Effect of pre and postnatal Exposure to Arsenic on the Immune and Gastrointestinal function: study in mice model

The thesis submitted for the Doctor of Philosophy (Science) in Life Science and Biotechnology, Jadavpur University

(2023)

By

MAINAK CHAKRABORTY
(Index No.: 79/19/Life Sc./26)

Department of Life Science and Biotechnology

Jadavpur University

Kolkata, India

2023

आई. सी. एम. आर. – राष्ट्रीय कॉलरा और आंत्र रोग संस्थान
ICMR - NATIONAL INSTITUTE OF CHOLERA AND ENTERIC DISEASES
स्वास्थ्य अनुसंधान विभाग, स्वास्थ्य और परिवार कल्याण मंत्रालय, भारत सरकार
Department of Health Research, Ministry of Health and Family Welfare, Govt. of India
WHO COLLABORATING CENTRE FOR RESEARCH AND TRAINING ON DIARRHOEAL DISEASES

CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled "**Effect of pre and postnatal exposure to Arsenic on the Immune and Gastrointestinal Function: study in mice model**" Submitted by Sri / ~~Smt~~ **Mainak Chakraborty** who got his / ~~her~~ name registered on **11.09.2019** For the award of Ph. D. (Science) degree of Jadavpur University, is absolutely based upon his own work under the supervision of **Dr. Moumita Bhaumik** and that neither this thesis nor any part of it has been submitted for either any degree / diploma or any other academic award anywhere before.

Moumita Bhaumik 02/02/2023

(Signature of the Supervisor(s) date with official seal)

Dr. Moumita Bhaumik
Scientist - C
Division of Immunology
ICMR-National Institute of Cholera
and Enteric Diseases
Beleghata, Kolkata

पी-३३, सी.आई.टी. रोड, स्किम - १०एम, बेलियाघाटा, कोलकाता - ७०००१०, भारत
P-33, C.I.T. Road, Scheme - XM, Beliaghata, Kolkata - 700010, India
निदेशक / Director : 91-33-2363 3373, 2370 1176, पि.बि.एक्स / PBX : 91-33-2353 7469 / 7470, 2370 5533 / 4478 / 0448
फैक्स / Fax : 91-33-2363 2398, 2370-5066, वेब / Website : www.niced.org.in

Index No.: 79/19/Life Sc./26
Title of the Thesis: Effect of pre and postnatal exposure to Arsenic on the Immune and Gastrointestinal Function: study in mice model

ABSTRACT

Arsenic is a highly potent environmental pollutant which affects more than 200 million people globally particularly through ingestion of contaminated food and drinking water. Arsenic has been known to exert deleterious effects not only in adult life but also in prenatal and early life. As Arsenic can easily cross the placenta a fetus becomes particularly vulnerable to toxic effects of the metalloid increasing the risk of infant mortality, and other associated impacts later in life including the maternal-offspring microbiota exchanges and development and maturation of the neonatal microbiome and the gastrointestinal system. This thesis aims to better understand how prenatal arsenic exposure may change the gut microbiome and its consequences on gut function, if any, in later life.

To witness how arsenic exposure in adult life may affect immune and gastrointestinal physiology we undertook initial approach by treating Balb/c mice with 4 ppm and 10 ppm of arsenic trioxide in drinking water respectively for 30 days. Mice exposed to Arsenic (As-mice) showed reduced CD4+ and CD8+ T-cells in spleen, IgG1 and IgG2a in serum and increased susceptibility to enteric *Shigella* infection. Shigella infection in As-mice increased bacterial burden in colon, expression of mucin-2, iNOS and cytokines IL-6 and TNF- α compared to control. This is an interesting observation where we found correlative association between reduced T cells and increased susceptibility to gut infection.

Next we investigated if and how prenatal arsenic exposure affects all three arms of immune system (innate, humoral, and cellular) and if it leads to any long lasting influence in the postnatal host immune repertoire. Pregnant mice were exposed to arsenic trioxide through their drinking water from time of conception until parturition. The 4-week-old pups that had not been exposed to Arsenic after birth were used for functional analyses of gastrointestinal and immune function. We uncovered that prenatal arsenic exposure significantly decreased splenic CD4+T-cells and CD8+ T-cells, while mice also failed to produce and decrease in IL-2 and IFN-γ upon proper stimulation. There were marked significant reductions in inducible early T-cell activation markers CD44 and CD69 while serum IgG2a levels were decreased and thus ultimately leading to decreased immunity as observed by increased susceptibility to septicemic *E. coli* infection. Thus prenatal arsenic exposure induced a generalized defect in immune function that then persisted even in the absence of further Arsenic exposures.

Lastly, we delved into the study to understand if prenatal arsenic exposure affects the gastrointestinal function in post natal life in mice. The prenatally arsenic exposed mice (pAs-mice) showed a striking reduction in *Firmicutes* to *Bacteroidetes* (F/B) ratio coupled with decrease in tight junction protein, occludin resulting in increase in gut permeability, increased infiltration of inflammatory cells in the colon and decrease in common SCFAs in which butyrate reduction was quite prominent. Further studies with supplementing butyrate, to pAs-mice we observed reversal of the arsenic induced changes in the gut. Our investigation on the molecular mechanism revealed that reduction in butyrate production in pAs-mice leads to increase in miR122 expression in gut which in turn decays Occludin mRNA, one of the important tight junction proteins.

Thus in conclusion, we can state that prenatal and postnatal exposure to Arsenic hampers the development of immune system and gastrointestinal system which could lead to increased susceptibility to diseases and other variety of health complications later in life.

Mainak Chakraborty
02/02/2023

Dr. Moumita Bhaumik
02/02/2023
Scientist - C
Division of Immunology
ICMR-National Institute of Cholera and Enteric Diseases
Beleghata, Kolkata

DECLARATION

I do, hereby declare that the work embodied in this thesis entitled "**Effect of pre and postnatal exposure to Arsenic on the Immune and Gastrointestinal Function: study in mice model**" submitted for the award of Doctorate of Philosophy (Science) in Life Science and Biotechnology, is the completion of work carried put under the supervision of **Dr. MoumitaBhaumik, Scientist- C, at the Division of Immunology, ICMR-National Institute of Cholera and Enteric Diseases, Kolkata**. Neither this thesis nor any part of it has been submitted for either any equivalent degree/diploma or any other academic award elsewhere.

Date: 02/02/2023

Place: Kolkata

Mainak Chakraborty

Signature of the candidate

Mainak Chakraborty

I dedicate this thesis to
My parents, my brother Bikramjit and my friend Nilotpal

For without them this would have not been possible…

Acknowledgements

.

I would like to express heartfelt gratitude to all those who have contributed in countless ways to the success of this thesis work and made this journey a memorable one for me.

Firstly, I would like to express my enormous gratitude to Dr. Moumita Bhaumik for her invaluable advice in these last five years. As my mentor, her relentless enthusiasm and support have made it possible for me to achieve my goals. I have been extremely lucky to have a practical, judicious, efficient, supportive and inspiring supervisor like her. Her countless advice was instrumental in shaping the overall direction of my research work.

I also take this opportunity to offer my sincerest thanks to Council of Scientific and Industrial Research (CSIR) for providing financial assistance in the form of Junior and Senior Research Fellowship that enabled me to be financially independent during my PhD tenure.

I am also grateful to the Director of ICMR-National Institute of Cholera and Enteric Diseases, Dr. Shanta Dutta for providing me the opportunity to work in this institute.

The work in my thesis work would not have been possible without close collaboration with a few laboratories. My sincere thanks go to Dr. Amit Pal (Division of Pathophysiology, ICMR-NICED), Dr. SantaSabuj Das (Division of Clinical Medicine, ICMR-NICED) and Dr. Nabendu Sekhar Chatterjee (Department of Biochemistry, ICMR-NICED) for providing helpful suggestions and comments and reagents that were immensely helpful in completion of the thesis work.

I would also like to extend my gratitude to Dr. Debmalya Mitra whose valuable advice has helped me overcome many a hurdles I faced during my PhD. Also I would like to thank Ananda Pal for his patience and help with my flow cytometry

experiments.

My special thanks go to Narayan Da for his help with animal experiments and Swapan Da for helping me with administrative work regarding my fellowship.

I extend my sincere thanks to all my lab mates: Oishika, Sohini, Aaheli, Ankita, Niraj, Saibal, Suparna, Prolay, Indranil, Debjyoti, Suman, Mahadev and Sangita for making the journey an absolute memorable one for me. I would also like to thank my seniors: Ashim Da, Dwiprohi Da, Tanmoy Da, Vipul Da, Rima Di, Tanushree Di, Upayan Da, Rakesh Da for their help and advice.

I am forever indebted to my good friends Nilotpal, Debarshi, Niksh, Wrick, Rajdeep, Pushpendu, Kaushik, Debasish, Paromita and Purbasha for they have stood by me in the toughest of times.

I would also like to thank my teachers Dr. Sayak Ganguli, Dr. Deba Prasad Mondal, Mrs. Bhaswati Talukdar Mukhopadhyay, Mr. Deepak Sutradhar and Mr. Abhijit Mazumdar for installing a passion for learning in me and playing a major role in me pursuing me a Doctorate.

Last but not the least I would like to thank my parents and my brother Bikramjit for inspiring me not only during my PhD but also in life. Without them it would have been impossible for me to have come this far in life. I am forever indebted to them.

Contents

Abbreviations

Ab,	antibody
Ag,	antigen
APC,	Ag-presenting cell
APC (stain)	Allophycocyanin
As_2O_3	Arsenic Trioxide
BALB/c,	a mouse strain
BSA,	bovine serum albumin
bw,	body weight
cDNA,	complementary DNA
Con A,	concanavalin A
CTL,	cytotoxic T lymphocyte
CFU	Colony forming Unit
DMEM,	Dulbecco's modified Eagle's medium
DMSO,	dimethylsulfoxide
DTT,	dithiothreitol
DNase,	deoxyribonuclease
EDTA,	ethylenediaminetetraacetic acid
ELISA,	enzyme-linked immunosorbent assay
FACS,	fluorescence-activated cell sorter
FBS,	fetal bovine serum
FITC,	fluorescein isothiocyanate

gm, gram (only with numbers)

GAPDH or G3PDH, glyceraldehyde-3-phosphate dehydrogenase

HBSS, Hanks' balanced salt solution

HRP, horseradish peroxidase

IFN, interferon (e.g., IFN-γ)

Ig, immunoglobulin (e.g- IgA, IgD, IgE, IgG, IgM)

IL, interleukin (e.g., IL-2)

i.p., intraperitoneal

LPS, lipopolysaccharide

BME, 2-mercaptoethanol

Mϕ, macrophage

mg, milligram (only with numbers)

MHC, major histocompatibility complex

min, minute (only with numbers)

ml, milliliter (only with numbers)

MLN Mesenteric Lymph Nodes

MLR, mixed leukocyte reaction

mRNA, messenger RNA

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide

µg, microgram (only with numbers)s

µl, microliter (only with numbers)

NS, not significant

OD, optical density

p,	probability
PAGE,	polyacrylamide gel electrophoresis
PEC,	peritoneal exudates cells
PBS,	phosphate-buffered saline
PCR,	polymerase chain reaction
PE	Phycoerythrin
PMSF,	phenylmethylsulfonyl fluoride
rpm,	revolutions per minute
RPMI 1640	Roswell Park Memorial Institute medium 1640
RT-PCR,	reverse transcriptase polymerase chain reaction
s,	second (use only with numbers)
SD,	standard deviation
SDS,	sodium dodecyl sulphate
SE,	standard error
SEM,	standard error of the mean
TCR,	T cell receptor for Ag
Th cell,	T helper cell
TLR,	Toll-like receptor
TNF,	tumor necrosis factor
Tris,	tris(hydroxymethyl)aminomethane
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with 0.1% Tween 20.
UV,	ultraviolet

U,	unit (only with numbers)
v/v,	volume to volume ratio (%)
v/w,	volume to weight ratio (%)
wk,	week (only with numbers)

Preface

Arsenic is a highly potent environmental pollutant which can induce a wide range of adverse effects. It affects more than 200 million people globally particularly through ingestion of contaminated food and drinking water. Arsenic have been known to exert deleterious effects on the immune system however the specific effects and mechanisms and how it leads to increased susceptibility to various disease remain poorly understood. As arsenic can easily cross the placenta a fetus becomes particularly vulnerable to toxic effects of the metalloid increasing the risk of infant mortality, health risks and impaired intellectual development with associated impacts later in life. Oddly enough there are no reports till date on how *in utero* arsenic exposure may change the gut microbiome and its consequences on gut function, if any, in later life. All the studies conducted to construct this thesis attempt to answer impacts on the gut physiology and immune system in prenatal and postnatal Arsenic exposure.

Chapter 1 of this thesis deals with the effects of postnatal Arsenic exposure. There are increasing evidences that suggest that arsenic has a detrimental effect on the immune system (Dangleben, Skibola, & Smith, 2013).Thus the exposure could lead to increased susceptibility to many diseases and carcinogenesis as a result of the impaired immune surveillance. However, the mechanism of such effects and whether it could lead to any potential increase in enteric infections remain poorly understood. Given that the population of T-cells, B-cells and NK cells along with other splenic and mesenteric lymph node (MLN) parameters indicate the state of immune surveillance, we endeavored to look for changes in the immune parameters in mice exposed to arsenic (4 ppm and 10 ppm) postnatal and whether they were more vulnerable to an enteric pathogen *Shigella* challenge.

Immunosupression and prevalence of infectious diseases due to prenatal exposure to arsenic have been reported in human cohorts. While majority of the epidemiological and experimental evidences of impairment of the immune system is due to de facto

exposure of arsenic, in **Chapter 2** of the thesis we set out to uncover the effects of prenatal arsenic exposure on the developing immune system which encompasses all three arms of immune system i.e. innate, humoral, and cellular and if it leads to any long lasting influences in the postnatal host immune repertoire.

Maternal–offspring microbiota exchanges play a significant role in the development and maturation of the neonatal microbiome and development of the gastrointestinal system and arsenic has been known to alter gut microbiome composition of children in affected areas and cause gut microbial dysbiosis in mice. In **Chapter 3** we shift our focus on effect of prenatal arsenic exposure on gastrointestinal system. We investigated whether prenatal arsenic exposure had any effect on the gut microbiome composition and secretion of short chain fatty acids (SCFA) in juvenile mice. We also investigated whether such changes could influence gut permeability by modulating functions of tight junction proteins.

Review of Literature

Background:

The advent of industrialization has been one of the major events that have shaped the history of humanity. While it has benefited mankind in regards to employment and general advent of civilization, it has come at a cost of deteriorating environmental quality and previously unseen consequences on health. The basic requirements of food, water and air have been contaminated with chemical and biological agents that possess an unnerving threat to our health when we are exposed to them by breathing, ingestion or simple contact. One of this environmental toxicant is arsenic which is distributed globally. Arsenic related health problem in humans have been on the rise in different parts of the world as a result of its cosmopolitan distribution and is alarming enough to land the toxicant the number one spot in the Agency for Toxic Substances and Disease Registry Priority List of Hazardous Substances (ATSDR, 2005).

