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Perinatal Arsenic Exposure-Induced Modulation of Pu.1/Csf1r Axis Leads to Microglial Proliferation in the Mouse Hippocampus

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Abstract

Earlier we showed that perinatal arsenic exposure induces microglial activation, proliferation and cognitive impairment in mouse pups. The present study is focused to identify the role of PU.1/CSF1R axis in arsenic-induced microglial proliferation. For *in vivo* study six to eight-week-old male and female BALB/c mice were used. For mating male and female mice were kept in the same cage at a ratio of (1:2). After mating pregnant females were divided into vehicle control and arsenic treatment group. Sodium arsenite was dissolved in reverse osmosis water and given to the treatment group. The treatment regimen continued from gestational day 5 (GD5) until the weaning period of pups around post-natal day 22 (PND22). Following the exposure regimen, pups were sacrificed for each of the western blot analysis, qRT-PCR and immunohistochemical staining. For *in vitro* studies, mouse micrglial cell line, BV2 was used. BV2 cells were treated with arsenic, L-methionine or siRNA as required in various combinations. Following *in vitro* treatment cells were harvested and used for western blot analysis or qRT-PCR. It was observed that perinatal arsenic exposure (0.38 mg/kg bd wt) from gestational day 5 till the sacrifice of the pups on PND 22 increased the microglial number. The expression of CSF1R was observed to be up-regulated. The expression of CSF1R transcription factor PU.1 was also found to be increased in the arsenic-exposed group. The role of arsenic-induced PU.1 was confirmed by checking the expression of CSF1R following inhibiting PU.1 using siRNA in BV2 microglia. Reversal of PU.1 expression in the L-methionine-supplemented arsenic-exposed BV2 microglia indicates possible demethylation of the *in silico* predicted CpG island in the upstream regulatory region of the PU.1 promoter. Taken together, the study reveals that arsenic exposure increases microglial proliferation by modulating the PU.1/ CSF1R axis in mouse hippocampus.

Keywords: Arsenic, CSF1R, Hippocampus, L-methionine, Microglia, PU.1

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1. Introduction

Arsenic (As) is an environmental chemical which is known for its global distribution and its toxicity in every sphere of life[1]. Contamination of arsenic in drinking water is a challenging issue in different parts of the world including India[2]. Globally more than 230 million people are getting exposed to inorganic arsenic through contaminated drinking water with an arsenic concentration more than the WHO-prescribed limit of 10 µg/L[3,4]. Long-term exposure to inorganic arsenic leads to various health hazards like cancer, neurotoxicity, nephrotoxicity, and hepatotoxicity[5]. It is also associated with health problems like diabetes and cardiac dysfunction[5] as well as impaired learning and memory function[6,7]. Arsenic exerts its toxic effect either by inhibiting enzyme activity or by altering the epigenetics[8,9].

Microglia are the brain resident macrophages which help in the development of the brain by secreting various growth factors as well as help in the maintenance of brain homeostasis by performing various functions like clearance of pathogens, neuronal debris and synaptic pruning[10,11]. Thus, the precise maintenance of the microglial population is very crucial at early developmental stages as well as in the adult brain. The colony-stimulating factor-1 receptor (CSF1R), a key transmembrane receptor tyrosine kinase, is expressed by macrophages and microglia having two natural ligands – CSF1 and IL-34[12,13]. Signalling downstream to CSF1R plays a pivotal role in the proliferation, differentiation, and survival of microglia in the CNS, whereas, the expression of CSF1R is regulated by a transcription factor, PU.1 which is also associated with the activation of microglia[14,15]. PU.1 is encoded by the *Sfpi1* gene which has a large CpG island 17kb upstream from the start point[16,17]. The CpG region controls gene expression depending on its methylation status. The DNA methylation process is controlled by enzymes like DNA methyl transferase 1 (DNMT1). Interestingly arsenic has been reported to deplete the level of cellular methyl group[18] and its exposure has been shown to decrease the expression of DNMT1 as well as the level of DNA methylation in the regulatory region of various genes[19]. Although several studies have reported that early-life arsenic exposure activates microglia[20], there is a lack of study regarding how arsenic exposure alters microglial proliferation following early-life arsenic exposure. Earlier we have shown that perinatal exposure to an environmentally relevant dose of arsenic (0.38 mg/kg bd wt) increases the expression of CSF1R and increased co-localization of Ki67and microglial marker Iba1in mouse brains[20]. Proliferative microglia remain in an activated condition and therefore may have an adverse impact on the neuronal health of the offspring leading to cognitive impairment[6].

In the present study, we have tested the hypothesis that perinatal arsenic exposure increases microglial proliferation through the PU.1/CSF1R axis in the mouse hippocampus. Pregnant BALB/c mice were exposed to arsenic from Gestational Day 5 (GD) and continued until pups were sacrificed on PND22. Microglial proliferation was checked in the hippocampus through immunohistochemistry using Iba1 and Ki67 antibodies. Expression of PU.1 and CSF1R was checked in the hippocampus as well as BV2 microglia following arsenic treatment through RT-PCR and western blot analysis. The expression of DNMT1 has also been checked. Arsenic-exposed BV2 microglia cultures were supplemented with the methyl group donor, L-methionine and possible reversal of PU.1 and CSF1R expression were also checked. In the present study, an effort was made to unravel the underlying mechanism of arsenic-induced enhanced microglial proliferation mediated through PU.1/CSF1R axis.

2. Materials and Methods

2.1 Reagent and Chemicals

Detailed catalogues and vendors' information on the reagents and laboratory chemicals have been provided in Table 1.

2.2 Antibodies

Details of antibody specificity, dilution, vendor information and catalogue numbers are listed in Table 2.

