there is any alteration in the permeability of the blood–brain barrier using Evans blue dye. The optical density of Evans blue in the brain shows a significant dose-dependent increase to 1.3 and 1.8-fold in 0.038 and 0.38 ppm arsenic-exposed group with respect to control (Fig. 6a). Although very faint, but still blue tinch of Evans blue is visible on the brain isolated from 0.38 ppm arsenic-exposed animals (Fig. 6b). Representative fluorescence image of brain cryosection of control as well as arsenic treatment group showing increased fluorescence of Evans blue dye (Fig. 6c). Brain sections were also analyzed in the in vivo imaging system (IVIS, Caliper Life Sciences/ Perkin Elmer). Red region corresponds to the more intense

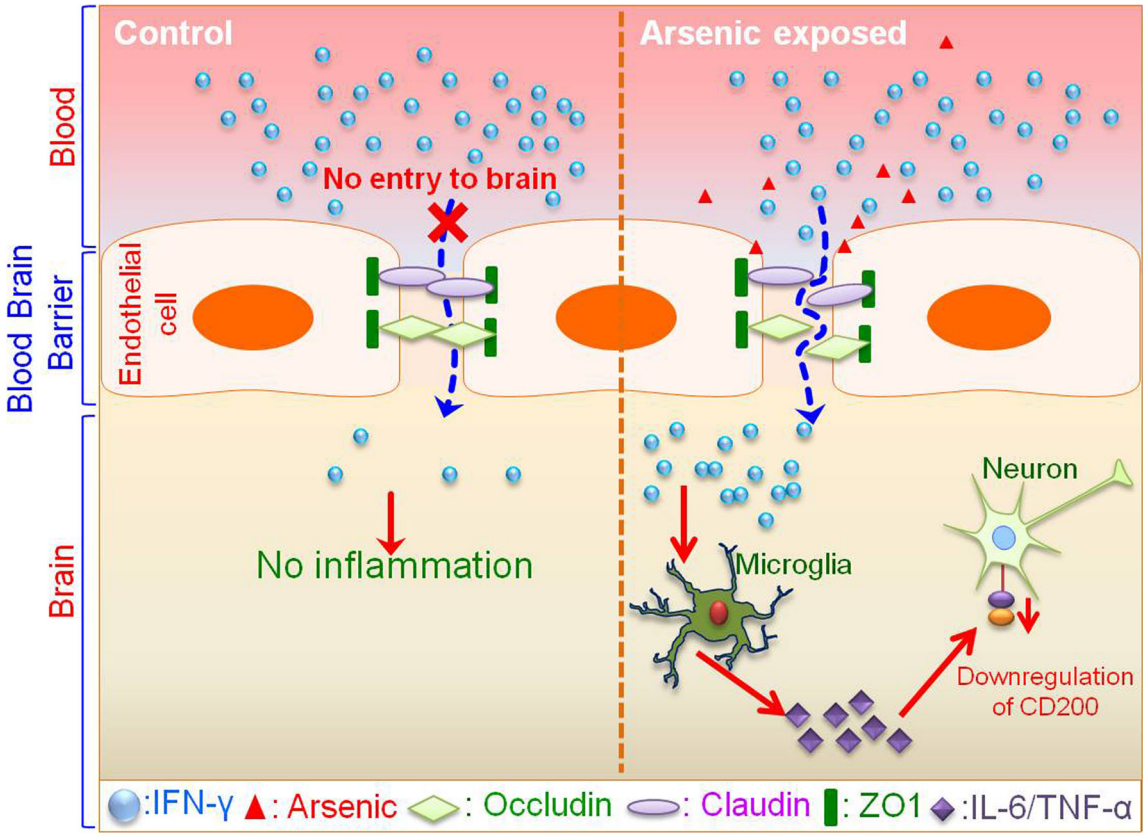

Fig. 8 Schematic representation of the role of arsenic in suppressing the expression of CD200. Exposure to environmentally relevant dose of arsenic compromises the permeability of the BBB. Blood IFNγ enters the brain through leaky BBB and induces microglial inflammation, which in turn suppresses the expression of CD200 in neurons

fluorescence of Evans blue dye which was observed in the arsenic-exposed group (Fig. 6d).

In Vivo Neutralization of IFNγ Brings Downregulated IL-6 and TNF-α, CD68 in Ex Vivo Microglia from Arsenic-Exposed Mice

All the results described above proved that in vivo arsenic exposure compromised the blood–brain barrier. Blood IFNγ enters through the leaky vasculature and thereby induced microglial pro-inflammatory cytokines. To validate the involvement of IFNγ in arsenic-induced microglia inflammation as well as suppression of CD200, IFNγ was neutralized in vivo by injecting anti-IFNγ antibody directly into the cortical region of the brain by stereotaxy. We have observed a significant decrease in the cytokine level in ex vivo cortical microglia culture supernatant, following inhibition of IFNγ in arsenic-exposed animals. The IL-6 levels determined were around 154 pg/ml in control, 155 pg/ml in sham control, 346 pg/ml in the arsenic-exposed group, and 152 pg/ml in the IFNγ-inhibited group. Similarly, TNF-α level detected were around 31, 33, 104, 46 pg/ml in control, sham control, arsenic-exposed group, and in IFNγ-inhibited group respectively (Fig. 7a, b). In parallel, in vivo neutralization of IFNγ brought the expression of CD200 similar to control (Fig. 7c, d). At the same time, we also observed a decrease in CD68 staining in brain sections in case of IFNγ neutralization (Fig. 7e).