History of Arsenic:

Arsenic is a naturally occurring metalloid with the atomic number 33. It is found in both organic and inorganic forms. It is the twentieth most abundant element in the earth's crust where the average content varies between 2 mg to 3 mg/kg (W. R. Cullen & Reimer, 1989; Tanaka, 1988). Throughout history arsenic has achieved notoriety and infamy as poison. Many arsenic compounds lack taste, odor or color, are ubiquitously found in the environment and are difficult to detect as it causes little to no symptoms prior to death. These factors have led arsenic to be widely used as poison. The term arsenic is derived from the ancient Greek word '*arsenikon*' which means potent (Bentley & Chasteen, 2002). While the existence of arsenic compounds such as orpiment (As_2S_3) and realgar (As_4S_4) has been known since ancient times, however Albertus Magnus is credited as the first person to obtain pure elemental arsenic by heating the oxide (As_2O_5) with soap (Buchanan, 1962). Arsenic compounds such as realgar have been recommended in the treatment of ulcers by Hippocrates in the 5th century BC and Galen in 2nd century AD (Waxman & Anderson, 2001). Thus arsenic has been traditionally used in wide ranging applications from cosmetics and paint to agricultural and modern electronics industry.

Chemistry of Arsenic:

Since Arsenic is a metalloid, it displays properties of both metals and non-metals. The electronic configuration of arsenic is [Ar] $3d^{10}$ $4s_2$ $4p^3$ while its outer shell has 5 orbiting electrons. Arsenic is mono-isotopic in nature and exists in four main valence states -3, 0, +3 and +5 which depends upon favorable environmental conditions. The two most common oxidation states of arsenic are arsenate (As V) and arsenite (As III) which exists in oxidizing and mildly reducing conditions respectively (Duker, Carranza, & Hale, 2005). Arsenic exists as a solid and is more commonly found in complexes with other elements like sulphur, lead, cobalt, copper, iron, nickel and silver. It forms colorless crystalline oxides that are readily soluble in water to form weak acids. The most common organic arsenic compounds are methylarsonic acid and dimethylarsinic acid whereas the most common inorganic arsenic compounds are arsenic trioxide, sodium arsenite and various arsenates (e.g.- Lead Arsenate) (WHO, 2000).

(A)  (B) 

Figure 1: (A) large sample of Native Arsenic (Natural History Museum, London. (B) Arsenic mineral realgar (Getchell Mine, Adam Peak, Potosi District, Humboldt County, Nevada, United States)

Arsenic in the human environment:

Arsenic is found in the environment ubiquitously as a result of both natural and anthropogenic consequences. Primary natural sources of arsenic include volcanic eruptions, wind erosion, forest fires and marine aerosols (Chilvers & Peterson, 1987; Smedley & Kinniburgh, 2001). The primary anthropogenic causes that result in

arsenic in the atmosphere are burning of fossil fuels and production of various metals like copper, nickel, zinc and steel. Mining, smelting and subsequent refining of metallic sulfides releases arsenic in the air. Burning of coal also contributes to the amount of arsenic released in the air. Bituminous coal on average contains 9.0 ± 0.8 mg As/kg (Yudovich & Ketris, 2005). Despite this, some coals used in China can contain several hundreds or thousands of mg As/kg (Ng, Wang, & Shraim, 2003; Zhao et al., 2008). Copper smelting and coal burning make up almost 60% of the emission of arsenic by anthropogenic sources. China is one of the biggest emitters of arsenic in the air with a reported average value of 51 ng As/m^3 in air of 44 main Chinese cities (Duan & Tan, 2013).

(A)  (B) 

Figure 2: (A) An active volcano erupting. (B) Coal power plant

Apart from air arsenic also accumulates in water. The concentration of arsenic in fresh water greatly depends on the local geochemical environment which mainly includes water rock interactions and conditions of the water aquifers. The concentrations of arsenic in the ocean vary greatly ranging from 0.15 and 6 μg/L (A. H. Welch, Lico, & Hughes, 1988) to 1 and 2 μg/L (Chappell, Abernathy, & Calderon, 2001). In major parts of the world the arsenic that is present in groundwater is found in oxidized forms. However, arsenic found in many alluvium aquifers of West Bengal and Bangladesh occurs in reduced condition. Arsenic content in the soil are greatly altered by human activities. Wastes from mines, smelting plants and run-offs from pesticide manufacturing plants can lead to a rise in arsenic levels in the soil as high as 27 g/kg-35 g/kg soil (Chappell et al., 2001). Arsenic can be found in the soil in both organic and inorganic forms with the latter being more predominant. These forms can then be methylated into monomethyl arsenic acid (MMA), dimethyl arsenic acid (DMA) and trimethyl arsenic oxides (TMAO) by microbes present in the soil. Arsenic can also

undergo alkylation and demethylation resulting in organic arsenic compounds (Bentley & Chasteen, 2002; Garcia-Manyes, Jimenez, Padro, Rubio, & Rauret, 2002).

Arsenic contamination of groundwater has emerged as a problem for many countries across the world which includes India, Bangladesh, China, Nepal, Pakistan, Bolivia, Cambodia, Chile, El Salvador, Iran, Hungary, Laos, Mexico, Myanmar, Ghana, Greece, Poland, Peru, Romania, South Africa, USA, Uruguay, Vietnam, Thailand and Mongolia (Chowdhury et al., 2000). The Environmental Protection Agency in 2001 estimated that nearly 160 million people in the world are exposed to dangerous levels of arsenic through contaminated drinking water (IARC (International Agency for Research on Cancer), 2004). The estimated risk of arsenic consumption through drinking water is illustrated in Figure 3.

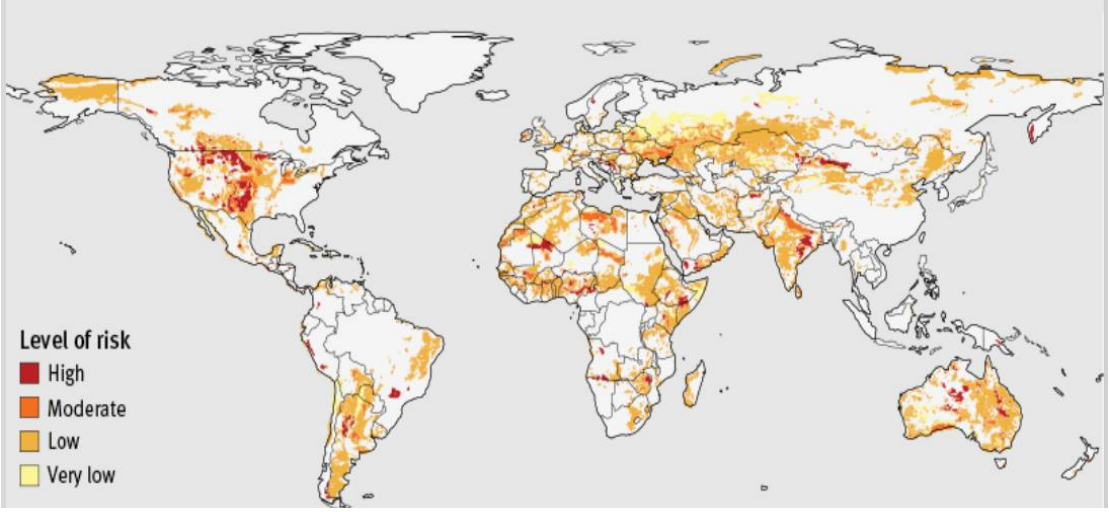


Figure 3: Risk of Arsenic in drinking water at significant levels (Schwarzenbach, Egli, Hofstetter, Von Gunten, & Wehrli, 2010)

The arsenic scenario in Bangladesh paints a very grim picture where almost 57 million people are vulnerable as they are exposed to concentrations of arsenic in drinking water far exceeding the WHO limit (10 µg/L) (K. Ahmad, 2001). In 1998 only 41 out of 64 districts in Bangladesh exceeded a concentration of 50 µg/L arsenic in drinking water; however by 2005 the number of districts has risen to 50 out of the 64 districts (Ahamed et al., 2006; Mukherjee et al., 2006). Arsenic contamination had significant impact on economy, agriculture and public health in Bangladesh (K. Ahmad, 2001). The already grim situation is only exacerbated by malnutrition and

poverty which adds woes especially in rural areas of the country.

Arsenic Contamination in the Indian Context:

Since groundwater plays a major role in various important sectors like agriculture, domestic consumption and industry, contamination by arsenic possess a major challenge to it (D. Saha & Ray, 2019; Suhag, 2016). Contamination of groundwater by arsenic mainly occurs in the alluvial aquifers of the Gangetic Delta which is the wealthiest groundwater province in the country. The contamination is mainly caused by sediments from the sulphide-rich mineralized areas of the state of Bihar. In India; an estimated 50 million people are at risk of arsenic contamination from groundwater. The first instance of arsenic contamination of groundwater was detected in 1976 in Chandigarh (Datta & Kaul, 1976) however it is widely believed that the problem was exacerbated as a result of digging several tube wells as a part of irrigation program (Bagla & Kaiser, 1996; Guha Mazumder et al., 1988). In India at least 20 states (West Bengal, Jharkhand, Bihar, Uttar Pradesh, Assam, Gujarat, Haryana, Madhya Pradesh, Panjab, Arunachal Pradesh, , Karnataka, Tamil Nadu, Himachal Pradesh, Telangana, Andhra Pradesh, Orrisa, Nagaland, Tripura, Manipur, Chhattisgarh) and 4 Union territories (Delhi, Daman and Diu, Puducherry, Jammu and Kashmir) are affected by presence of Arsenic in groundwater (Shaji et al., 2021). The affected states are shown in detail in Figure 4 and Table 1.

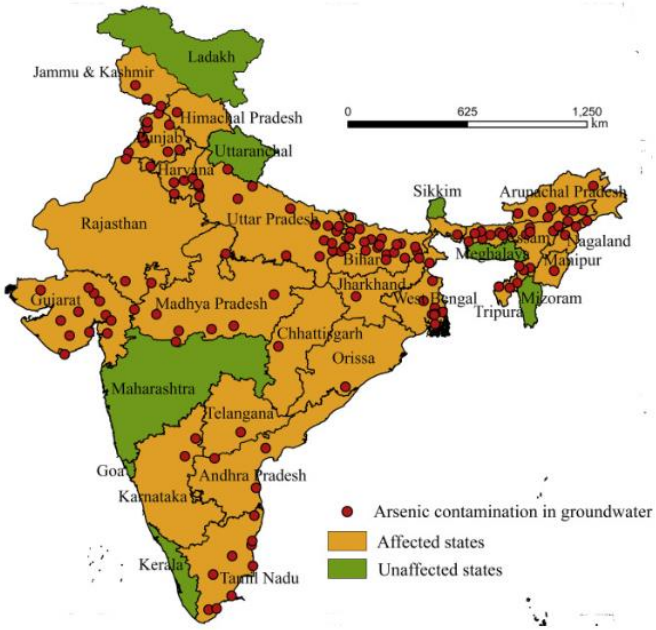


Figure 4: States in India with arsenic contaminated groundwater, (Shaji, 2021)

State	Year of Detection	Arsenic affected districts (above 0.05 mg/L)	Level of Arsenic Contamination(in mg/L)
West Bengal	1984	North-24-Parganas , South-24- Parganas , Murshidabad, Nadia, Maldah, Howrah , Hoogly , Kolkata,Bardhaman	Up to 3.000*
Bihar	2002	Begusarai, Bhagalpur, Bhojpur, Buxar, Darbhanga, Katihar, Khagaria, Kishanganj, Lakhisarai, Munger, Patna, Purnea, Samastipur, Saran and Vaishali	Up to 0.178
Uttar Pradesh	2003	Bahraich, Balia, Balrampur, Bareilly, Basti, Bijnor, Chandauli, Ghazipur, Gonda, Gorakhpur, Lakhimpur Kheri,Meerut, Mirzapur, Muradabad, Raebareli, Santkabir Nagar, Shajahanpur, Siddharthnagar, Unnao,SantRavidas Nagar	Up to 0.150
Jharkhand	2003	Sahibgunj	Up to 0.090
Haryana	2003	Ambala, Bhiwani, Faridabad, Fatehabad, Hissar, Jhajjar, Jind, Karnal, Panipat, Rohtak, Sirsa, Sonepatand Yamunanagar	Up to 0.070
Punjab	2003	Mansa, Amritsar, Gurdaspur, Hoshiarpur, Kapurthala and Ropar	Up to 0.400
Chhattisgarh	1999	Rajnandgaon	Up to 0.720
Karnataka	2009	Raichur and Yadgir	Up to 1.000
Assam	2003	Sivsagar, Jorhat, Golaghat, Sonitpur,Lakhimpur, Dhemaji, Hailakandi, Karimganj, Cachar, Barpeta, Bongaigaon, Goalpara, Dhubri, Nalbari, Nagaon, Morigaon, Darrang and Baksha.	Up to 0.996
Manipur	2004	Vishnupur, Thoubal	Up to 0.500

:

Table 1: Arsenic contamination in different states of India as published by the Ministry of Water Resources and River Development and Ganga Rejuvenation, 2014.

Arsenic contamination in West Bengal:

Arsenic contamination in groundwater in West Bengal was first reported in 1978. A recent report suggests that approximately 16.66 million people in 8 districts of West Bengal are at significant risk (D. Das et al., 1996; T. K. Das, 2019). The main affected areas are located in the upper delta plain. Chakraborti and colleagues classified different districts of West Bengal depending on groundwater arsenic concentration: (i) **Highly affected.** These include places where arsenic concentration in groundwater exceeded 300 µg/L. It includes 9 districts Murshidabad, Nadia, Maldah, Howrah, North-24-Parganas, South-24-Parganas, Hooghly, Bardhaman and Kolkata. These areas are mainly located on the eastern side of the river Bhagirathi. (ii) **Mildly affected.** These include areas where the groundwater contains arsenic concentrations mostly below 50 µg/L. They include 5 districts mainly Dinajpur-North, Dinajpur-South, Jalpaiguri, Darjeeling and Kochbihar. (iii) **Unaffected.** These include areas where all the recorded concentrations of arsenic were below 10 µg/L. This includes 5 districts consisting of Birbhum, Purulia, Medinipur-East, Medinipur-West and Bankura (Chakraborti et al., 2009). The details of arsenic concentrations in different districts are illustrated in figure 5.