2.3 Animals and Treatment

Six to eight-week-old male and female BALB/c mice were procured from the animal facility of the CSIR-Indian Institute of Toxicology Research (CSIR-IITR). We used the standard protocol for the study permitted by the Institutional Animal Ethics Committee (IAEC) of CSIR-IITR (Ref. no. IITR/IAEC/60/21). Mice were housed at 25 ± 3°C, 12h/12h light and dark cycle with rodent chow diet (Cat. no: 1324, Altromin, Germany) and reverse osmosis (RO) water supplied ad libitum. For mating, male and female animals were kept in the same cages in a ratio of 1:2 (male:female)/ cage. After timed mating, pregnant

Table 1. List of reagents and chemicals

Reagents and Chemicals	Cat. No	Vendor
Sodium arsenite (SA) ($NaAsO_2$)	90110K02	SDFCL (Chennai, India)
SDS	L3771–100 G	Sigma (St. Louis, MO, USA)
PBS	D5652–50 L	Sigma (St. Louis, MO, USA)
Trizma base	T6066–1Kg	Sigma (St. Louis, MO, USA)
L-Methionine	M9625	Sigma (St. Louis, MO, USA)
Bromophenol blue	B5525–10 G	Sigma (St. Louis, MO, USA)
Protease inhibitor cocktail	P8340–1 mL	Sigma (St. Louis, MO, USA)
Triton X-100	T8787–250 mL	Sigma (St. Louis, MO, USA)
Paraformaldehyde (PFA)	P6148–1Kg	Sigma (St. Louis, MO, USA)
Ponceau S stain	P-3504	Sigma (St. Louis, MO, USA)
Chloroform	34854	Sigma (St. Louis, MO, USA)
Glycerol	DK7P672935	Merck-Millipore (USA)
Isopropanol	DI6P662807	Merck-Millipore (USA)
Ethanol	K48191683632	Merck-Millipore (USA)
Polyvinylidenedifluoride membrane	IPVH00010	Merck-Millipore (USA)
Immobilon	WBKL50500	Merck-Millipore (USA)
Methanol	439193	Merck-Millipore (USA)
Acetone	1.00014	Merck-Millipore (USA)
Sfpi1 siRNA	mm.Ri.Spi1.13.7	Integrated DNA Technology (USA)
DMEM/F12	12500096	Thermo Scientific (MA, USA)
cDNA kit	4368814	Thermo Scientific (MA, USA)
FBS	A4766801	Thermo Scientific (MA, USA)
TRIzol™ Reagent	15596026	Thermo Scientific (MA, USA)
siPOR T™ NeoFX™ Transfection Agent	AM4511M	Thermo Scientific (MA, USA)
Antibiotics	15140-122	Thermo Scientific (MA, USA)
Protein ladder	PG-PMT2922	Puregene, (India)
SYBR Green Master Mix (2X)	PGK022B	Puregene, (India)
Tank buffer	1610772	Biorad (California, USA)
Transfer buffer	1610771	Biorad (California, USA)
Bradford	500–0006	Biorad (California, USA)
30% Acrylamide/Bis Solution	1610156	Biorad (California, USA)
β-mercaptoethanol	GRM2895-100 mL	Himedia (India)
Trypsin EDTA1x (6 ×500 mL)	TCL048	Himedia (India)
Sucrose (1KG)	PCT0607-1KG	Himedia (India)
Vectashield Antifade Mounting Medium	H-1200	Vector Laboratories (California, USA)

females were randomly assigned to either the vehicle control or arsenic-treatment groups (0.38 mg/kg body weight) [20]. Sodium arsenite was dissolved in RO water and the pregnant females of the treatment group were gavaged daily with arsenic. The treatment regimen continued from gestational day 5 (GD5) until the weaning period of pups around post-natal day 22 (PND22). Following the exposure regimen pups were sacrificed, hippocampus

Table 2. List of antibodies

Antibodies	Reactivity	Vendor	Cat. No	Lot No	Dilution
Primary Antibodies					
Iba1	Human, Mouse, Rat	Millipore (USA)	MABN92	325663 3	IHC:1:1,000
Ki67	Human, Mouse	Abcam (UK)	ab15580	GR309731–1	IHC: 1:1,000
PU.1	Human, Mouse, Pig	Invitrogen (USA)	MA5-15064	XJ3738404	WB: 1:2,500
CSF1R	Mouse	Thermo Scientific (USA)	14-1152-82	4313368	WB: 1:2,000
Beta Actin	Human, Mouse	Abcam (UK.)	ab8227	GR3195358-1	WB: 1:5,000
Secondary Antibodies					
Goat anti-rabbit IgG-HRP	-	Invitrogen (USA)	656120	UA276828	WB: 1:10,000
Goat anti-rat IgG-HRP	-	Santacruz (USA)	Sc2006	H0211	WB:1:10,000
Goat anti-Rabbit IgG-Alexa Fluor 594	-	Thermo Scientific (USA)	A -11037	1915919	IHC:1:1,000
Goat anti-Mouse IgG-Alexa Fluor 488	-	Thermo Scientific (USA)	A -11029	1911843	IHC:1:1,000

was isolated and processed either for western blot analysis, quantitative real-time PCR (qRT-PCR) or immunohistochemical staining.

2.4 Cell Line Maintenance and Treatment

Mouse microglia cell line (BV2) was cultured in complete DMEM/F12 medium supplemented with 1% penicillin-streptomycin (Pen-Strep) and 10% Fetal Bovine Serum (FBS) in a CO_2 incubator maintaining 37°C temperature, 5% CO_2 and 95% humidity. Cells were passaged when they reached 80-90% confluency. BV2 cells were seeded either 6 well culture plate for PCR and western blot analysis or 96 well culture plate for viability assay. Cells were treated with a non-cytotoxic dose of arsenic (sodium arsenite, 1µM) for 72h[19]. For L-methionine supplementation, BV2 cells were exposed to a maximum non-cytotoxic dose (500 µM) of L-Methionine (L-Met). L-Met was added 1h before the addition of the arsenic treatment[21,22]. For all *in vitro* treatments 4% FBS was supplemented along with 1% Pen-strep.

2.5 Transfection of *Sfpi1* siRNA

BV2 microglia were transfected with *Sfpi1* siRNA using siPORTNeoFX transfection reagent[19]. Transfection agent, siPORTNeoFX mixed with opti-MEM in one microcentrifuge tube, while *Sfpi1* siRNA was mixed with opti-MEM in another tube and incubated. Then the siRNA-opti-MEM mixture was transferred into the other transfection reagent tube and the final transfection complex was added to BV2 cells followed by incubation in a standard CO_2 incubator for 72 h.