Discussion

Neuronal surface protein CD200 physically interacts with CD200R1 (CD200 receptor) present on microglia, thereby keeping it in resting state. It has been demonstrated that absence of anti-inflammatory cytokines like IL4 or presence of inflammatory stimulus like Aβ downregulates CD200 [8]. Reports suggest that downregulation of CD200 acts as a trigger for several neurodegenerative diseases like Alzheimer's [2]. Interestingly, arsenic has been predicted to induce Alzheimer's like neurodegenerative disease in human [24] as well as reported to induce pre-onset of Alzheimer's like symptoms in rats, [14, 25] which is very much associated with neuroinflammation [26]. Therefore, we focused to deduce the underlying mechanism of arsenic-induced suppression of CD200 in the brain through microglial inflammation. The present study will provide insight into the mechanism of the environmental toxicants-induced neuroinflammation and

neurodegeneration which is appearing to be a real problem with increasing pollution worldwide [27–29].

In the present study, in vivo arsenic exposure reduced the expression of CD200 but unable to alter it in primary cortical neuron culture in vitro. Differential expression pattern of CD200 following in vivo and in vitro arsenic exposure signifies the involvement of non-cell autonomous mechanism in the event. Reversal of CD200 expression by administration of minocycline indicated the participation of microglial inflammatory response in the reduction of CD200 expression. Minocycline suppress microglial activation by inhibiting MAP kinases thereby reduces the microglia-mediated inflammation which helps in restoring the expression of CD200 [30]. Arsenic exposure was found to induce microglial activation both in vivo as well as in vitro as observed by the increased expression of microglia activation marker, CD68. Arsenic could even increase the level of IL-6 and TNF-α as detected in ex vivo microglial culture isolated from arsenic-exposed mice. Simultaneously, increased expression of IFNγ was observed in the arsenic-exposed brain lysate. Conversely, arsenic alone was unable to increase the level of IL-6 and TNF-α in arsenic-exposed microglia mono-culture as well as microglia-neuron co-culture in vitro. Interestingly, the addition of IFNγ could increase the IL-6 and TNF-α level in both the monoculture as well as co-culture. Thereby, this data shows the direct involvement of IFNγ in arsenic-induced neuroinflammation [5], at the same time indicating the indirect involvement of arsenic in the event. Arsenic could activate microglia, but for the activation to the level of cytokine secretion, it needs IFNγ help.

However, the question remains regarding the source of IFNγ in the arsenic-exposed brain. Th1 lymphocytes mainly secrete IFNγ; none of the brain cells namely microglia, astrocytes, and endothelial cells were responsible for the increased level of IFNγ in the brain. There is a detectable level of IFNγ in serum, and interestingly IFNγ is least capable of crossing intact blood–brain barrier which suggests that impairment of blood–brain barrier is responsible for IFNγ accumulation in the brain [31]. Therefore, IFNγ might be entering from the blood to brain crossing the blood–brain barrier. Entry of IFNγ into the brain prompted us to check any alteration in the expression of blood–brain barrier proteins following arsenic exposure. We were unable to detect any significant alterations in blood–brain barrier proteins, i.e., occludin and claudin-5 in the arsenic-exposed brain, but could detect the presence of Evans blue dye. This fact shows the compromised permeability of blood–brain barrier following arsenic exposure which could be resulted from the altered localization of BBB protein, as shown in case of tight junction structure of human airway epithelial cells [32]. An epidemiological study in Taiwan [33] indicated the increased risk of cerebrovascular diseases where mean level of arsenic exposure through drinking water is 0.5 ppm which is close to the dose used in our study (0.38 ppm). To confirm the role of the IFNγ in the arsenic-mediated induction of microglial IL-6 and TNF-α as well as CD200 expression, we have given a stereotaxic injection of IFNγ neutralizing antibody. It was observed that IFNγ neutralizing antibody could suppress the arsenic-induced IL-6, TNF-α, and at the same time rescued CD200 expression. Taken together, we have shown that environmentally relevant dose of arsenic exposure, facilitate the entry of IFNγ by altering blood–brain barrier permeability, which in turn induce microglial pro-inflammatory response leading to suppression of CD200 (Fig. 8). We have demonstrated that chronic low-level arsenic exposure suppresses CD200 expression in the brain through a non-cell autonomous mechanism. Our study showcase that IFNγ entry in the brain plays a key role in microglia activation-mediated CD200 downregulation.

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Compliance with Ethical Standards

Competing Interests The authors declare that they have no conflict of interest.

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