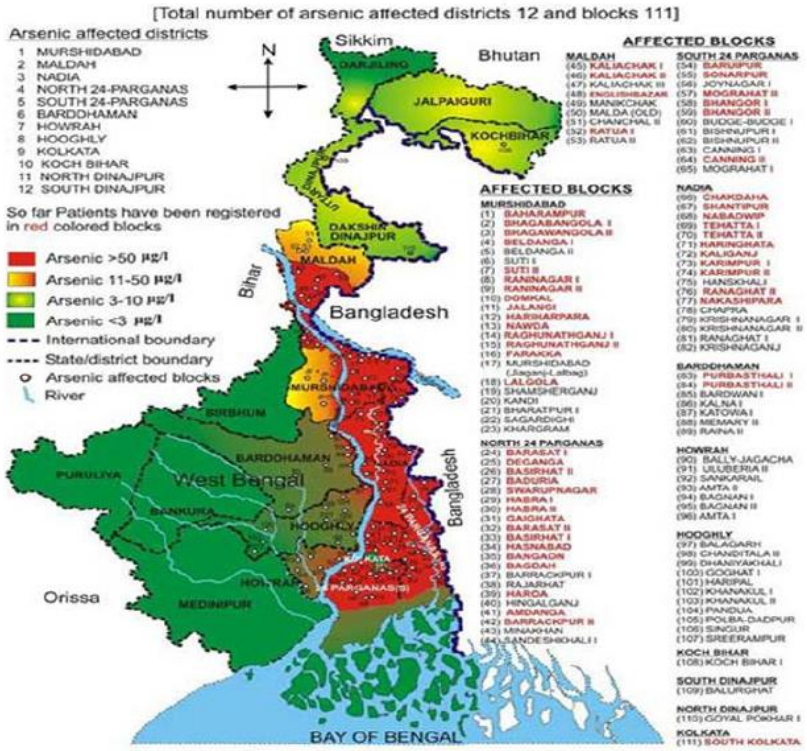


Figure 5: Groundwater arsenic contamination situation in the state of West Bengal, India (till Sep 2006), (Source: SOES and Chakraborti et al., 2009)

Metabolism of Arsenic in the body:

Once ingested orally, arsenic is readily absorbed through the gastrointestinal tract. It primarily undergoes reduction and methylation in the body and excreted out through urine (Hughes, 2006). Arsenate (As V) is better absorbed in the body than Arsenite (As III). Since arsenic in drinking water is present as arsenate, it is readily absorbed in the body through the gastrointestinal tract. Many mammals possess the capacity to methylate inorganic arsenic however the rate and extent to which they can methylate vary greatly between species and even between human populations (Hughes, 2002; Marie Vahter, 1994). After absorption arsenic is carried to different parts of the body through blood. The toxicokinetics of arsenic in the human body is not well understood. Development of robust kinetics and dynamics of arsenic pharmacology in the human body is of paramount importance to understand and eventually remedy ill effects of arsenic exposure in humans. Some of the toxicologically relevant arsenic compounds are illustrated in Figure 6.

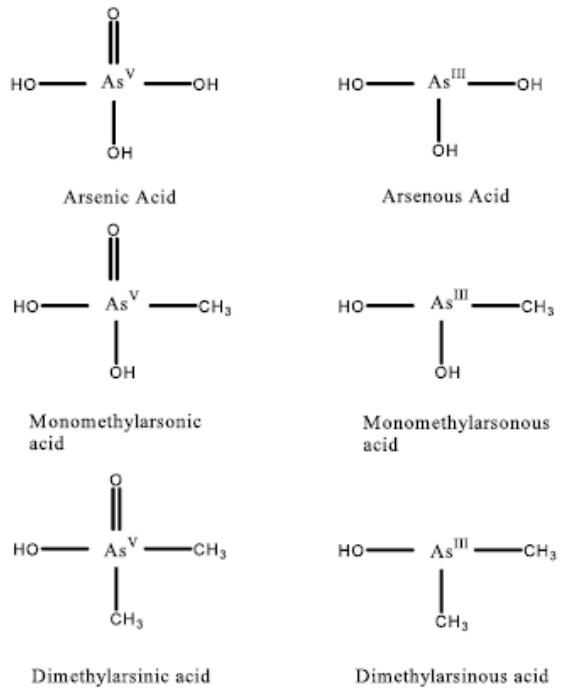


Figure 6: Some toxicologically relevant Arsenic Compounds, (Hughes, 2002)

Methylation of inorganic arsenic can either be oxidative or reductive. In oxidative methylation, arsenate (As V) is transformed to arsenite (As III) which then undergoes further oxidative methylation to give rise to products like monomethylarsonic acid (MMA^V), monomethylarsonous acid (MMA^{III}), dimethylarsinic acid (DMA^V) and finally dimethylarsinous acid (DMA^{III}) (Challenger, 1945). In some cases DMA^V can also get converted to trimethylarsine oxide ($TMAO^V$) (Pinyayev, Kohan, Herbin-Davis, Creed, & Thomas, 2011). The methylation of arsenic primarily occurs in the liver under the influence of the enzyme arsenic methyltransferase (AS3MT) and glutathione (GSH). The methyl group is donated by S-adenosylmethionine (SAM). Following this methylation arsenic is usually excreted through urine (Khairul, Wang, Jiang, Wang, & Naranmandura, 2017). Human liver arsenate reductase (Radabaugh & Aposhian, 2000) and MMA^V reductase (Robert A Zakharyan & Aposhian, 1999) have been shown to catalyze the reduction of inorganic AsV to inorganic AsIII and methylated AsV to methylated AsIII respectively. Oxidative methylation pathway however cannot fully explain why DMA^V is the major arsenical found in human urine (Dheeman, Packianathan, Pillai, & Rosen, 2014).

Methylation of arsenic is widely considered to be a detoxifying process. Methylated products of inorganic arsenic are readily excreted and are much less toxic than their inorganic counterparts (Buchet, Lauwerys, & Roels, 1981; Gebel, 2002; Hughes & Kenyon, 1998; Moore, Harrington-Brock, & Doerr, 1997). However trivalent arsenicals have been found in urine samples in various epidemiological studies (Aposhian et al., 2000; Del Razo, Styblo, Cullen, & Thomas, 2001; Le, Ma, et al., 2000; Mandal, Ogra, & Suzuki, 2001; Z. Wang, Zhou, Lu, Gong, & Le, 2004) which are more toxic than the pentavalent forms which may suggest that methylation may not always be a detoxifying process.

In reductive methylation, trivalent arsenicals (AsIII) are first conjugated with GSH following which then undergo methylation. The products generated in the pathways are arsenotriglutathione ($As^{III}(GS)3$), monomethylarsenodiglutathione ($MMA^{III}(GS)2$) and finally dimethylarsenoglutathione ($DMA^{III}(GS)$) (Hayakawa, Kobayashi, Cui, & Hirano, 2005). The oxidation state of arsenic continues to be trivalent throughout the entire duration of the catalytic cycle. The end products of this pathway are MMA^V and DMA^V which are less toxic in nature and are formed non-enzymatically (Drobna

et al., 2013; Le, Lu, et al., 2000). The oxidative and reductive methylation pathways are illustrated in detail in figure 7.

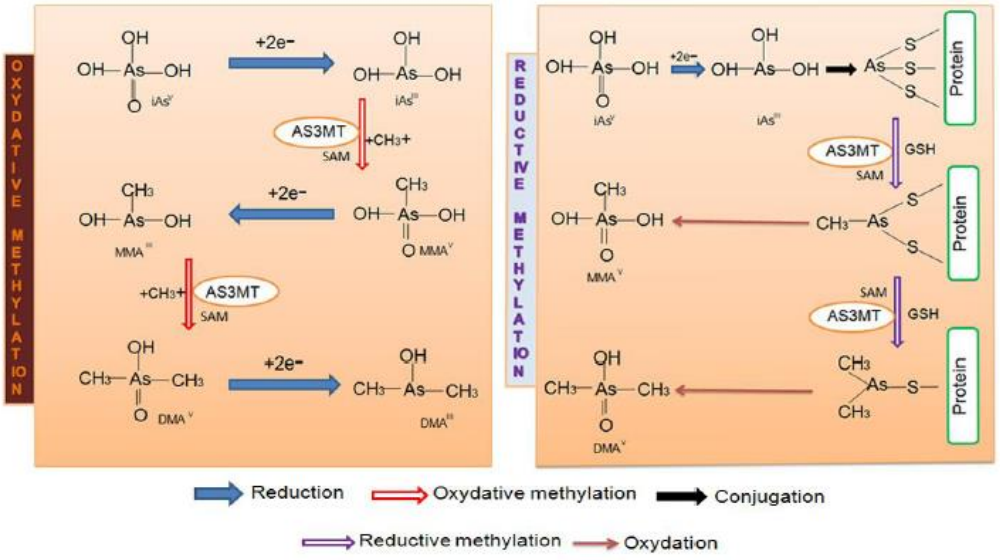


Figure 7: Oxidative and reductive metabolic pathways of inorganic arsenic (Drobna, et al, 2006)

The primary enzyme that takes part in metabolism of arsenic is **Arsenite (As^{III}) S-adenosyl-L-methionine methyltransferase (AS3MT)**. The gene for the enzyme in humans is located on Chromosome 10. AS3MT catalyzes the transfer of methyl group from SAM to trivalent arsenic forms (Dheeman et al., 2014; Drobna, Xing, Thomas, & Styblo, 2006). The other groups of enzymes that take part in the biotransformation of arsenic are **Glutathione S-transferases (GSTs).** They are important members of a family of eukaryotic metabolic isozymes. They catalyze the conjugation of reduced form of glutathione (GSH) to xenobiotic substrates for detoxification (Hayes, Flanagan, & Jowsey, 2005; Nebert & Vasiliou, 2004; P. Yang, Ebbert, Sun, & Weinshilboum, 2006). Members of GST superfamily possess extremely diverse amino acid sequences (Atkinson & Babbitt, 2009). GSTs detoxify xenobiotics primarily by catalyzing the nucleophilic attack on electrophilic carbon, sulfur, or nitrogen atoms. There are eight subclasses of GST's: Alpha, Mu, Pi, Theta, Zeta, Sigma, Kappa and Omega. The most recently described subclass is the Omega which contains two genes *GSTO1* and *GSTO2* (L. Wang et al., 2005). These two enzymes are found in a wide range of human tissues and catalyze the reduction of trivalent arsenicals to DMA^V and MMA^V (Schmuck et al., 2005; R. A. Zakharyan et al., 2001).

The metabolism of arsenic gives rise to formation of reactive oxygen species that has been known to cause damage to DNA (T. C. Lee & Ho, 1994; Matsui et al., 1999). The common forms of DNA damage include chromosomal breaks and aberrations and formation of micronuclei (Basu et al., 2002; A. R. Choudhury, Das, & Sharma, 1997)
.

Biomarkers of Arsenic toxicity:

The most common biomarker of arsenic exposure is the excretion of arsenicals and porphyrins through the urine (J. P. Wang et al., 2002). However, since arsenic is metabolized relatively quickly in blood, measurement of arsenic levels in blood may not be an accurate indicator of long term exposure to the metalloid. The presence of skin lesions is another common biomarker of long term arsenic exposure (C. J. Chen et al., 2005). Arsenic also gets deposited in keratinized tissues (e.g. - hair and nail) as they bind to sulfhydryl groups in keratin. So analysis of arsenic concentrations in hair and nail could also provide an indication of chronic arsenic exposure (Hinwood et al., 2003). Arsenic has also been known to cause micronuclei formation in bladder cells and oral mucosal cells (Biggs et al., 1997), clastogenicity in peripheral lymphocytes (Maki-Paakkanen, Kurttio, Paldy, & Pekkanen, 1998) and hemeoxygenase induction (Del Razo, Quintanilla-Vega, et al., 2001). Appropriate biomarkers for arsenic greatly depend on metabolism of the arsenicals in the human body and variability in genes metabolizing arsenic makes the choice of one standard biomarker difficult.

Toxicity of Arsenic:

Toxicity to arsenic is dependent on many factors which include dosage, individual susceptibility, ability to metabolize arsenic and even age of individuals who are being exposed to the metalloid. Toxicity caused by the metalloid can be acute i.e. which results from either a single exposure to arsenic or multiple doses in a very short period of time or it can be chronic which results from long term exposure to the metalloid.

A basic assumption about acute toxicity always has been that trivalent arsenic is potentially form harmful than pentavalent ones. In adult humans, the lethal amount of arsenic is estimated to be at 1–3 mg As/kg. In humans symptoms of severe acute arsenic toxicity include vomiting, diarrhea, convulsions, anuria, shock,

gastrointestinal discomfort, coma and death (Khairul et al., 2017). Different arsenic compounds have different toxicities depending largely on animal species rate of uptake and cell types. The details of LD_{50} values of several arsenic compounds in laboratory animals are described in Table 2.

Chemical	Species (sex)	Route	LD_{50} (mg As/kg)	Reference
Arsenic trioxide[a]	Mouse (m)	Oral	26	Kaise et al. (1985)
Arsenic trioxide	Mouse (m)	Oral	26–48[b]	Harrison et al. (1958)
Arsenic trioxide	Rat (m/f)	Oral	15	Harrison et al. (1958)
Arsenite	Mouse (m)	im	8	Bencko et al. (1978)
Arsenite	Hamster (m)	ip	8	Petrick et al. (2001)
Arsenate	Mouse (m)	im	22	Bencko et al. (1978)
MMA^{III}	Hamster (m)	ip	2	Petrick et al. (2001)
MMA^{V}	Mouse (m)	Oral	916	Kaise et al. (1989)
DMA^{V}	Mouse (m)	Oral	648	Kaise et al. (1989)
$TMAO^{V}$	Mouse (m)	Oral	5500	Kaise et al. (1989)
Arsenobetaine[c]	Mouse (m)	Oral	>4260	Kaise et al. (1985)

[a] Arsenic trioxide, As_2O_3 is a trivalent arsenical.
[b] Four strains of mice were treated.
[c] Pentavalent organic arsenic.

Table 2: Acute toxicity of arsenic in laboratory animals (Hughes, 2002)

Chronic exposure to arsenic affects many organ systems in humans. The effect of chronic arsenic toxicity on humans is termed as arsenicosis. This term was first coined by DN Guha Majumder (Guha Mazumder et al., 1988). This term was then later put to common use by WHO (WHO, 2005) and others to describe effects of long term exposure to arsenic. The hallmark sign of chronic arsenic toxicity in humans from oral exposure are skin lesions. It is characterized by hyperpigmentation, hyperkeratosis, and hypopigmentation (Cebrian, Albores, Aguilar, & Blakely, 1983; Yeh, How, & Lin, 1968). Chronic toxicity to arsenic has been linked with many diseases such as diabetes, hypertension, ischemic heart diseases, hepatotoxicity, and cancer of the skin, bladder, and lungs (Khairul et al., 2017).

Dermatological Manifestations: The most telltale sign of chronic arsenic exposure is development of lesions on the skin that includes hyperkeratosis and hyperpigmentation. These symptoms are often used as a diagnostic characteristic to identify arsenicosis (McCarty et al., 2007). Men seem more susceptible to arsenic induced skin disorders than women (Lindberg, Rahman, Persson, & Vahter, 2008). Hyperpigmentation manifest itself as raindrop-shaped discolored spots, diffused dark

brown spots or diffused darkening of the skin on the limbs and trunk (Guha Mazumder et al., 1988). Dermal effects due to arsenic exposure indicate early stages of arsenic poisoning while cancers induced by arsenic may take decades to develop. Dermal effects of exposure to organic arsenic have not been observed. Arsenic induced skin lesions are illustrated in figure 8.