2.6 Preparation of Cell/Tissue Lysate and Western Blot Analysis

BV2 cells were lysed with RIPA buffer. For the preparation of tissue lysate, the hippocampus was isolated from the PND22 mouse brains and homogenized in lysis. Finally, the cell/tissue lysate was centrifuged to collect supernatant for western blot. Bradford reagent was used to estimate protein concentration. Protein was mixed with loading dye and an equal amount of protein was loaded on each well SDS-PAGE followed by transfer to the PVDF membrane. Then membranes were probed with various primary antibodies at 4°C overnight. After incubation, membranes were incubated with secondary antibodies followed by washing with washing buffer. Immobilon Western Chemiluminescent HRP Substrate was used to develop a blot. Protein band intensities were quantitated using Image-J software from the captured images and the densitometric data was presented as a scatter plot with bar[19].

2.7 Quantitative Real-Time PCR (qRT-PCR)

TRIzol reagent was added to cells and lysate was collected. In the case of hippocampus tissue 1ml TRIzol reagent was added and homogenized using a tissue homogenizer. Cell/tissue homogenate was collected in microcentrifuge tubes, chloroform was added and centrifuged. The transparent

upper layer was collected and mixed with an equal volume of isopropanol and centrifuged. The pellet was washed with 75% ethanol by centrifugation and the RNA was dissolved in nuclease-free water. A Nanodrop spectrophotometer (ND-1000 Spectrophotometer, Thermo) is used to quantify RNA. cDNA was synthesized followed by qRT-PCR was run using SYBR green master mix and specific forward-reverse primers. Changes in mRNA expression were calculated using the Relative Quantity (RQ) equation, RQ¼2_DDCT; where Delta Cycle Threshold (DCT) denoted a difference in CT values between the target gene and the housekeeping gene (beta-actin), and DDCT signified the differences in the DCT value of treated sets compared to control[20]. The forward and reverse primer sequences are given in Table3.

2.8 Immunohistochemistry (IHC)

For immunohistochemistry, animals were deeply anaesthetized at PND22 and perfused with 0.01 M PBS followed by 4% PFA. Brains were fixed in 4% PFA followed by transferred to 15% and 30% sucrose solution. Tissue blocks were prepared using Cryomatrix and 14 µM coronal cryo-sections were cut. To start the immunostaining, free-floating sections were washed with 0.1% PBST. Antigen/epitope retrieval was performed using 0.01M sodium citrate buffer followed by probing with primary antibodies at 4°C for 48 h. Following incubation, sections were incubated with AlexaFluor-conjugated secondary antibodies. Finally, sections were counterstained with DAPI containing mounting medium and slides were observed and images were captured in an EVOS-M5000 microscope. For quantifying Iba1 and Ki67 staining National Institutes of Health ImageJ software (NIH, https://imagej.nih.gov/ij/) was used. To count the number of microglia Iba1-positive cells in the hippocampus region were counted and expressed as the number of Iba1+ cells/mm[23].

2.9 Statistical Analysis

Data were presented as mean ± SD (standard deviation). Statistical analysis was performed in GraphPad Prism. For comparing the control and arsenic-exposed group "A Mann-Whitney test" was performed. "One-way ANOVA" was used for more than two groups followed by "Tukey's multiple comparison test" to determine which means amongst a set of means differ from the rest. $p<0.05$ was considered a statistically significant change.

3. Results

3.1 Perinatal Arsenic Exposure Enhanced Microglia Proliferation in the Mouse Hippocampus

Arsenic exposure activates microglia in mouse brain and microglial activation is closely associated with its proliferation. In the present study, the microglia proliferation was checked by enumerating the microglia number in the Iba1 immunostained hippocampus section as well as Ki67 collocalization with Iba1. Coronal cryo-sections (14 µM) were cut (-1.8 mm to -2.3 mm from bregma, hippocampus region) through the hippocampus region of the brain (Figure 1A). Iba1 associated immunofluorescence was measured using imageJ software and the arsenic-exposed group showed higher Iba1 associated immunofluorescence compared to the control group (Figure 1B). Numbers of microglia in the hippocampus were also enumerated from the Iba1 immunostained sections using image J and similar to the Iba1-1 associated immunofluorescence, the arsenic-exposed group showed higher microglial number compared to the control group in each unit area (mm^2) of the cryosection (Figure 1C). Figure 1D shows the representative image of Iba1-immunostained hippocampus sections used to generate Figure 1B and C (Figure 1D).

Microglial proliferation was also checked by studying the co-localization of proliferation marker, Ki67 with microglial marker, Iba1 in brain cryosection (Figure 2A). Overlapping regions in the images of Iba1 and Ki67 co-stained sections were quantitated as the coefficient of co-localization of Iba1 and Ki67-associated fluorescence using the JAcoP plugin (just another Co-localization Plugin) in ImageJ software after converting the image into 8-bit grayscale. It was observed that the coefficient of

Table 3. List of primers

Target Gene	Forward Primer 5' > 3'	Reverse Primer 5' > 3'
PU.1	ATCGGATGACTTGGTTAC	CAGTAGTGATCGCTATGG
CSF1R	TACAAGTGGAAGTGGAGGAGCCAT	AGTCCTGTGTGCCCAGCATAGAAT
β-actin	TATGGAATCCTGTGGCATC	GTGTTGGCATAGAGGTCTT