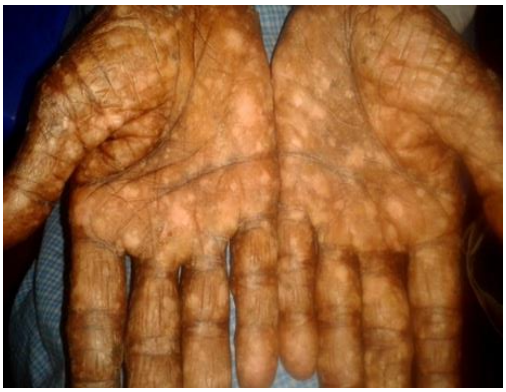
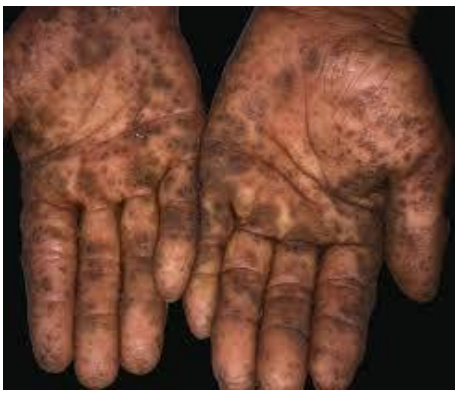
(A)  (B) 

Figure 8: (A) Arsenical pigmentation (spotty rain drop like) (B) Arsenical keratosis (nodular and confluent thickening) (Source: Guha Mazumder, 2008)

Cancers: Arsenic is a potent environmental carcinogen. It has been linked with cancers of the skin, lung, bladder, liver and kidney (Rossman, 2003). It is thought that they cause cancer by generating oxidative stress in which the body's antioxidant capacity is overwhelmed by ROS (Reactive Oxygen Species) leading to damages to proteins, lipids and DNA (S. X. Liu, Athar, Lippai, Waldren, & Hei, 2001). Trivalent arsenic have also been known to detach iron from the storage protein ferritin (Salnikow & Zhitkovich, 2008) which allows the free iron to catalyze the decomposition of hydrogen peroxide giving rise to reactive hydroxyl radical which then causes DNA damage. Epidemiological studies indicate that exposure to inorganic arsenic increases risk of lung cancer (Wall, 1980; K. Welch, Higgins, Oh, & Burchfiel, 1982). Apart from lung cancers several studies also indicate significantly increased bone and large intestine cancer with inhalation of arsenic (Enterline, Day, & Marsh, 1995). While it is generally accepted that methylated arsenicals are significantly less toxic than their inorganic counterparts, the process of methylation could lead to the formation of carcinogenic methylated arsenicals (Cohen, Yamamoto, Cano, & Arnold, 2001). Various animal studies indicate that MMA (V), DMA (V) is capable of inducing bladder tumor in rats when administered in very high doses (100 ppm) in the diet for two years (Gemert M, 1998). Since they are both stored in the

bladder, it is more susceptible to arsenic induced carcinogenesis. The exact molecular mechanism by which arsenic causes carcinogenesis is under active study by many researchers. Involvement of genetic and epigenetic changes, formation of Reactive Oxygen Species, enhanced cell proliferation, changing pattern of expression of various genes, and induction of hypoxia are generally accepted mechanisms (Galanis, Karapetsas, & Sandaltzopoulos, 2009).

Epigenetic modifications: Epigenetic modifications in mammals are vital for many important functions like maintaining stability of the genome, block retrotransposon activity and maintain parental imprinting etc. (Z. D. Smith et al., 2012). Arsenic toxicity can alter gene methylation therefore contribute to erroneous formation of proteins and even cancer (Reichard & Puga, 2010; Ren et al., 2011). A recent study indicates positive association between arsenic exposure and gene specific differential white blood cell DNA methylation (Argos et al., 2015). Higher doses of arsenic are also known to alter the miRNA expression in Sprague–Dawley rats (Ren et al., 2015). *In vitro* analysis of human bronchial epithelial cells indicates that chronic exposure to arsenic can lead to tumorigenesis by down regulation of miR-199a-5p (He et al., 2014).

Cardiovascular Diseases: Many epidemiological studies link exposure to arsenic with adverse effects on the cardiovascular system (Navas-Acien et al., 2005; States, Srivastava, Chen, & Barchowsky, 2009). Exposure to arsenic has been known to lead to altered myocardial depolarization and cardiac arrhythmias (N. M. Cullen, Wolf, & St Clair, 1995; Mumford et al., 2007). Long term exposure to arsenic has also been known to cause severe ventricular wall hypertrophy (Quatrehomme, Ricq, Lapalus, Jacomet, & Ollier, 1992). A study in USA found that there was a significant increase in the number of deaths from arteriosclerosis, aneurysm and other related diseases in areas with arsenic concentration in the groundwater exceeding 20µg/L (R. R. Engel & Smith, 1994). Arsenic has also been known to increase the mRNA transcripts of growth factors like GM-CSF and TNF-α which has been known to induce atherosclerosis (Germolec et al., 1997; Kitchin, 2001). A study also associates hypertension with increased arsenic exposure (H. T. Yang, Chou, Han, & Huang, 2007). Exposure to arsenic resulted in enhanced myosin light-chain phosphorylation leading to an increase in calcium-sensitization in blood vessels.

Liver Diseases: Inorganic arsenic is known to cause hepatic injury in humans after oral exposure (J. Liu et al., 2002). Blood tests show elevated levels of enzymes like globulin, alkaline phosphatase, alanine amino transferase and aspartate amino transferase in the liver while histological slides of the liver also reveal presence of portal tract fibrosis (Mazumder et al., 2005). People who underwent arsenic exposure have also suffered from cirrhosis and damage to the hepatic blood vessels. Hepatic lesions have also been reported occurring in the later stages of toxicity (Kapaj, Peterson, Liber, & Bhattacharya, 2006). Enlargement of liver and bleeding from esophageal varices and jaundice have also been linked with arsenic exposure (Kapaj et al., 2006).

Renal Toxicity: Accumulation of arsenic occurs in kidney cells during the process of elimination of the metalloid from the body through excretion. This accumulation of arsenic leads to cytotoxicity in renal tissues (Madden & Fowler, 2000). Increased levels of serum creatinine, blood urea nitrogen, hypourea and proteinuria are also observed in association with arsenic exposure (Sasaki, Oshima, & Fujimura, 2007). Albuminuria and proteinuria is also known to have a positive correlation with arsenic exposure (Zheng et al., 2014). Arsenic can also cause damage to kidney glomeruli and capillaries (M. M. Rahman, Ng, & Naidu, 2009).

Haematological Toxicity: Individuals exposed to arsenic have been known to suffer from anaemia, leucopaenia and thrombocytopaenia (Guha Mazumder, 2008).

Respiratory Toxicity: Complications in the respiratory system are one of the most common clinical manifestations of arsenic exposure. Distress in the respiratory system due to arsenic exposure includes shortness of breath, blood in sputum, chest sounds and chronic cough (Parvez et al., 2010). Bronchiectasis is also positively correlated with arsenic exposure via contaminated drinking water (Guha Mazumder, 2007).

Neurological Toxicity: Studies in animals show that exposure to arsenic can cause changes in the levels of neurotransmitters such as dopamine, norepinephrine and 5-hydroxytryptamine (Kannan, Tripathi, Dube, Gupta, & Flora, 2001). Studies also

indicate possible positive correlation between arsenic and neurobehavioral alterations in children (Tsai, Chou, The, Chen, & Chen, 2003). The most common neurological feature as a result of arsenic induced toxicity is peripheral neuropathy which may persist for several years (Mathew, Vale, & Adcock, 2010). Reduced nerve conducting velocity in peripheral nerves has become a hallmark symptom in people suffering from arsenic induced neurotoxicity. Low level arsenic exposure have also been found associated with Alzheimer‘s disease and its accompanying abnormalities (O'Bryant, Edwards, Menon, Gong, & Barber, 2011). Arsenic induced neurotoxicity is primarily caused by the inactivation of enzymes that are important for cellular energy metabolism.

Arsenic and the Immune System:

Extensive research has been conducted on the various toxic effects of Arsenic on different organ systems and its potential role as a carcinogen, however recent studies (Selgrade, 2007; M. Vahter, 2008) have only begun to unearth the sinister hold of the metalloid on the immune system. Arsenic induced immunomodulation most likely promotes and adds to systems toxicity. Arsenic induced immunotoxicity potentially plays a vital role in aiding arsenic related carcinogenesis in various tissues through reduced immune surveillance. Recent studies have begun to show the immunotoxicological effects of arsenic in animals, *in vitro* models and in humans and how these responses could modulate the activity of both innate and adaptive immune responses through various molecular mechanisms which could lead to increased risk of infections and chronic diseases, including various cancers (Selgrade, 2007). Possible mechanisms of arsenic induced immunotoxicity are outlined in figure 9.

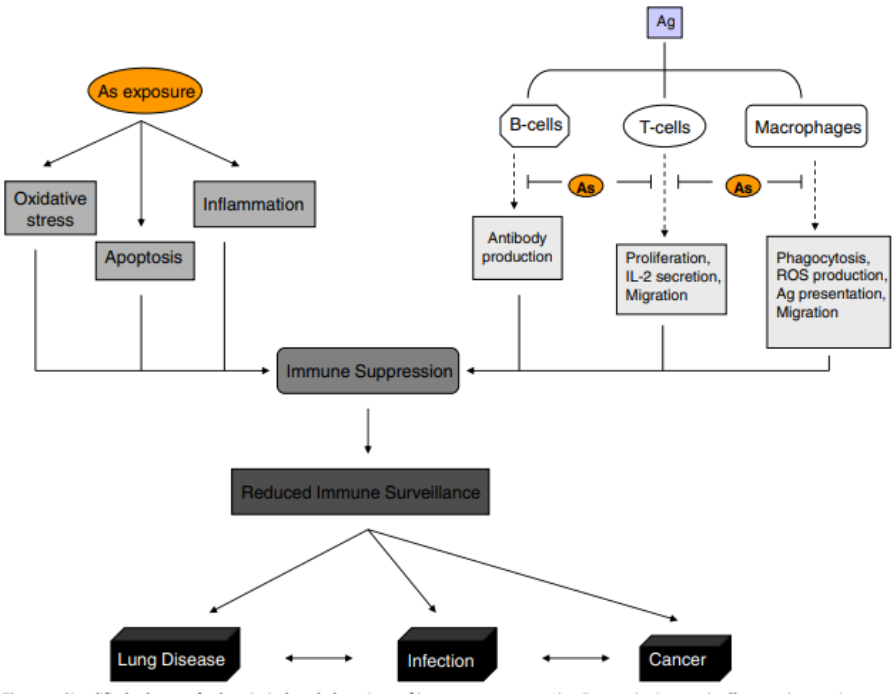


Figure 9: Arsenic induced alterations in immune responses and their possible contributions to various illnesses (Dangleben, 2013)

Gene Expression: A microarray-based genome wide expression study of peripheral blood mononuclear cells (PBMC) from 21 subjects in New Hampshire whose drinking-water As averaged 0.7 µg/L (range 0.007–5.3 µg/L, n = 10) and 32 µg/L (range 10.4-74.7 µg/L, n = 11) showed significant differences between exposure groups in transcripts with functions in T-cell receptor signaling, cell cycle regulation and apoptosis, and most strikingly defense and immune response (Andrew et al., 2008). Increased As exposure resulted in increased expression of killer cell immunoglobulin-like receptors that inhibit natural killer cell-mediated cytotoxicity, as well as decreased expression of MHC class II molecules, HLA-DQB1, HLADPA1, and HLA-DRB1; defense response genes, CD69, HSPA9B and MALT1; and inflammatory genes, IL2RA and IL1B.

Another microarray study in Bangladesh from PBMC of arsenic induced population showed down regulation of IL1B (Argos et al., 2006). Another cDNA microarray study in Mexico from individuals having urinary arsenic levels between 117.23 and 435.12 mg/g showed significant differences in expression of genes involved in inflammation. There was a significant downregulation in expression of genes that

includes TNF, IL11, IL10RB, CCR1, and CXCL2 (Salgado-Bustamante et al., 2010). These findings contradict those from a microarray analysis of PBMC from 24 individuals in Taiwan with low (0–4.32 μg/L), intermediate (4.64–9 μg/L), and high (9.6–46.5 μg/L) blood As levels (Wu, Chiou, Ho, Chen, & Lee, 2003). Among 62/708 significantly altered genes were several inflammatory molecules that were up-regulated, including IL1B, IL6, CCL2 and CD14, indicating that prolonged exposure may induce ongoing inflammation contributing to As-associated disease (Wu et al., 2003).

In animal models, arsenic exposure altered expression of various genes involved in immune response. When mice were exposed to trivalent arsenic for 5-6 weeks there were significant downregulation of genes involved in the humoral immune responses, antigen binding, TLRs, cytokines, cytokine receptors and genes involved in cell adhesion and migration (Andrew et al., 2007; Kozul et al., 2009). In zebra fish arsenic significantly inhibited expression of genes involved in mounting immune responses against viral and bacterial infections (Hermann & Kim, 2005; Nayak, Lage, & Kim, 2007). While changes in gene expression may not necessarily be a toxic response, exposure to arsenic could lead to changes in adaptive responses and may alter cellular function and ultimately the immune system's ability to defend the host (Tokar et al., 2010).

Lymphocyte subpopulations and activation: Exposure to Arsenic significantly impacts different immune cell subpopulations. A study in India showed that arsenic exposed individuals with skin lesions (n= 70) significantly increased eosinophil numbers and decreased monocytes when compared with unexposed individuals (Maiti et al., 2012). T and B lymphocytes mediate the cellular and humoral responses of adaptive immunity, respectively. The activation of T lymphocytes by Dendritic Cells (DCs) stimulates their proliferation and differentiation into various CD4+ T helper cell subtypes and CD8+ T cytotoxic cells.

Numerous studies have demonstrated that arsenic markedly alters both the number and function of human T lymphocytes. Specifically, chronic arsenic exposure is correlated with decreased percentages of peripheral blood CD4+ T cells and CD4/CD8 ratios in children and adults and with reduced ex-vivo proliferation of

activated T lymphocytes (R. Biswas et al., 2008; Liao et al., 2009; Soto-Pena et al., 2006). Substantial childhood morbidity that has been documented in As-polluted areas is probably due to compromised immune status. Studies in mice and chickens show that arsenic exposure resulted in decreased weight, index and/or cellularity of major immunocompetent organs like spleen and thymus (Aggarwal, Naraharisetti, Dandapat, Degen, & Malik, 2008; Sikorski, Burns, Stern, Luster, & Munson, 1991).

Exposure to arsenic also causes impaired T-cell activation. A study in Mexico shows that chronic exposure to arsenic significantly reduced proliferation of mitogen activated T- lymphocytes (Ostrosky-Wegman et al., 1991). This observation was late supported by another study which identified delayed cell cycle progression from S- to M-phase in immune cells of persons exposed to high arsenic concentrations (412 μg As/L) (Gonsebatt et al., 1994). A study in West Bengal also showed that 20 As-exposed individuals with skin lesions had significant reductions in proliferation of lymphocytes and secretion of cytokines like IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF-α (R. Biswas et al., 2008). *In vitro* experiments show that low and non-cytotoxic arsenite concentrations significantly down regulate the proliferation of T-cells and secretion of IL-2 (Burchiel et al., 2014; Morzadec, Bouezzedine, Macoch, Fardel, & Vernhet, 2012). Arsenic also inhibits differentiation of T_H17 lymphocytes by repressing IL-17 expression which is an important cytokine that not only increases defenses against infection but also an important regulator in auto-immune diseases (Morzadec, Macoch, et al., 2012). Exposure to low doses of arsenic (< 0.1 mL/L) increased the numbers of CD4+ CD25+ FoxP3-expressing cells natural regulatory T (nTreg) cells in Swiss albino mice (S. Choudhury et al., 2016). In utero exposure to As has been reported to reduce CD4/CD8 ratios in offspring (Soto-Pena et al., 2006). In pre-school students in rural Bangladesh, As exposure appeared to cause reductions in cell-mediated immunity and in formation of T-helper (TH) Type-1 cytokines (Raqib et al., 2017).

B-cells mediate humoral immunity by producing and secreting antibodies. While there have been extensive studies observing the effect of arsenic on the function of T-cells, there have been very little observations on the effect arsenic has on B-cells. A study has shown that low concentrations of arsenic (0.1 to 2 μM), seemed to have little or no effect on *in vitro* CD40L-dependent Immunoglobulin M antibody-forming

cell response of human isolated naïve B lymphocytes however the same amount of arsenic significantly reduces the same responses in murine B cells (H. Lu, Crawford, North, Kaplan, & Kaminski, 2009). Arsenic and monomethylarsonous acid have also been known to reduce development of pre B-cells from hematopoietic stem cells both *in vivo* and *in vitro*. This is achieved by suppression of IL-7 signaling and ultimately resulting in suppressed humoral immunity in mice (Ezeh et al., 2014; Ezeh et al., 2016).

Studies evaluating antibody levels in As-exposed individuals yield conflicting results. One study observed no changes in serum IgM, IgA or IgG in 47 adult male workers exposed to As in a coal-burning power plant compared to 27 workers from another plant in the same district whose As coal content was > 10 times lower (Bencko, Wagner, Wagnerova, & Batora, 1988). It should be noted that exposure duration and internal As doses are unknown; thus, negative results could be due to acute or low-dose internal As levels. In contrast, Bangladeshi subjects (n = 125) chronically exposed to drinking water As demonstrated significantly elevated serum IgA, IgG and IgE compared to unexposed persons (Islam, Nabi, Rahman, & Zahid, 2007). IgG and IgE levels were significantly higher during initial stages of skin manifestations, and IgE continued to increase with prolonged exposure. Moreover, increased prevalence of respiratory complications including cough, chest sound, bronchitis and asthma were evident in exposed individuals, and mean serum IgE was higher in subjects with respiratory symptoms relative to exposed subjects without (Islam et al., 2007). Arsenic has been known to increase the levels of reactive oxygen (ROS) in several cell types including lymphocytes (J. Biswas et al., 2010) thus leading to DNA damage and ultimate death of the cells.

Macrophages and Dendritic Cells: Macrophages and dendritic cells are professional phagocytes that play an important role in bridging the innate and adaptive immune system. Monocytes are the precursor cell of both these population of cells. It is only when monocytes migrate to different tissues they differentiate into mature phagocytes. Macrophages engulf and digest microbes, cellular debris and cancer cells in a process that is known as phagocytosis. They also release different cytokines in response to different infections and tissue damage. Chronic arsenic exposure significantly hampers ex-vivo differentiation of peripheral blood monocytes into

mature macrophages. Studies have shown that monocyte-derived macrophages from individuals exposed to arsenic exhibited decreased cell adhesion capability, F-actin expression, nitric oxide production and phagocytic activity (N. Banerjee et al., 2009).

Arsenic has also been known to block the differentiation of human peripheral blood monocytes into functional macrophages by repressing NF-κB-related pathways (Lemarie et al., 2006). Treatment of human macrophages with low arsenic concentrations (1 to 5 μM, 6 h) resulted in decreased transcriptional activity of the liver X receptor which resulted in decreased expression of its target genes ABCA1 and SREBP-1c ultimately resulting in impaired cholesterol efflux (Padovani, Molina, & Mann, 2010). Prolonged treatment with arsenic resulted in macrophages being de-differentiate into CD14 CD14-expressing monocytic-like cells by reorganization of its actin skeleton (Lemarie, Bourdonnay, Morzadec, Fardel, & Vernhet, 2008). Arsenic exposed macrophages also showed increased expression of various pro-inflammatory cytokines like CXCL2 and CCL18 coupled with decreased expression of several macrophage specific markers like metalloproteinase 7, 9 and 12 (Bourdonnay et al., 2009; Lemarie et al., 2008). These macrophages also exhibited an enhanced pro-inflammatory response to the lipopolysaccharide (LPS) endotoxin.

Increased exposure to arsenic through contaminated drinking water have been shown to positively correlate with high CD14 expression in monocytes coupled with increased levels of pro inflammatory cytokines like TNF-α and IL-8 in plasma (Dutta, Prasad, & Sinha, 2015; Wu et al., 2003). Recent studies also indicate that arsenic hampers the function of microglia. They are the resident macrophages of the central nervous system (G. Chen et al., 2016; V. Singh, Mitra, Sharma, Gera, & Ghosh, 2014). There was an increase in the secretion of IL-6 and TNF-α from microglia isolated from mice that were exposed to 0.38 mg/kg arsenite for 7 days (V. Singh et al., 2014). *In vitro* experiments also showed that supernatant from arsenic-exposed-microglial cultures induced murine neuronal cell death (V. Singh et al., 2016). The death of these cells did not stem from increased inflammation but from critical cystine/glutamate imbalance induced by arsenic in the microglia. These observations could provide an insight into increased developmental neurotoxicity in children exposed to arsenic (Tsuji, Garry, Perez, & Chang, 2015).

Dendritic cells (DC) are the primary antigen presenting cells that link innata and adaptive immunity together. Once they are activated by infectious or tumor cell antigens, they migrate to the peripheral lymph nodes and activate T-cells leading to their polarization. Low concentrations of arsenic (0.1 to 0.5 μm), did not seem to block the ability of mature DCs to activate the proliferation of human T cells *in vitro* however it significantly inhibited the secretion of IL-12p70 and IL-23, the two cytokines which are known to play an important role in T-cell polarization (Macoch, Morzadec, Fardel, & Vernhet, 2013). High concentrations of arsenic (1 μM) induced monocyte necrosis and consequently impaired myeloid DC differentiation (Macoch et al., 2013). Exposure to arsenic also hampered phagocytic activity of immature DCs (Bahari & Salmani, 2017). These results have also been somehow substantiated epidemiologically where Langerhans cells, isolated from individuals chronically exposed to arsenic showed quantitative loses and structural lesions (C. H. Lee et al., 2012; B. J. Wang et al., 1991). While it is clear that arsenic negatively impacts DC mediated polarization of T-cells, there are no report on how arsenic modulates antigen presentation in dendritic cells.

Inhibition of Inflammasomes: Inflammasomes are multiprotein complexes in the cytoplasm that control the activation of the caspase 1-dependent cleavage of the pro-inflammatory cytokines IL-1β and IL-18 into their active forms. These complexes are formed in response to stimuli that can be pathogen derived (bacterial toxins), environmental contaminants (silica, asbestos) or any self-molecules. These are primarily generated in macrophages and are composed of nucleotide-binding oligomerization domain-like receptor (NLR) proteins that act as sensors (Howrylak & Nakahira, 2017). Arsenic inhibits the formation of NLRP1, NLRP3 and NAIP5/NLRC4 inflammasomes in murine bone marrow-derived macrophages (Maier, Crown, Liu, Leppla, & Moayeri, 2014). It reduces NLRP3-dependent inflammation in mice treated with monosodium urate crystals and also blocks the maturation of pro-IL-1β preventing the autoproteolytic and substrate cleavage activities of caspase-1 in murine macrophages. These observations suggest that arsenic directly hampers the activity of Inflammasomes in human macrophages thus constituting an important target for their toxicity.

As described, arsenic exerts immunotoxic effects on immune cells, which may

initially result in the suppression of major immune functions. arsenic decreases bacterial phagocytosis by macrophages, reduces T cell proliferation and markedly represses the secretion of pro-inflammatory cytokines by activated DC and T cells. These lead to an increase in pathogen susceptibility by limiting pathogen eradication. Arsenic-induced immunosuppression likely increases the incidence of lower respiratory tract infections and diarrhea, which frequently develop in young exposed children (A. Rahman, Vahter, Ekstrom, & Persson, 2011). Multiple mechanisms mediate arsenic induced immunotoxicity such as: ROS production, alteration of redox-sensitive signaling pathways, DNA damage, epigenetic effects and inflammasome inhibition. A complete picture of arsenic induced immune effects is illustrated in Figure 10 and Table 3.

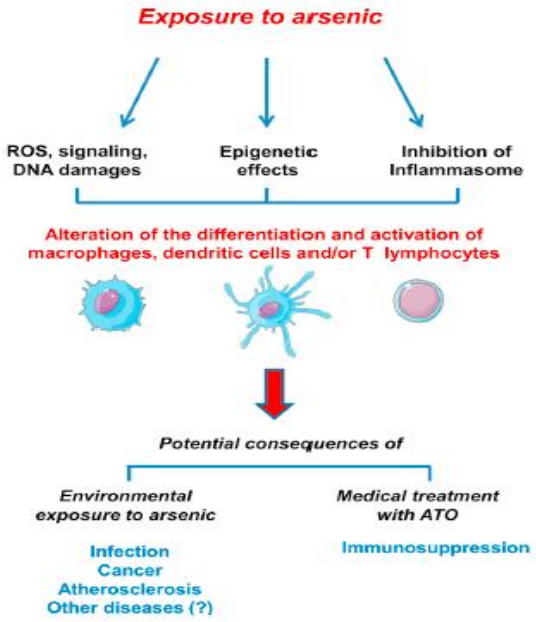


Figure 10: Effects of arsenic on immune cells and their potential consequences (Bellamri, Morzadec, Fardel, & Vernhet, 2018)

Immune parameter	Major findings	Study model	Description
Defense genes/ proteins	↓ MHC class II	Humans	PBMC mRNA
		Animals	Mouse macrophage surface expression
	↓ CD69	Humans	PBMC mRNA
		Human cells	PBMC surface expression
		Animal cells	Mouse SMC surface expression
	↓ IL-1β	Humans	PBMC mRNA
		Animals	Mouse lung mRNA & protein
			Zebrafish mRNA
	↑ CD14	Humans	PBMC mRNA & surface expression
		Human cells	Macrophage surface expression
	↓ TNF-α	Humans	PBMC mRNA
			PBMC secretion
		Animals	Zebrafish mRNA
			Rat PAM secretion
			Mouse lung fluid protein
Inflammation	↑ Expression of inflammatory mediators	Humans	↑ PBMC *IL1B*, *IL6*, *CCL2* & *CD14* mRNA in adults
			↑ PBMC CD14 surface expression & TNF-α secretion in adults
			↑ PBMC GM-CSF secretion in children
			↑ Placental & cord blood IL-1β, TNF-α and IFN-γ in neonates
		Human cells	↑ Macrophage mRNA & secretion of TNF-α & IL-8
Lymphocyte activation	↓ Stimulated proliferation	Humans	PBMC in adults
			PBMC in children
		Animals	Chicken SMC & PBMC
			Mouse SMC
			Catfish SMC
		Human cells	PBMC
		Animal cells	Mouse SMC
			Chicken SMC
	↓ Stimulated IL-2 secretion	Humans	PBMC in adults
			PBMC in children
		Animals	Mouse SMC
			Catfish SMC
		Human cells	PBMC
		Animal cells	Mouse SMC
			Chicken SMC
			Harbor seal 11B7501 lymphoma B-cells
Humoral immunity	↓ AFC response to antigen	Animals	Mouse SMC Rat SMC
		Animal cells	Mouse SMC

Immune parameter	Major findings	Study model	Description
Hypersensitivity reaction	↓ Response to cutaneous sensitization	Animals	↓ LC migration to lymph nodes & subsequent T-cell activation in mice Rats
			Chickens
Monocytes/ macrophages	↓ Number/survival	Humans	↓ Monocyte count
		Animals	↓ Mouse splenic macrophage count
			↓ Catfish HK macrophage count
			↑ Apoptosis of mouse splenic macrophages
		Human cells	↑ Apoptosis of blood monocytes & U937 promonocytic cells
	Impaired development	Human cells	↓ Differentiation of monocytes into macrophages
			Induced differentiation of macrophages into DC-like cells
	Diminished function	Humans	Cell rounding; ↓ adhesion/CD54 adhesion molecule, F-actin, $NO^{\cdot}$ production & phagocytosis; altered Rho A-ROCK signaling
		Animals	↓ Rat PAM stimulated TNF-α secretion
			↓ Mouse peritoneal macrophage $NO^{\cdot}$ & $O_2^{\cdot}$ production
			↓ Mouse splenic macrophage adhesion, chemotactic index, phagocytosis, $NO^{\cdot}$ production, MHC class II surface expression & antigen presentation
			↓ Chicken SMC & PBMC $NO^{\cdot}$ production
			↓ Molluscan haemocyte phagocytosis & $NO^{\cdot}$ production
		Human cells	Cell rounding; ↓ adhesion & macrophage-specific markers; reorganized F-actin cytoskeleton resembling that of monocytes; ↑ monocytic marker CD14; ↓ endocytosis & phagocytosis via activated Rho A-ROCK signaling
Survival	↑ Induction of apoptosis	Humans	PBMC in adults
			↑ PBMC *BCL2L1* & *CASP2* mRNA in adults
			PBMC in children
			↑ CBMC *CASP2* mRNA in neonates
		Animals	Mouse splenic macrophages
		Human cells	Blood monocytes & U937 promonocytic cells
			PBMC
			B-cells, T-cells, macrophages & neutrophils
		Animal cells	Mouse TA3 antigen-presenting B-cells
			Rat T-cells
ROS production	Induced oxidative stress	Humans	↑ Serum SOD & PBMC MDA in adults
			↑ Basal PBMC/monocyte $NO^{\cdot}$ & $O_2^{\cdot}$ in children
			↑ Placental 8-oxoguanine in neonates
			↑ Cord blood 8-hydroxy-2′-deoxyguanosine in neonates
	↓ Stimulated ROS production	Humans	↓ Macrophage $NO^{\cdot}$ in adults
			↓ Monocyte $NO^{\cdot}$ & $O_2^{\cdot}$ in children
		Animals	↓ Mouse peritoneal macrophage $NO^{\cdot}$ & $O_2^{\cdot}$
			↓ Mouse splenic macrophage $NO^{\cdot}$
			↓ Chicken SMC & PBMC $NO^{\cdot}$
			↓ Molluscan haemocyte $NO^{\cdot}$
			Zebrafish embryos & larvae

Immune parameter	Major findings	Study model	Description
Microbial challenge	↓ Clearance of pathogens	Animals	↑ Viral & bacterial loads in zebrafish embryos and larvae
			↑ Pathogen colonization & ulcers/septicemia following bacterial infection in catfish
			↓ Splenic clearance of *S. aureus* in mice
			↑ Morbidity & respiratory viral titers following H1N1 viral infection in mice
Pulmonary health	Altered lung features	Humans	Altered airway protein expression in adults
		Animals	Altered mouse airway protein expression
			↓ Rat PAM stimulated TNF-α secretion
			↓ Mouse lung expression of genes involved in cell adhesion/migration
			↓ Killifish gill chloride secretion via ↑ CFTR degradation
		Human cells	↓ CFBE41o- AE cell chloride secretion via ↑ CFTR degradation
			↓ 16HBE14o- bronchial epithelial cell migration and wound repair
		Animal cells	↓ Rat PAM stimulated TNF-α secretion & $NO^{\cdot}$ & $O_2^{\cdot}$ production
	↑ Risk of infection/ disease	Humans	↑ RTI & tuberculosis in adults
			↓ Lung function; ↑ prevalence/mortality from lung cancer and non-malignant lung disease, including bronchitis & bronchiectasis in adults
			↑ RTI in infants
		Animals	↑ Morbidity & respiratory viral titers following H1N1 infection in mice

Note: all cells are primary cells unless otherwise stated. Subjects of human studies are adults unless otherwise stated.
↓ decreased; ↑ increased.