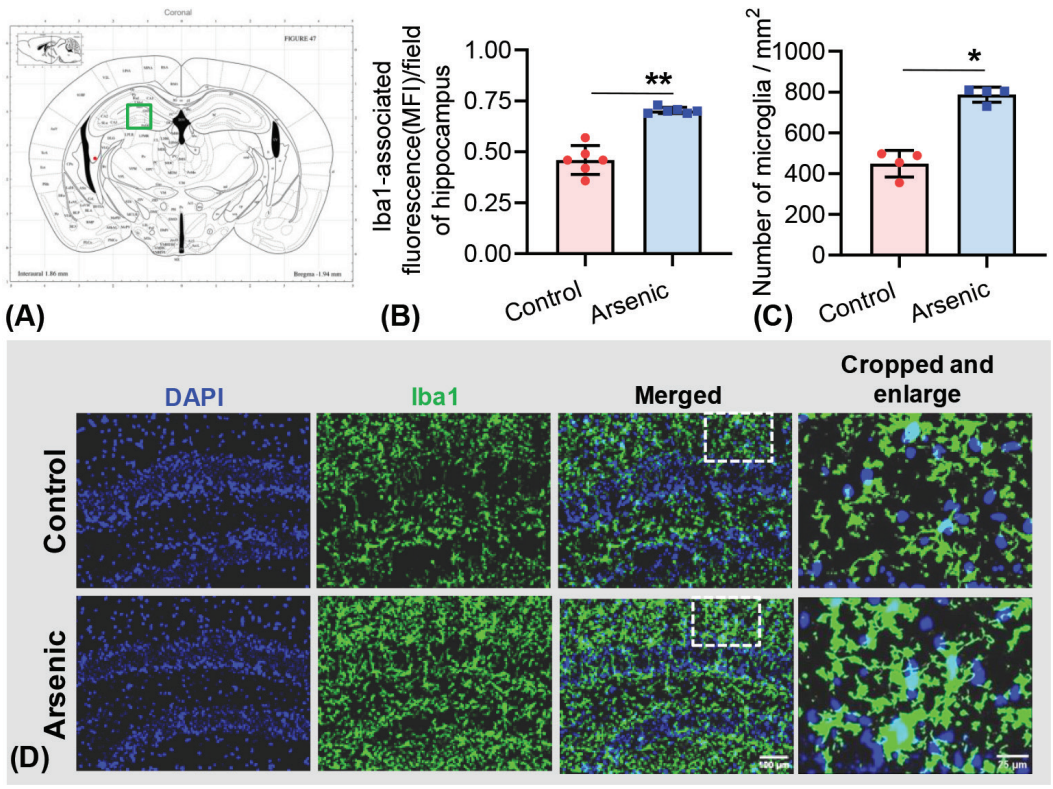


Figure 1. Effect of arsenic on the microglial density in the hippocampus of Balb/c mouse brain. Pregnant females were gavaged with sodium arsenite (As, 0.38 mg/kg body weight) from the gestational day 5 (GD5) until PND21. Pups were sacrificed on PND22, brains were isolated, 14 µM coronal cryo-sections were cut (-1.8 mm to -2.3 mm from bregma) and processed for immunostaining Iba1 antibody followed by evaluation of microglial number in the hippocampus region. **(A)** Schematic of a coronal section of mouse brain showing the hippocampus region (green box) used for evaluation of microglia number. **(B)** Iba1-associated fluorescence (mean fluorescence intensity; MFI) in each field of view was measured and represented as a scatter plot with a bar. **(C)** Microglia numbers were enumerated following Iba1 staining and represented as the number of microglia/ mm^2 of the hippocampus in a scatter plot with a bar. **(D)** Representative images of Iba1 immunostained hippocampal coronal cryo-section captured using EVOS5000 microscope. Immunostaining with Iba1 showed an increased microglial density in the hippocampus of the arsenic-exposed mouse brain. Scale bar: 100 µm for un-cropped images and 25 µm for the cropped. N= 6 pups (2 pups from each of 3 pregnant females) in each group and 3-4 coronal sections from each animal were analyzed for both fluorescence intensity calculation and microglial number. For comparing the control and arsenic-exposed group Mann-Whitney test was performed. *"p"* denotes the level of significance; *p<0.05, **p<0.01.

Iba1/Ki67 co-localization was significantly higher in an arsenic-exposed group compared to control (Figures 2A, B). Therefore it is evident that perinatal arsenic exposure induces microglial proliferation which results in an increased number of microglia in the hippocampus region.

3.2 Arsenic Exposure Increased the Expression of CSF1R in Mouse Hippocampus and BV2 Microglia

Arsenic exposure increased the Ki67 expression and microglial number in mouse hippocampus as observed in (Figures 1 and 2). Signaling through CSF1R is a vital regulator for microglial proliferation, development and survivability in CNS. Therefore, the expression of CSF1R was checked through qRT-PCR and western blot analysis in mouse hippocampus as well as in BV2 microglia. It was observed that mRNA and protein level of CSF1R was significantly increased in the hippocampus of PND22 pups following perinatal arsenic exposure compared to control (Figures 3A, B). BV2 microglia also showed a similar pattern of CSF1R expression following *in vitro* arsenic (1µM, 72h) exposure (Figure 3C, D).

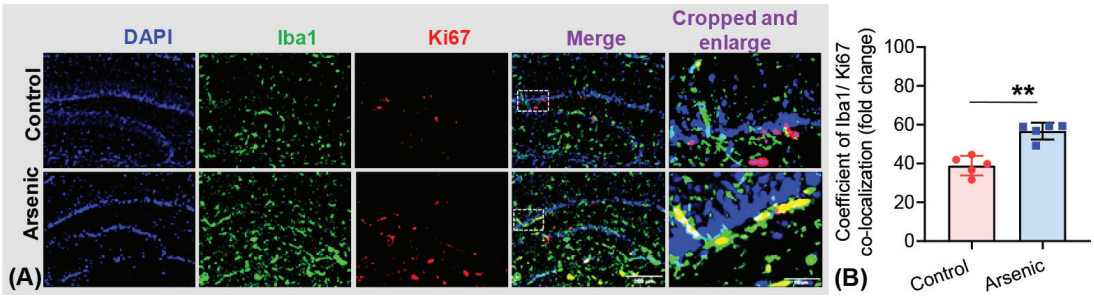


Figure 2. Effect of arsenic on the proliferation marker, Ki67, in the hippocampal microglia of Balb/c mouse brain. Pregnant females were gavaged with sodium arsenite as described in Figure 1 and pups were sacrificed on PND22. **(A)** Representative image of the Iba1 and Ki67 immunostained coronal cryo-section of the hippocampus. Scale bar: 200 µm for un-cropped images and 20µm for the cropped images. **(B)** The coefficient of co-localization was measured in the coronal section of control and arsenic-exposed animals and represented as a scatter plot with a bar. N=6 pups (2 pups from each of 3 pregnant females) in each group and 3-4 coronal sections from each animal were analyzed for calculation of the coefficient of co-localization. For comparing the control and arsenic-exposed group Mann-Whitney test was performed. *"p"* denotes the level of significance; *p<0.05, **p<0.01.