Table 3: Major Arsenic associated effects on immunity summarized (Dangleben et al., 2013)

Prenatal Exposure to Arsenic:

As arsenic is reported to cross the placenta, the fetus is particularly vulnerable to toxic insult in utero (Concha, Vogler, Lezcano, Nermell, & Vahter, 1998; Davis et al., 2015). There are very few studies that delve into the effect of arsenic on development of fetus hence thus the relationship between arsenic and developmental anomalies in humans is not well established. Studies in animals show that rats receiving doses of 8 mg of As_2O_3 from 14 days prior to mating showed no discernible changes in their reproductive activity which was measured in terms of mating index, a fertility index and the pre-coital interval (time before mating) index (Holson, Stump, Ulrich, & Farr, 1999). Another comprehensive study observed the effects of three generation intake of arsenic in drinking water in mice. The study observed small litters and a trend toward a smaller number of pups per litter in each of the three generations that were exposed to arsenic (Schroeder & Mitchener, 1971). These changes in reproductive capability maybe due to several changes in the reproductive system including reduced weights of ovary and uterus, reduced ovarian and uterine peroxidase activities,

inhibition of steroidogenic enzymes and decreased estradiol levels relative to the controls (Chattopadhyay, Ghosh, Debnath, & Ghosh, 2001; Chattopadhyay, Pal Ghosh, Ghosh, & Debnath, 2003).

In humans there have been reports of increased frequency of stillbirth among pregnant women who have been exposed to arsenic chronically in contaminated drinking water (Cherry, Shaikh, McDonald, & Chowdhury, 2008; von Ehrenstein et al., 2006). Another study in Bangladesh finds strong association of arsenic exposure (range 0-1710 μg/L) with spontaneous abortion (Milton et al., 2005). A study in an endemic area in Northeastern Taiwan found positive correlation between ingestion of arsenic through contaminated drinking water (range 0.15−3585 μg/L) and low birth weight (C. Y. Yang et al., 2003). However, in contrast there were several other studies that found no correlation between arsenic and low birth weight or even frequency of stillbirth (Kwok, Kaufmann, & Jakariya, 2006; Myers et al., 2010).

Immunotoxic effects of arsenic in early life have been widely-studied in human and animal models. For instance, immunosuppression due to prenatal As exposure was identified (i.e., poor cellular immune function reported among cord blood cells) in human cohorts (Nadeau et al., 2014). Impaired function of T-cell subsets has also shown to be associated with prenatal As exposure. The presence of maternal cells in the cord blood limits the observation to be conclusive on immunosuppression in early childhood. The prevalence of infectious diseases has been reported to be increased dramatically in As-impacted regions of the world (Ferrario, Gribaldo, & Hartung, 2016; Gera et al., 2017).

Chronic low-level As exposure has been reported to increase host risk of lung infections, influenza, diarrhea, etc. (Farzan et al., 2016; A. Rahman et al., 2011; Raqib et al., 2009; P. M. Smith et al., 2013; Z. D. Smith et al., 2012), especially among children. While in many cases these outcomes might be associated with ongoing As exposure of the children, it should be recalled that As exposure can affect fetal development (Attreed, Navas-Acien, & Heaney, 2017; Kippler, Skroder, Rahman, Tofail, & Vahter, 2016; M. Vahter, 2008) and even cause long-term epigenetic re-programming of the fetus (Bailey & Fry, 2014). One epidemiological study showed an association between in utero As exposure and development of adult diseases

(Young, Cai, & States, 2018). If one of the results of these changes was alterations in child immunocompetence, this might mean these hosts could be impacted even if they were no longer exposed to As in their environments. Prenatal Arsenic effects are illustrated in Figure 11.

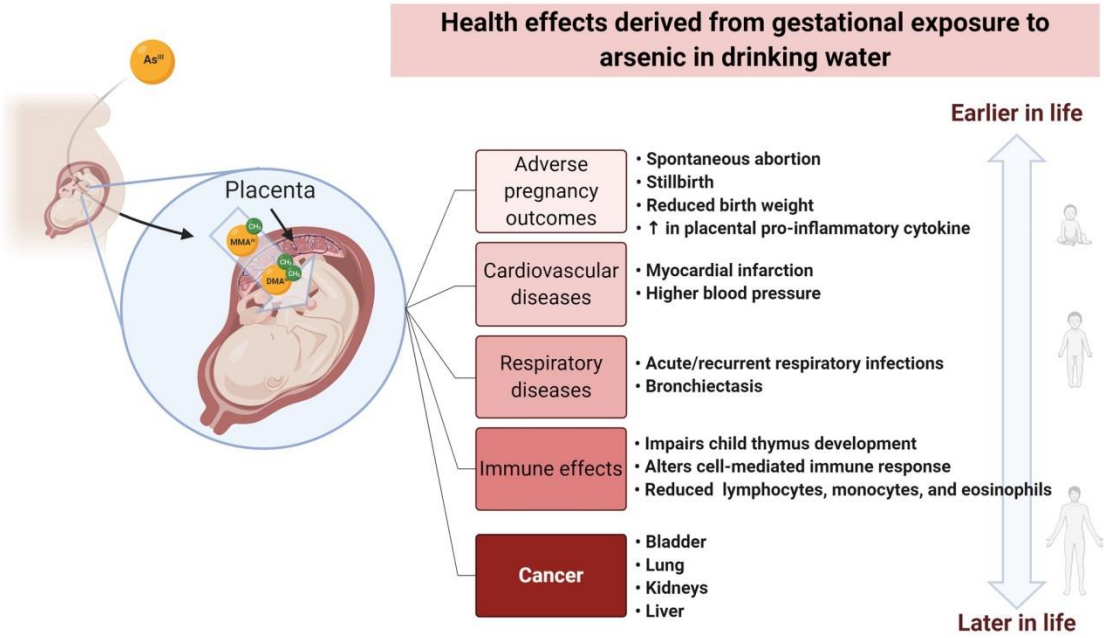


Figure 11: Health effects derived from prenatal exposure to arsenic in drinking water. (Martinez & Lam, 2021)

Arsenic and the Gut Microbiome:

Gut microbiota are the microorganisms that are present in the digestive tract of animals including humans. They include bacteria, archaea, fungi, and viruses (P. Engel & Moran, 2013; Moszak, Szulinska, & Bogdanski, 2020). The gut microbiota has far reaching implications in human health that includes resistance to pathogens, maintaining the intestinal epithelium, metabolizing dietary and pharmaceutical compounds and even controlling the behavior through the gut-brain axis. The composition of the gut microbiota varies between regions of the digestive tract with the colon having the highest density representing between 300 and 1000 different species (Guarner & Malagelada, 2003). Bacteria are the most studied component of the gut microbiota and 99% of gut bacteria come from only about 30 or 40 species (Beaugerie & Petit, 2004).

In humans the gut microbiome establishes and flourishes alongside the intestinal epithelium and intestinal barrier (Faderl, Noti, Corazza, & Mueller, 2015; Sommer & Backhed, 2013). The relationship between the host and gut microbiota ranges from commensal to highly mutualistic. Many microorganisms in the gut ferment dietary fibers to produce short chain fatty acids (SCFA's) like acetic acid and butyric acid which are then absorbed by the host (Clarke et al., 2014; Quigley, 2013). They also play a vital role in synthesizing vitamin B and vitamin K as well as metabolizing bile acids, sterols, and xenobiotics (Clarke et al., 2014). Dysbiosis of gut flora has been linked with many (Shen & Wong, 2016). The composition of the human gut microbiota changes over time and depends heavily on diet and overall health (Quigley, 2013; Shen & Wong, 2016).

The most common bacterial phyla in the in the human gut are *Firmicutes*, *Bacteroidota, Actinomycetota,* and *Pseudomonadota* (Khanna & Tosh, 2014). Gut microbiome composition depends on the geographic origin of populations (Yatsunenko et al., 2012). The gut flora plays an active role defending against pathogens by fully colonising the space, making use of all available nutrients, and by secreting compounds that kill or inhibit unwelcome organisms (Yoon, Lee, & Yoon, 2014). The gut microbiome also helps the host to digest carbohydrates because have enzymes that human cells lack for breaking down certain polysaccharides (Clarke et al., 2014). Bacteria turn carbohydrates into short-chain fatty acids by fermentation to produce short chain fatty acids like acetic acid, propionic acid and butyric acid which are then used by host cells, providing a major source of energy and nutrients (Beaugerie & Petit, 2004). Gut microbiota can also metabolize other xenobiotics such as drugs, phytochemicals, and food toxicants (Laukens, Brinkman, Raes, De Vos, & Vandenabeele, 2016; Sousa et al., 2008). Roles of gut microbiome are illustrated in Figure 12

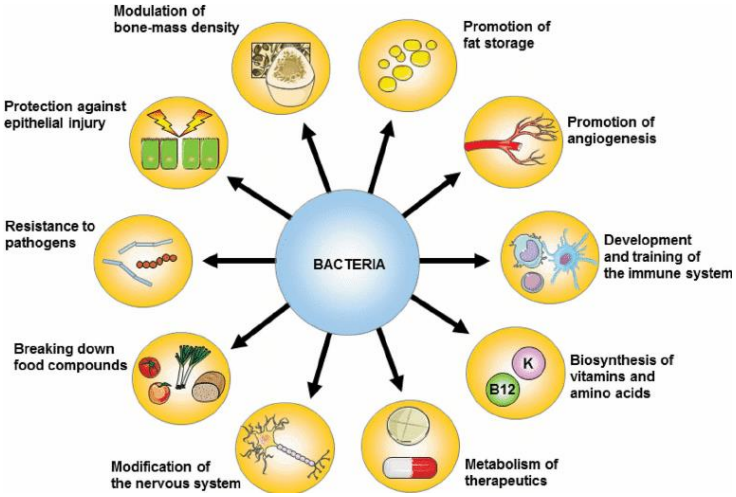


Figure 12: Important roles of Gut Microbiome, (Laukens et al, 2008)

There have been recent studies that studied the effect of arsenic on the gut microbiome (Brabec et al., 2020; Chi et al., 2017). Contaminated drinking water and food are the major sources of arsenic in both humans and animals, and the gastrointestinal tract is therefore nearly always the first location at which arsenic exposure and its biotransformation occur. Many previous studies have also demonstrated that presystemic metabolism of arsenic in the gut by gut bacteria changes the pattern of arsenic species and altered both the bioaccessibility and the toxicity of arsenic (Laird et al., 2007; Van de Wiele et al., 2010).

Much of what is known about arsenic-microbe interactions comes from environmental microbiology and in ecosystems such as soil and the subsurface, where microbial metabolisms are the primary determinants of arsenic speciation, mobility, and toxicity. Many of the same principles used in these environmental microbiology studies of arsenic can be directly applied to understand arsenic interactions with the human microbiome. Arsenic-microbiome interactions can have three general and sometimes overlapping outcomes: no noticeable effect, perturbation of microbiome taxonomic structure and function, and alteration of the pharmacological and/or toxicological properties of the toxicants (Naranmandura, Rehman, Le, & Thomas, 2013). More than a few studies argue that host metabolism is as important or perhaps more important than microbiome metabolism with respect to biotransformation and toxicity of arsenic (Bu et al., 2011; Nemeti, Anderson, & Gregus, 2012). The microbiome clearly has the potential to alter host arsenic metabolism and disease outcomes in mice. Thus, future research is needed to quantify the net influence of

potentially important mechanisms for arsenic detoxification in the human body. The microbiome should be a target for arsenicosis prevention and treatment strategies.

The development of the infant microbiome is influenced by several factors like fetal life, birth and early feeding (Dogra et al., 2015; Kerr et al., 2015; C. Y. Lu & Ni, 2015; Meropol & Edwards, 2015). Hence, it is important to understand how prenatal exposure to arsenic shapes the neonatal microbiome. Thus a deeper understanding how arsenic shapes the microbiome by in utero exposure is necessary to unearth the development of gastrointestinal health and diseases in chronically exposed populations. Given the scale and scope of global arsenicosis, probiotics with active arsenic metabolisms capable of mitigating arsenic toxicity may represent feasible, low-cost therapeutics. Figure 13 illustrates potential interactions of arsenic with the gut microbiome.

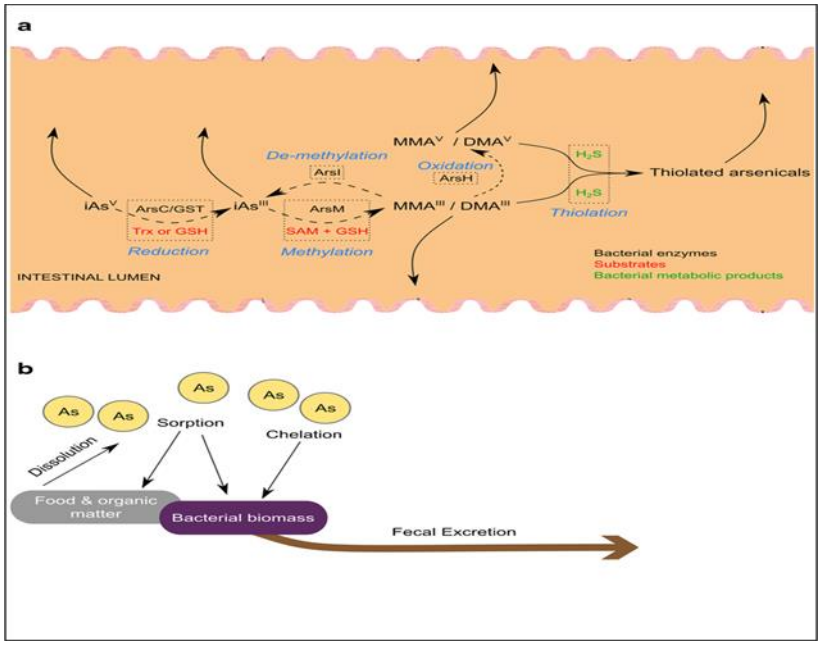


Figure 13: (A) Overview of supported and potential arsenic-microbiome interactions in the mammalian gut. (B) The overall fate of arsenic in the gut, (Coryell, Roggenbeck, & Walk, 2019)

Arsenic and intestinal barrier function:

Apart from the microbiome, another rapidly emerging marker of gut barrier function and eventual gastrointestinal health is intestinal permeability (Assimakopoulos, Triantos, Maroulis, & Gogos, 2018). Intestinal barrier is the property of the intestinal mucosa that ensures adequate containment of undesirable luminal contents within the intestine while preserving the ability to absorb nutrients. It provides separation between the body and the gut and prevents the uncontrolled translocation of luminal contents into the body. It provides protection to the mucosal tissues and the circulatory system from microorganisms, toxins, and antigens (S. H. Lee, 2015; Sanchez de Medina, Romero-Calvo, Mascaraque, & Martinez-Augustin, 2014; Turner, 2009).

The intestinal mucosal barrier is composed of physical, biochemical, and immune elements. The central component is the intestinal epithelial layer, which provides physical separation between the lumen and the body. It secretes various molecules into the lumen that reinforces the barrier function (De Santis, Cavalcanti, Mastronardi, Jirillo, & Chieppa, 2015; Yan, Yang, & Tang, 2013). Mucus is a form of highly glycosylated hydrated gel formed by mucin molecules which is secreted by goblet cells. The mucus allows small molecules to pass, while prevents large particles from contacting the epithelial cell layer (Marquez, Fernandez Gutierrez del Alamo, & Giron-Gonzalez, 2016). The bile produced by the liver to aid in the digestion of lipids has bactericidal properties (Marquez et al., 2016). Paneth cells secrete abundant quantities human α-defensins, lysozyme, Reg3γ which is an antibacterial lectin and various other antimicrobial peptides into the intestinal lumen (Jager, Stange, & Wehkamp, 2013; Marquez et al., 2016; Ouellette, 2011). A brief overview of the intestinal barrier is illustrated in Figure 14.

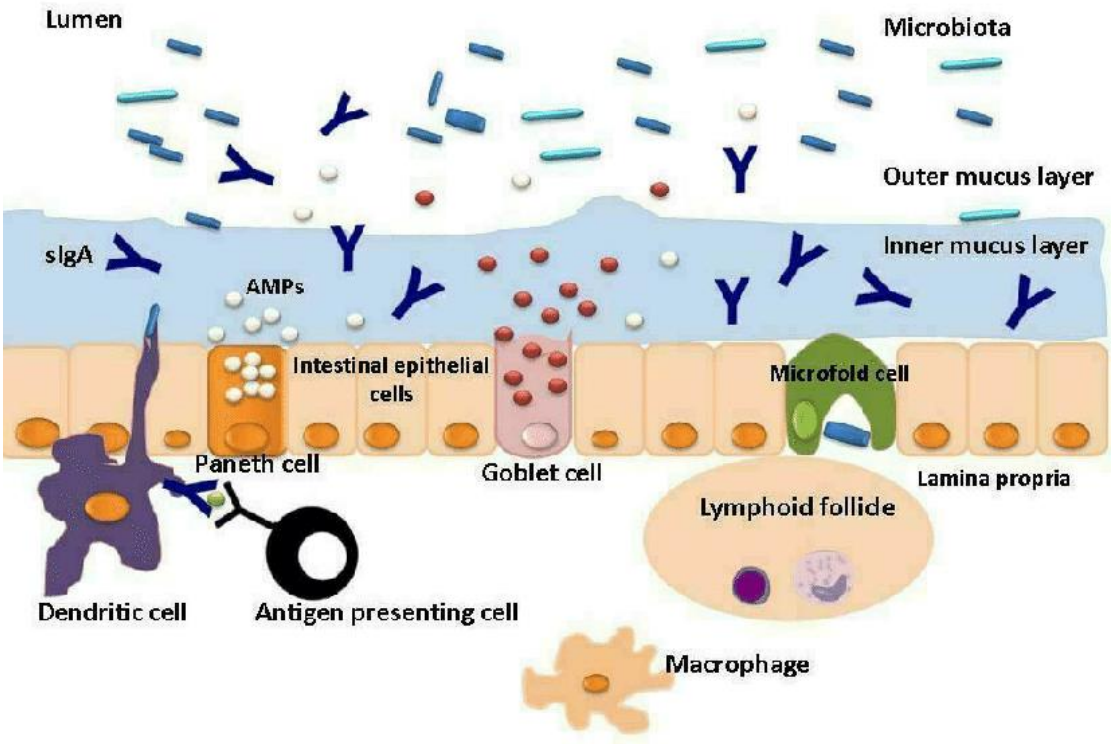


Figure 14: A brief overview of intestinal barrier, (Gerova, 2016)

The foremost component of the intestinal barrier is the intestinal epithelium. It consists of the layer of epithelial cells lining the intestine. It consists of the layer of epithelial cells lining the intestine. The space between the adjacent cells is maintained by junctional complexes formed by protein connections elaborated by each individual cell (Turner, 2009). The most important junctional complex are the tight junctions. These junctions prevent leakage of solutes and water and seals between the epithelial cells. Tight junctions are present mostly in vertebrates (with the exception of Tunicates (S. Banerjee, Sousa, & Bhat, 2006). Tight junctions are composed of network of sealing strands, each strand acting independently from the others. Each strand is formed by a row of trans-membrane proteins embedded in both plasma membranes, with extracellular domains joining one another directly. There are at least 40 different proteins composing the tight junctions (Anderson & Van Itallie, 2009). The three major proteins are: occludin, claudins, and junction adhesion molecule (JAM) proteins. These proteins then associate with another peripheral membrane protein such as ZO-1 located on the intracellular side of plasma membrane, which anchor the strands to the actin component of the cytoskeleton (Anderson & Van Itallie, 2009).

Occludin was the first integral membrane protein to be identified. It consists of four transmembrane domains and both the N-terminus and the C-terminus of the protein are intracellular. It forms two extracellular loops and one intracellular loop. These loops help regulate paracellular permeability. It plays vital role in barrier function and cellular structure. (W. Y. Liu, Wang, Zhang, Wei, & Li, 2012). Claudins are a family of over 27 different members in mammals (Schneeberger & Lynch, 2004). They have a structure similar to occludin play a significant role in the tight junction's ability to seal the paracellular space (Mitic, Van Itallie, & Anderson, 2000). Junctional Adhesion Molecules (JAM) are part of the immunoglobulin superfamily. They only have one transmembrane domain instead of four. JAM's are involved in helping to maintain cell polarity (Luissint, Artus, Glacial, Ganeshamoorthy, & Couraud, 2012). Tight junctions in cells are depicted in Figure 15.

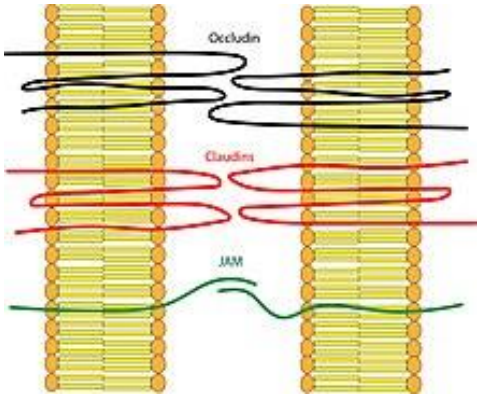


Figure 15: Depiction of the transmembrane proteins that make up tight junctions (Sanchez de Medina et al., 2014)

As the main route through which inorganic Arsenic enters the body is via oral exposure, the gastrointestinal tract becomes susceptible to any changes induced by the metalloid. There have been reports of gastroenteritis and dyspepsia symptoms in populations chronically exposed to arsenic (Borgono, Vicent, Venturino, & Infante, 1977; Majumdar, Guha Mazumder, Ghose, Ghose, & Lahiri, 2009). Various in vitro studies with colon epithelial cell lines have shown that acute as well as sub-chronic exposure to arsenic results in increasing oxidative stress and pro-inflammatory response in addition to disruption of proteins that form tight junctions in epithelial cells. (Calatayud, Devesa, & Velez, 2013; Calatayud, Gimeno-Alcaniz, Velez, & Devesa, 2014; Chiocchetti, Velez, & Devesa, 2019; Jeong, Seok, Petriello, & Han,

2017), while in vivo exposure to arsenic has also contributed to increased intestinal permeability (Chiocchetti, Domene, et al., 2019). Hence, it is evident from existing literature that de facto exposure to arsenic affects the gut microbiome and modulates intestinal permeability by disrupting tight junction proteins; however, there is a dearth of information in as to what may happen due *to in utero* exposure to Arsenic.

Materials and Methods

1. Chemicals and Reagents:

Cell culture reagents were purchased from Thermo Fisher Scientific (Waltham, MA) and Sigma (St. Louis, MO). Arsenic trioxide (As_2O_3) was purchased from MP Biomedicals (Irvine, CA). The As(III) standard (1000 mg/L) was prepared by dissolving 1.320 g of As_2O_3 in 25 mL of KOH (MP Biomedicals) at 20% (m/v),neutralizing with 20% H_2SO_4 (v/v) (Merck) and making up to a final volume of 1 L with 1%H_2SO_4 (v/v). The ELISA kits used for analyses of TNF-α, IL-12, IFN-γ and IL-2 were purchased from BD Biosciences (San Jose, CA). PerCP (peridinin-chlorophyll-protein)-conjugated-anti-CD4, APC (allophycocyanin)-anti-CD8, FITC (fluorescein isothiocyanate)-anti-CD25, APC-anti-CD44, FITC-anti-CD69 and FITC-anti-CD11b were each purchased from eBiosciences (San Diego, CA). Easy Sep CD4 T-cell enrichment kits were bought from Stem cell Technologies (Vancouver, Canada).FITC-Dextran, MTT, RPMI, DMEM and FBS and other cell culture reagents were obtained from Sigma. BCA protein assay kit was purchased from Thermofisher (Waltham, MA, USA). Penicillin, streptomycin, Triton X100, PMSF, leupeptin, glycine, acrylamide, bis-acrylamide para-formaldehyde, sodium butyrate, sodium propionate, sodium acetate, Hoechst 33342, were purchased from Sigma (St. Louis, MO, USA). PVDF membrane, Trizol, was purchased from Invitrogen (Carlsbad, CA, USA). Super Reverse Transcriptase MuLV Kit, RT2 SYBR® Green qPCR Mastermix were purchased from Qiagen (Hilden, Germany). Ripa lysis buffer, Occludin (rabbit, polyclonal) were purchased from Abcam (Cambridge, UK). Anti-GAPDH antibody (rabbit polyclonal) was purchased from Bio-Bharati (Kolkata, India). All primers were purchased from IDT (Lowa, USA). MiR122 expression plasmid was a kind gift from Dr. Suvendranath Bhattacharya (CSIR-IICB).

2. Animals:

Male and female BALB/c and C57BL/6 mice (all 6 weeks of age) were procured from the ICMR-National Institute of Cholera and Enteric Diseases (ICMR-NICED; Kolkata, India). All mice were housed in cages containing straw bedding that were maintained in pathogen-free facilities maintained at 24°C with a 50% relative humidity and a 12-hr light:dark cycle. All mice had ad libitum access to standard rodent chow. After 2 weeks of acclimatization, the BALB/c (H-2d) mice were bred by housing two females with a male. The breeding trios were given ad libitum access to

drinking water containing 4 ppm arsenic trioxide (As_2O_3). The As-containing water was changed twice weekly. After birth, the mothers were then given ad libitum access to clean As-free water, while one group of juveniles received rodent chow supplemented with Sodium Butyrate for the fourth week. For the experiments, when pups reached 4 week-of-age, groups were randomly collected, and processed for biomaterials. Age-matched juvenile mice whose mothers were never exposed to Arsenic were processed in parallel as controls. For each experiment, 5-6 juvenile mice randomly chosen without any sex bias were evaluated. For direct exposure, male BALB/c mice were divided into three groups with each group containing 5 animals. Two of the groups were given 4 ppm and 10 ppm of Arsenic Trioxide via drinking water for a period of 30 days after which the animals were sacrificed and they were randomly collected and processed for biomaterials.

All protocols for these studies were approved by the Institutional Animal Ethics Committee of ICMR-NICED. All experiments were done in accordance with the guidelines set by the committee for the purpose of control and supervision of experiments on animals (CPCSEA), Ministry of Environment and Forests (Government of India), New Delhi, India.

3. Cell culture and Treatment:

Murine macrophage cell line RAW 264.7 obtained from National Centre for Cell Science (NCCS), Pune, India and human colon carcinoma cell line HT-29 cells which were a kind gift from Dr. Amit Pal (ICMR-NICED, India) were used for in vitro experiments. The cells were maintained in 25 cm2 flasks to which 10 mL of Dulbecco's Modified Eagle Medium (DMEM) with high glucose (4.5 g/L) and L-glutamine (0.6 g/L) was added. DMEM was supplemented with 10% (v/v) of fetal bovine serum (FBS), 1% (v/v) of non-essential amino acids, 1 mM of sodium pyruvate, 10 mM of HEPES (N-2-hydroxyethylpiperazine-N¢-2-ethanesulfonic acid), 100 U/mL of penicillin, 0.1 mg/mL of streptomycin. The cells were incubated at 37 °C in an atmosphere with 95% relative humidity and a CO_2 flow of 5%. The medium was changed every 2-3 days. When the cells reached 80% confluence, the cells were detached with a solution of trypsin (0.5 g/L) and EDTA (ethylenediaminetetraacetic acid, 0.2 g/L) (HyClone). The assays were performed with cultures between passages 5 and 25. The cells were treated with either As (III) or SCFA's prepared in Hanks

Balanced Salt Solution (HBSS). The treatment was kept for 24 hours. For LPS treatment the cells were incubated with LPS for 24 hours before the exposure to arsenic.

4. Cell viability and cytotoxicity analysis by MTT:

RAW 264.7 cells were treated with varying concentrations of Arsenic Trioxide and incubated for 24 hours at 37 °C in an atmosphere with 95% relative humidity and a CO2 flow of 5%. The viability of cells after concentration was determined using the standard MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. All the treatments were done using 2×104 cells/well in 48 wells plate. The purple formazan crystals were dissolved in DMSO, transferred in a 96 well plate (100 µL/well), and the absorbance was recorded on a microplate reader at a wavelength of 570nm.

5. Histopathologic examination of offspring spleen, thymus, colon and MLN:

The animals after their experimental duration were sacrificed and after their body weights were recorded, five mice in each group were selected, blood was drawn from their tail vein, and then they were euthanized by cervical dislocation. At necropsy, both macroscopic observations and weights of the spleen and thymus from each mouse were taken. Each organ was then fixed in 4% paraformaldehyde for 48 h at 4ºC. The fixed tissues were then dehydrated through graded alcohols, embedded in paraffin, and routine microtomy then carried out to generate 5-mM sections. The sections, in turn, were stained with hematoxylin and eosin for later microscopic examination.