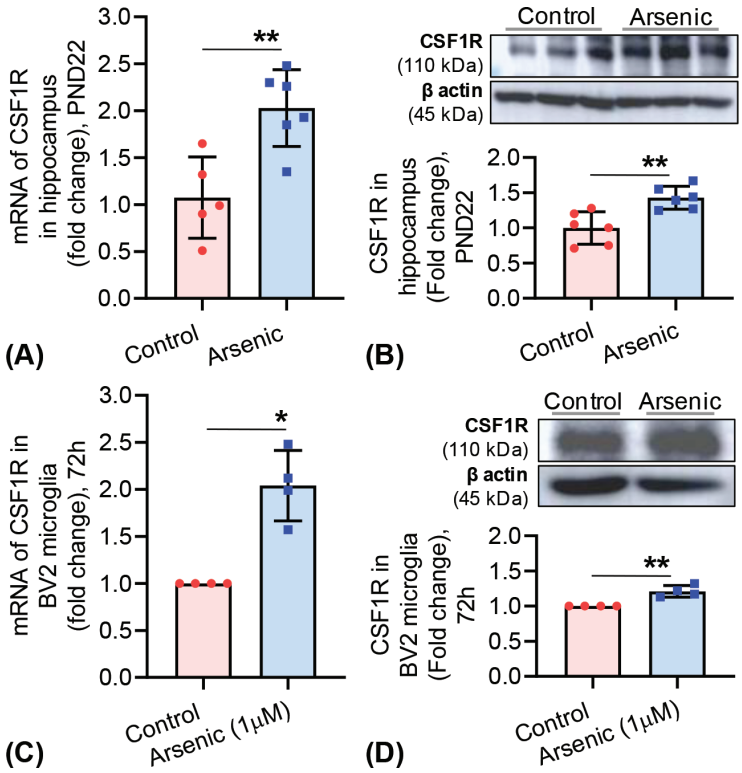


Figure 3. Effect of arsenic on the expression of CSF1R in hippocampus and BV2 microglia. Pregnant females were gavaged as described in Figure 1, hippocampus was isolated and processed for isolation of RNA as well as protein isolation. Similarly, RNA and protein were isolated from BV2 microglia treated with 1µM sodium arsenite *in vitro* for 72h. Expression of CSF1R was checked in the hippocampus **(A)** mRNA of CSF1R, **(B)** CSF1R protein. n=5-6 pups (1-2 pups from each of 3 pregnant females). Expression of CSF1R was also checked in BV2 microglia **(C)** mRNA of CSF1R, **(D)** CSF1R protein. N= 4 independent in vitro experiments. CSF1R band intensity in the western blots was quantified using ImageJ software and presented as fold change in a scatter plot with a bar. Levels of mRNA were also presented similarly as fold change. It was observed that expression of CSF1R was significantly increased in arsenic-exposed pups. For comparing the control and arsenic-exposed group Mann-Whitney test was performed. *"p"* denotes the level of significance; *p<0.05, **p<0.01.

3.3 Arsenic Exposure Increased the Expression of PU.1 in Microglia

It is evident from Figures 1-3 that arsenic exposure increased microglial number through up-regulation of CSF1R. The transcription factor PU.1 is the key regulator of CSF1R, therefore, to link the signalling cascade, the expression of PU.1 was checked in mouse hippocampus and BV2 microglia. It was observed that mRNA and protein level of PU.1 was significantly increased in the hippocampus of PND22 pups following perinatal arsenic exposure compared to control (Figure 4A, B). *In vitro,* exposure of arsenic (1μM, 72h) to BV2 microglia also showed a similar pattern of PU.1 expression (Figures 4C, D). This finding speculates a possible correlation between arsenic exposure, PU.1 activity and CSF1R expression in microglia.

3.4 Inhibition of PU.1 Simultaneously Decreased the Expression of CSF1R in Arsenic-Exposed BV2 Microglia

Arsenic exposure increased the expression of CSF1R as well as PU.1 in microglia (Figures 3 and 4). Therefore,

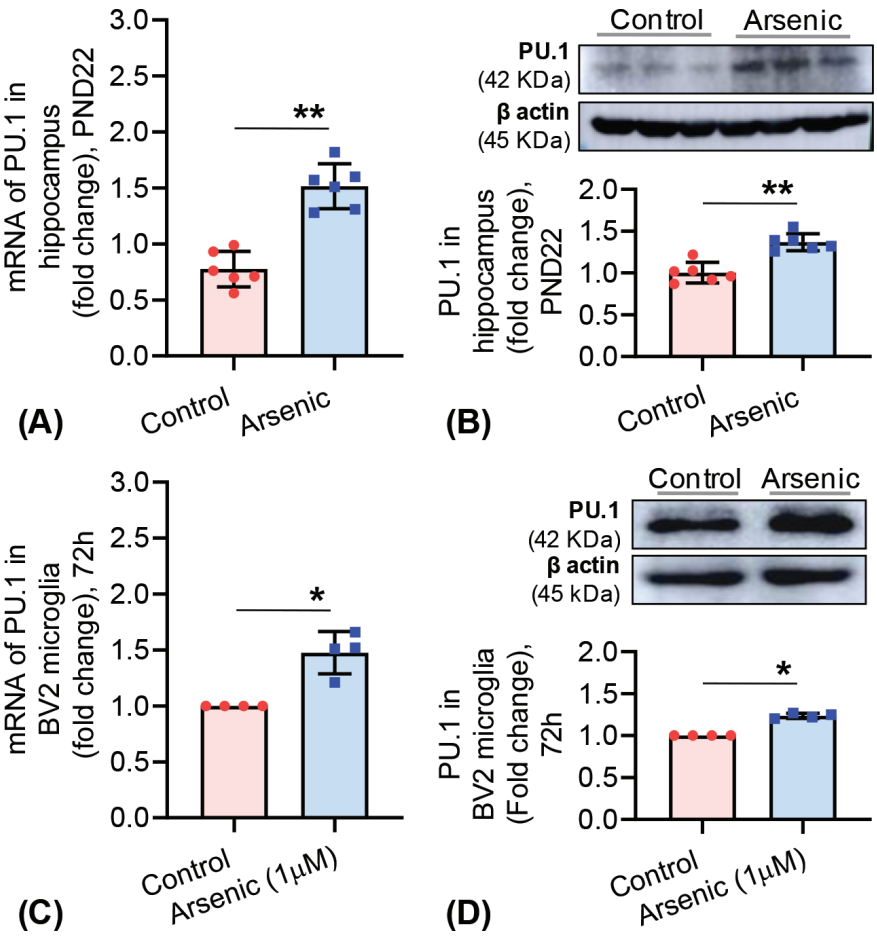


Figure 4. Effect of arsenic on the expression of PU.1 in the hippocampus and BV2 microglia. Pregnant females were gavaged as described in Figure 1, hippocampus was isolated and processed for isolation of RNA as well as protein isolation. Similarly, RNA and protein were isolated from BV2 microglia treated with 1μM sodium arsenite *in vitro* for 72 h. Expression of PU.1 was checked in the hippocampus **(A)** mRNA of PU.1, **(B)** PU.1 protein. n=6 pups (2 pups from each of 3 pregnant females). Expression of PU.1 was also checked in BV2 microglia **(C)** mRNA of PU.1, **(D)** PU.1 protein. It was observed that the expression of PU.1 was significantly increased in arsenic-exposed pups. N=4 independent in vitro experiments. PU.1 band intensity in the western blots were quantified using ImageJ software and presented as fold change in a scatter plot with a bar. Levels of mRNA were also presented similarly as fold change. For comparing the control and arsenic-exposed group Mann-Whitney test was performed. *"p"* denotes the level of significance; *p<0.05, **p<0.01.

to check the possible regulation of CSF1R by arsenic through PU.1, a knockdown experiment was performed using *Sfpi1* siRNA in BV2 microglia. BV2 microglia were transfected with *Sfpi1* siRNA and co-exposed to arsenic (1μM) followed by 72 h incubation. Following incubation, the levels of PU.1 and CSF1R were checked by western blot analysis. It was found that *Sfpi1* gene knockdown in arsenic-exposed BV2 cells significantly decreased the level of PU.1 in comparison to both the control and only the arsenic-arsenic-exposed group (Figure 5A). Similarly, the level of CSF1R was also significantly decreased in the arsenic and *Sfpi1* siRNA co-exposed group compared to only the arsenic-exposed group (Figure 5B). The data suggests the mechanistic involvement of PU.1 in the regulation of CSF1R expression in arsenic-exposed microglia.

3.5 Arsenic Regulates PU.1 Expression by Possible Demethylation of Upstream Regulatory Site of *Sfpi1* Gene

The next obvious question was how arsenic regulates the expression of PU.1. Increased expression of PU.1 mRNA suggests a potential transcriptional regulation of *Sfpi1* gene expression in arsenic-exposed microglia. Therefore, an *in silico* analysis using the MethPrimer online tool has been performed and two highly dense CpG islands were located at the 17kb upstream of the transcriptional start site. The two sites contain 33 and 11 CpG sites respectively (Figure 6A). CpG island methylation or demethylation plays a crucial role in the regulation of *Sfpi1* transcription in different cancers and during haematopoiesis. The methylation status of these sites plays a crucial role in

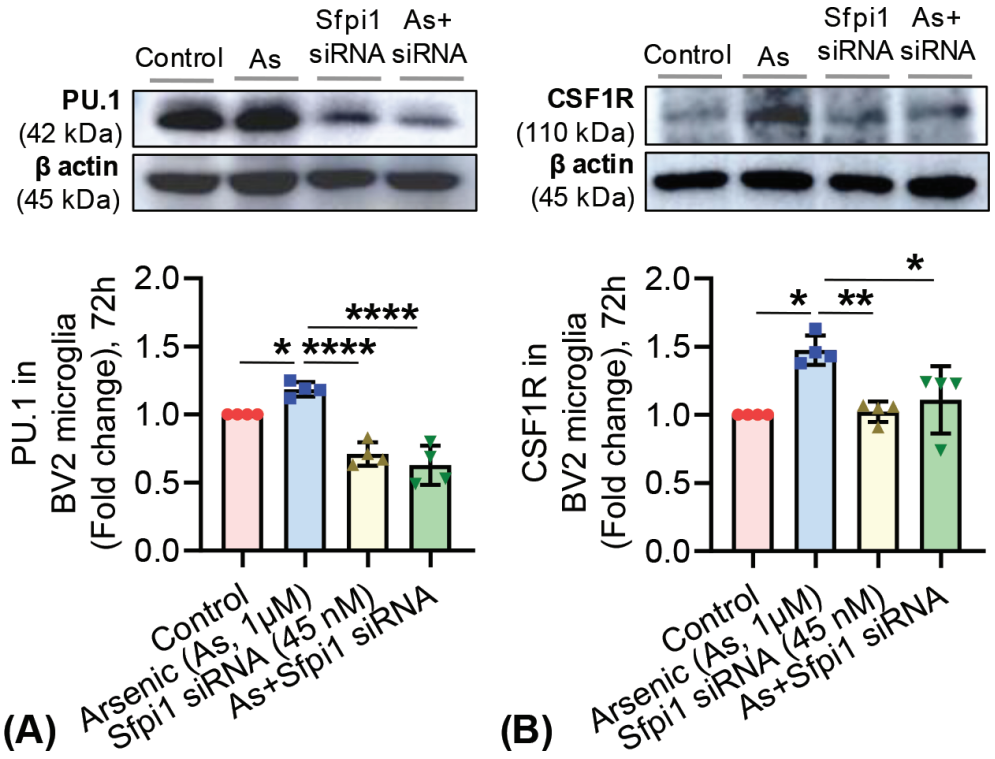


Figure 5. Effect of *Sfpi1 inhibition* on the expression of PU.1 and CSF1R in arsenic-exposed BV2 microglia. To ensure the involvement of PU.1 in the increased expression of CSF1R, BV2 microglia were exposed to arsenic and simultaneously transfected with *Sfpi1* siRNA (45 nM) and incubated for 72 h. Following incubation, the protein was isolated from BV2 microglia and run for western blot analysis and the levels of **(A)** PU.1 and **(B)** CSF1R were checked through western blot analysis. It was observed that the level of PU.1 and CSF1R were significantly reversed in arsenic and *Sfpi1*siRNA co-exposed BV2 microglia. N=4 independent *in vitro* experiments. PU.1 and CSF1R band intensity in the western blots were quantified using Image J software and presented as fold change in a scatter plot with a bar. For comparing control and experimental groups way-ANOVA was performed followed by Tukey's post hoc test. *"p"* denotes the level of significance; *p<0.05, **p<0.01; ***p<0.005; ****p<0.001.

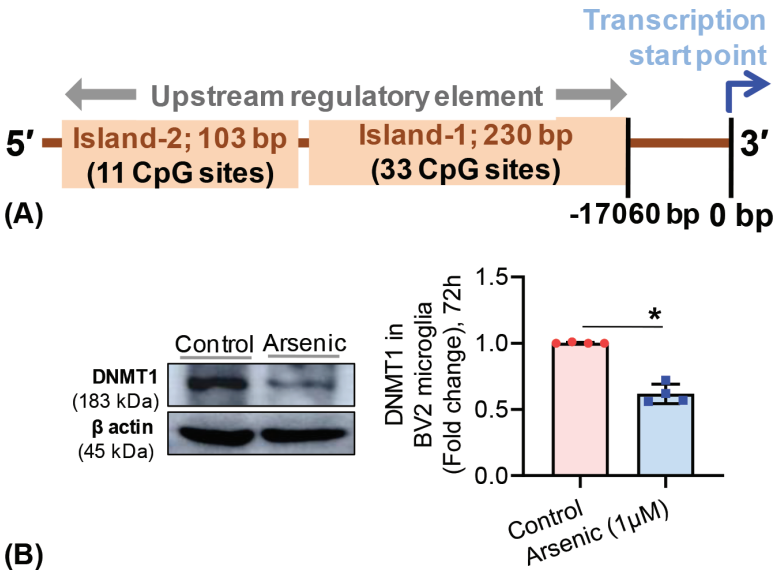


Figure 6. *In silico* analysis of CpG island for *Sfpi1* gene and effect of arsenic on the expression of DNMT1 in BV2 microglia. **(A)** *In silico* analysis was performed using the MethPrimer online tool to identify CpG sites for possible demethylation and Sfpi1 expression following arsenic exposure. Two CpG islands were detected 17Kb upstream from the transcription start point. The 1st contains 33 and the 2nd island contains 11 CpG sites. **(B)** To check the effect of arsenic on DNMT1 expression, BV2 microglia were exposed to arsenic and the level of DNMT1 was detected using western blot analysis. The band intensity of DNMT1 was quantified by ImageJ software to generate quantitative data and presented as fold change in a scatter plot with a bar. A significantly decreased level of DNMT1 was observed in the arsenic-exposed group. For comparing control and arsenic-exposed group Mann-Whitney test was performed. *"p"* denotes the level of significance; *p<0.05, **p<0.01; ****p<0.001.

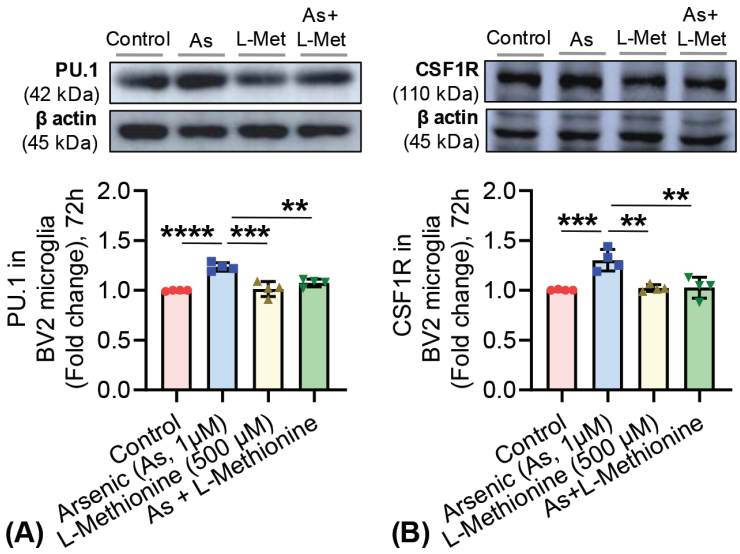


Figure 7. Effect of L-methionine supplementation on the arsenic-mediated altered expression of PU.1 in BV2 microglia. To check the effect of L-methionine (L-met) on the arsenic-mediated altered expression of CSF1R, BV2 microglia were exposed to arsenic and simultaneously supplemented with L-met (500 nM) and incubated for 72 h. Following incubation, the protein was isolated from BV2 microglia and run for western blot analysis and the levels of **(A)** PU.1 and **(B)** CSF1R were checked. It was observed that L-met supplementation decreased the level of both PU.1 and CSF1R in the arsenic+ L-met group compared to only the arsenic-exposed group. N= 4 independent in vitro experiments. PU.1 and CSF1R band intensity in the western blots were quantified using Image J software and presented as fold change in a scatter plot with a bar. For comparing control and experimental groups one way-ANOVA was performed followed by Tukey's post hoc test. "p" denotes the level of significance; *p<0.05, **p<0.01; ***p<0.005; ****p<0.001.

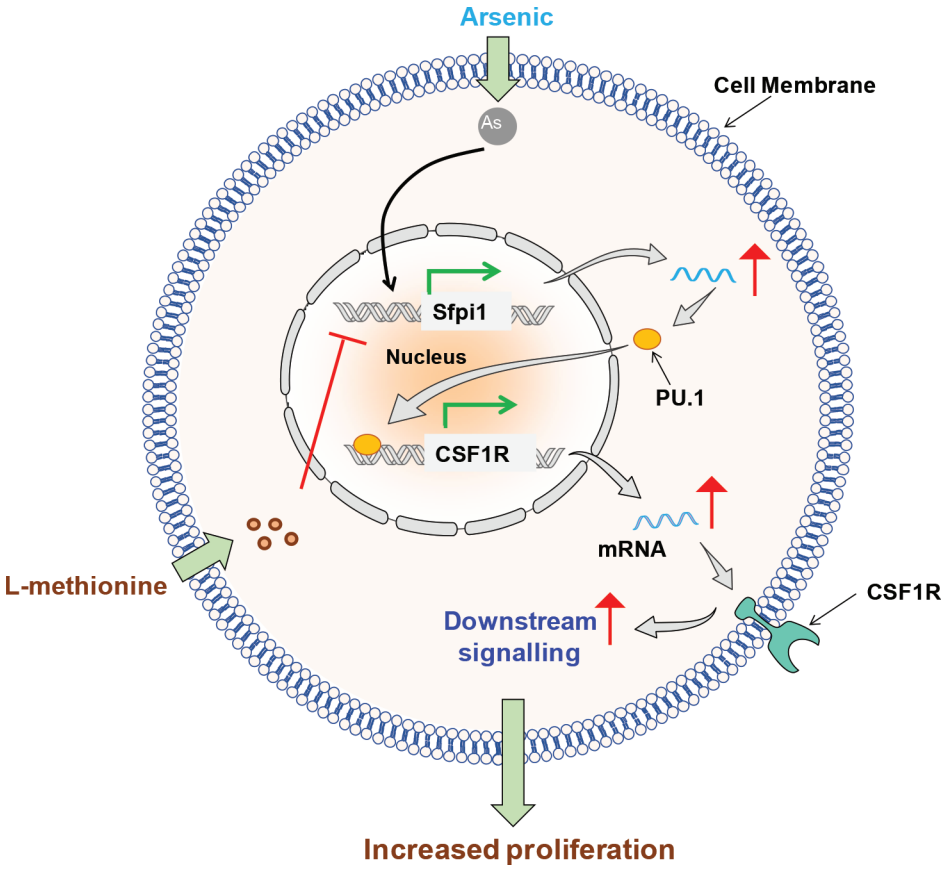


Figure 8. Schematic presentation of the summary of the work.

gene expression and the methylation is controlled by the enzyme DNA Methyl Transferase 1 (DNMT1). Therefore, the level of DNMT1 was also checked by western blot analysis and a decreased level was observed in arsenic-exposed BV2 microglia (Figure 6B).

The data in Figure 6 indicated a possibility of demethylation of the *Sfpi1* regulatory region leading to increased expression of *Sfpi1* in microglia. Therefore, as a confirmatory test, an experiment was performed to reverse arsenic-induced altered *Sfpi1* expression by methyl group supplementation using L-methionine along with arsenic treatment in BV2 microglia. A dose-response study (data not shown) was performed to detect the maximum viable does of L-methionine for BV2 microglia for 72 h exposure and 500 µM was selected for reversal study. The reversal study showed that 500 µM L-methionine significantly reversed the expression of PU.1 in arsenic-exposed BV2 cells (Figure 7A) which speculates a possible epigenetic mechanism that regulates the expression of PU.1 in arsenic-exposed-microglia by demythylating the CpG islands present in the *Sfpi1*gene. Simultaneously, expression of CSF1R was also checked and observed to be reversed similar to PU.1 in the arsenic and L-methionine co-exposed group (Figure 7B).

4. Discussion

The present study provides evidence that perinatal exposure to an environmentally relevant dose of arsenic leads to microglia proliferation in mouse hippocampus possibly through epigenetic regulation of PU.1/CSF1R axis.

During the development period microglial activation and proliferation occur simultaneously. Microglia activity reaches its peak around postnatal day 14 (PND14) and showed decreased activity around PND22. Interestingly, perinatal arsenic exposure induces microglial activation which is sustained even around PND22 leading to increased neuroinflammation as well as impaired learning and memory response[20,24,25]. We showed that perinatal arsenic exposure increased the number of microglia in the hippocampus. Our observation was supportive of the

fact that arsenic exposure increases the phosphorylation of STAT3[26] which plays an important role in cell proliferation[27]. Increased immunoreactivity of proliferation marker, Ki67 in hippocampal microglia of arsenic-exposed pups confirms the micrglial proliferation[20].

CSF1R, a class III receptor tyrosine kinase, is expressed by myeloid cells like microglia and macrophage[12]. CSF1R plays a direct role in controlling the development, survival and maintenance of microglia population in CNS[13]. In our study, an increased expression of CSF1R in the hippocampus and BV2 microglia was observed which indirectly indicates enhanced microglial proliferation[20,28]. An increased level of CSF1R mRNA in arsenic exposed group suggests a possible transcriptional regulation of the CSF1R gene. Transcription factors play an important role in the regulation of gene transcription. PU.1 is one of the key myeloid-specific transcription factors that is critically involved in CSF1R gene expression[15]. Therefore, we have checked the expression of *Sfpi1* (PU.1 gene) in PND22 pups and BV2 microglia. PU.1 expression was increased significantly in the arsenic-exposed group which is supportive of the report of increased PU.1 expression in activated microglia found in different disease[29]. To confirm the role of PU.1 in the arsenic-mediated regulation of CSF1R, the expression of PU.1 and CSF1R were checked following inhibiting *Sfpi1* using siRNA. The results suggested that the PU.1/CSF1R signalling axis mediates the arsenic-induced proliferation of microglia.

The increased expression of PU.1 following arsenic exposure in the present study prompted us to further investigate the mechanisms by which arsenic enhances Sfpi1 transcription. *In silico* analysis reveals that the *Sfpi1* gene carries a large CpG island at 17kb upstream from the transcriptional start point[30]. CPG island methylation and demethylation mediated transcriptional regulation of PU.1 is well reported in cancer and haematopoiesis[30]. Arsenic is a global demethylation agent and we have found that arsenic exposure significantly down-regulates the expression of DNMT1 enzyme, which in turn may demethylate the regulatory region of specific genes[16]. Therefore there is every possibility that arsenic could demethylate the CpG islands present in the *Sfpi1* gene to induce its transcription in microglia similar to miR-129-2 gene[19]. To ensure the arsenic-induced demethylation of the upstream regulatory region of the *Sfpi1* gene, PU.1 expression was checked following L-methionine supplementation in arsenic-exposed BV2 microglia. The reversal of arsenic-induced increased expression of PU.1 and CSF1R in the L-methionine-supplemented group suggested a possible demethylation of the Sfpi1 regulatory region[22,30]. Altogether, the study unravels that arsenic exposure induces microglial proliferation by modulating the PU.1/CSF1R signalling axis with a possible epigenetic regulation of *Sfpi1* expression (Figure 8).

5. Conclusion

The present study concludes that perinatal arsenic exposure increases the expression of the *Sfpi1* gene possibly through demethylation of the upstream regulatory region, which leads to increased microglia number in the hippocampus by modulating PU.1/CSF1R axis.

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7. References

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