6. Analysis of serum levels of IgG1, IgG2a and IgG3:

Each collected tail vein blood sample was allowed to stand for 3 h at room temperature and then serum was isolated by centrifugation at 1800 rpm. Serum samples were then analyzed to determine total IgG1, IgG2a and IgG3 titers. For this, an aliquot of each sample was diluted 1:10, 1:100, and 1:1000 with PBS (phosphate buffered saline, pH 7.4) þ 10% FBS solution. For each paired sets of diluted sample, an aliquot of the diluted serum was added to a 96-well plate whose wells had been pre-coated with biotin conjugated mouse anti-mouse IgG1 or mouse anti-mouse

IgG2a or anti-mouse IgG3. The plates were then incubated for 1 h at room temperature before unbound serum components were removed by repeated washings with PBS (and centrifugation). Detection reagent (avidin- conjugated horseradish peroxidase) was then added to each well and the plates were incubated a further 1 h. The absorbance in each tube was then evaluated at 450nm in an iMarkTM Microplate Absorbance Reader (BioRad, Hercules, CA). Results were reported in terms of mean OD values. Each mouse sample was analyzed in triplicate.

7. Isolation of peritoneal exudate macrophage cells (PEC/Mϕ):

BALB/c and C57BL/6 mice were each injected intraperitoneally (IP) once with 3 ml of 4% (w/v) starch solution in water. After 48 hr, Mϕ were isolated by peritoneal lavage, pelleted, re-suspended in RPMI medium supplemented with 10% Fetal Bovine Serum (FBS), 100 U penicillin/ml, and 100 μg streptomycin/ml, all from Gibco (Waltham, MA), and seeded into 24-well plates at 5 x 10^4 cells/ml (0.5 ml/well) (Zhang et al. 2008). The cells were then cultured for 48 hr at 37ºC in a humidified 5% CO_2 incubator to dampen the effect of starch. Non-adherent cell populations were removed thereafter by gentle washing in serum-free medium.

8. Ex vivo Mϕ phagocytic activity:

Mϕ isolated from each mouse were seeded into 96-well plates at 2.5 x 10^5 cells per/well in complete RPMI-1640. The cells were allowed to adhere overnight at 37°C and then non-adherent cells were removed by washing with RPMI-1640. Thereafter, sheep red blood cells (sRBC; source, city, state) that had been opsonized with IgG (REF) were added to the wells to yield a 5:1 ratio of RBC:Mϕ. Phagocytosis was allowed to proceed at both 37°C and 4ºC for 0.5, 1, 2, and 4 hr. The 4°C scenario was employed to create a "zero-ingestion" control. At the end of each period, non-ingested RBC in the wells were lysed by addition of a hypotonic solution of 0.2% NaCl and incubation at 37°C for 3 min.

9. Analysis of cytokines by ELISA:

RAW 264.7 cells and Mϕ from each mouse were plated (in triplicate) in 96-well plates at 5 x 10^5 cells/well. After allowing cells to adhere by incubation at 37°C, the cultures then received an addition of LPS (10 μg/ml) or medium only. Thereafter, the cells were incubated for 48 hr at 37°C in the 5% CO_2 incubator. The supernatant from

each well was then collected and ultimately, ELISAs performed to assess culture levels of IL-2, IL-12, IL-4 and TNFα. All samples were analyzed in triplicate.

10. Flow cytometric analysis of T-cell, B-cell and NK cell population in spleen and MLN:

10^6 splenocytes and lymphocytes from Mesenteric Lymph nodes (MLN) were stained in PBS with Per-CP-conjugated anti-CD4 and APC-conjugated anti-CD8 monoclonal antibodies, Percp-conjugated anti CD3, PE-conjugated anti CD19 and APC conjugated- anti NK 1.1 (1:500 dilutions). All the set of cells were analyzed in FACS Aria II. Data of ten thousand events were collected and analyzed using BD-FACSDiva software.

11. Analysis of T cell activation markers:

$CD4^+$ T cells were isolated from whole splenocytes using Easy Sep $CD4^+$ T cell enrichment kit from each group of BALB/c mice. The allogenic Mϕ were cultured with above purified $CD4^+$ T cells from exposed mice at a ratio of 1:5 respectively. As a control $CD4^+$ T cells was co-cultured with normal allogenic Mϕ. After 24 h incubation, the cells were analyzed for CD44 and CD69 expression by flow cytometry in BD FACS Aria II. In another set of the experiment was carried out after 72 h for CD25 expression by flow cytometry and the supernatant was collected for cytokine ELISA. CD69 is the early activation n marker and diminish within 48 h while CD25 is the late activator via TCR and last for 4-5 days. The FACS data was analyzed by FACS Diva and FCS express 6 (Han et al 2009).

12. Host resistance to infection:

To assess the impact of the prenatal As exposures on the intact host immune system, additional sets of control and prenatal As-exposed mice (n =3/group) were injected IP with a potent strain of Escherichia coli and then levels of the bacteria were assessed in several immunologically-active sites in the body (i.e., spleen, liver, peritoneal cavity). Prior to the injection, E. coli strain E14 was grown overnight in Luria Bertani broth at 37°C. After determining the concentration of bacteria by standard plate count method, the bacteria were washed by centrifugation and then re-suspended in PBS for injections. Specifically, each mouse was IP-injected with 10^8 *E. coli* in 100 µl PBS. At 24 hr post infection, each mouse was euthanized by cervical dislocation.

Thereafter, their peritoneal cavity was rinsed with PBS (2 ml) that was in turn collected and placed on ice; thereafter, the liver and spleen were also aseptically removed and then homogenized in PBS using a tissue homogenizer. Aliquots of the homogenates and of the peritoneal wash (20 µl) were then plated onto LB agar plates to estimate bacterial load. Colonies were counted after overnight incubation at 37ºC; all data are reported in terms of absolute counting colony forming units (CFU). To assess postnatal effect of arsenic exposure control and As-exposed pups were injected IP with an enteric pathogen of *Shigella Flexneri*. Specifically, each mouse was IP-injected with 10^8 CFU in 100 µl PBS. At 24 hr post infection, each mouse was euthanized by cervical dislocation. Thereafter the colon was isolated and then homogenized in PBS using a tissue homogenizer. Aliquots of the homogenates were then plated in SS agar plates to enumerate bacterial load. Colonies were counted after overnight incubation at 37ºC; all data are reported in terms of absolute counting colony forming units (CFU).

13. Evaluation of changes in the gut microbiome:

For extraction of fecal DNA, fecal pellets were incubated for 24 hr at 56°C with proteinase K. DNA was then isolated using QIAamp DNA Mini Kits (Qiagen) using ~25 mg of feces. Fecal pellets were collected from juvenile mice (irrespective of sex) from both the control and prenatally Arsenic exposed group. Bacterial genomic DNA was extracted using QIAamp Fast DNA Stool Mini Kit (Qiagen, Valencia, CA, United States) by following the manufacturer's protocol. DNA concentration was evaluated by NanoDrop spectrophotometer from Thermo Fisher Scientific (Waltham, MA). Illumina standardized V3-V4 regions of 16s rRNA library protocol were employed for the preparation of the library. The library which was generated contained V3-V4 amplicons were then sequenced on an Illumina MiSeq platform following the manufacturer's protocol. The generated reads (data) were processed by using QIIME2 (Bolyen et al. 2019) (version 2021.8.0). Filtering, merging and denoising was carried out by using DADA2 (Callahan et al. 2016) plugin within QIIME2. RESCRIPt (Robeson et al. 2021) plugin was used for processing and filtering of SILVA 138.1 (Quast et al. 2013) database to make it compatible with QIIME2 for carrying out taxonomy assignment. V3-V4 region primers were used for trimming SILVA sequencing and classify-sklearn (Pedregosa et al 2011) was used for taxonomical classification. Further, the biome and taxonomy table was exported and

diversity analyses were carried out by using phyloseq (McMurdie and Holmes 2013) in R Data. Alpha diversity and rarefaction curve were studied on samples (biom table) rarefy to a depth of 10,952 reads per sample. Samples generated during this experiment were submitted to Sequence Read Archives (SRA) of National Centre for Biotechnology Information (NCBI) under accession numbers (Control1=SRR19309430, Control 2= SRR19309429, pAs1= SRR19309428 and pAs2=SRR19309427). The raw data is available in bioproject_ accession PRJNA839617.

14. Estimation of fecal SCFA by GC/MS:

The faecal concentrations of butyrate were measured by GC/MS as previously described (Li et al. 2020). Briefly, 50 mg of faecal samples from both groups were homogenized in 200 µl of distilled water. The samples were then centrifuges at 4000 rpm for 5 minutes and the resulting supernatant was collected. 200 µL of a benzyl alcohol–pyridine mixture (3:2) and 100 µL DMSO was added to the supernatant and the mixture was vortexed for 5 seconds. 100 µl of benzyl chloroformate was added very carefully and the tube lids were kept open for 1 minute to release the gas formed during the reaction. The tube lids were then closed and the resulting mixture was vortexed for 3 minutes. After the derivatization, 200 µl of cyclohexane was added and the resulting mixture was vortexed for 1 minute which was then followed by centrifugation at 4000 rpm for 5 minutes. 100 µl of the resulting derivative extract (upper cyclohexane layer) was isolated and 1 µl was injected into GC-MS instrument for further analysis. The samples were analyzed using Shimadzu GCMS-QP2020 (Shimadzu Corporation, Kyoto, Japan) with an AOC-20i auto injector. An InertCap WAX capillary column (30 m × 0.32 mm × 0.25 µm; GL Sciences Inc., Tokyo, Japan) was used for separation. Helium was used as a carrier gas with a flow rate of 1ml/min. The temperature of the front inlet was set at 250 ºC while the temperatures of the transfer line and the ion source were set to 280 ºC and 230 ºC respectively. The initial column temperature was held at 70 °C for 3 min and then was increased to 200 °C at a rate of 10 °C /min and was finally increased to 290 °C at a rate of 35 °C /min and then was held at this temperature for 7 min. A single run took 25.5 min. The solvent delay time was set to 6.7 min. The electron energy was −70 eV and the gain factor was set to 2.0.

15. Dietary supplementation of sodium butyrate:

The dietary supplementation studies were performed as reported earlier with minor modification (Lin et al. 2012). Briefly, a group of 5 prenatally arsenic exposed mice pups (3 weeks old) were fed with 5% sodium butyrate (w/w) (Sodium butyrate in solid form were thoroughly blended into chow diet) for next 7 days (pAs-butyrate-mice) (Xu et al. 2018).

16. MiR122 overexpression in mice:

MiR-122 was overexpressed in mouse gut in an identical procedure as described earlier (Ghosh et al. 2013). The miR-122 expression plasmid or empty plasmid (mock) was injected through the tail vein of pAs-butyrate-mice at a dose of 25 µg plasmid DNA dissolved in 100 µl saline. Following sacrifice of the mice after 4 days, the expression of occludin and miR122 in colon was determined by qPCR.

17. Determination of in vivo gut permeability:

The gut permeability assay was performed following previously reported protocol (Rangan et al. 2019). Briefly, mice were starved 16 h prior to FD-4 administration. FD-4 (44 mg/100 g body weight) was administered by oral gavage with a needle attached to a 1 ml syringe. A gap of 30 min between each mouse was kept for the FD-4 oral gavage. After 4 h, the blood was collected from tail vein. The blood was immediately transferred to a Microtainer SST tube and was mixed by inverting the tube 3-4 times and was stored at 4 ℃ in the dark. Once blood was collected from all the mice, SST tubes are processed to separate the serum following the manufacturer's instruction. The serum was then diluted with an equal amount of PBS. The concentration of FD-4 in sera were determined by spectrophotofluorometry with an excitation of 485 nm (20 nm band width) and an emission wavelength of 528 nm (20 nm band width) in Fluoroskan 168 TM Microplate Fluorometer (Thermo Fisher Scientific).

18. Short Chain Fatty Acid treatment in HT29 cells:

HT-29 cells were cultured in DMEM supplemented with 10% FCS and 50 µg/ml penicillin and streptomycin at 37° C with 5% CO_2. Cellular viability was assayed by MTT. Cells (10^6 cells/ml) were treated with either sodium butyrate (butyrate) or sodium propionate (propionate) or sodium acetate (acetate) at concentrations

indicated in the figures for 24 h. Thereafter the cells were washed and processed for further analysis.

19. Immunofluorescence Staining and imaging:

Colon samples were collected from all the groups during necroscopy examination and were fixed in 4% paraformaldehyde. The sections were then embedded in paraffin and 5 μm sections were generated. The paraffin embedded sections were then de-paraffinized in xylene and were rehydrated by passing through a graded series of ethanol followed by rinsing with distilled water. Antigen retrieval was performed by immersing the slides in 1 mM EDTA buffer pH 8.0 for 5 minutes.at sub boiling temperatures. The slides were then washed with distilled water. The sections were then permeabilized by 0.1% sodium citrate and 0.5% Triton-X in TBST for 15 minutes. The sections were then blocked with 5% animal sera in TBST for 1 hour at room temperature. The primary antibody to Occludin was then diluted (1:200) in the blocking solution and added to the sections which were then incubated overnight in a humidified chamber at 4ºC. Following incubation, the sections were washed TBS and TBST alternatively for 5 minutes. The sections were then incubated with goat anti-mouse or goat-anti rabbit secondary antibody conjugated with Alexa Fluor 488 for 2 hours at room temperature while being protected from light. Following incubation the slides were again washed with TBS and TBST alternatively for 5 minutes. The sections were then mounted with 1μg/ml Hoechst 33342 stain which acted as a nuclear counterstain. Fluorescence images were then captured using Carl Zeiss microscope equipped with a CCD camera and controlled by Zen software (Carl Zeiss, Gottingen, Germany) (Gumber et al. 2014).

20. Tissue homogenisation and RNA and Protein isolation:

Colon samples (dissected into small pieces) or the cells were resuspended either in RIPA Lysis buffer (20mM Tris-HCl pH 7.5, 150mM NaCl,, 1mM EDTA, 1mM EGTA, 1% NP-40, 1% Sodium deoxycholate, 2.5 mM Sodium Pyrophosphate, 1mM β-glycophosphate, 1mM Na3VO4, 1 μg/ml leupeptin with 1mM of PMSF immediately before use) for protein isolation or in Trizol (Invitrogen, US) for RNA isolation. The tissue was homogenized using a micropestle and centrifuged at 13,000 g for 15 min at 4°C. The clear supernatant was collected and either stored as protein lysate in -80°C or further processed to isolate RNA using the standard protocol (Wu

et al. 2018). The concentration of the extracted RNA was analyzed by Nanodrop and RNA was stored at –80° C.

21. RNA extraction and reverse transcription:

cDNA was prepared from total RNA by reverse specific primers using Super Reverse Transcriptase MuLV Kit. The primers for the reverse transcription are listed in Table 1. U6 and GAPDH were normalized for the expressions of miR122 and other genes of interest. The total reaction volume for reverse transcription was 20 µl in which 1 µM of reverse primer, 5 ng of RNA template, 1 µl dNTP mix, 12 µl of DEPC treated water, 4 µl of 5X first strand buffer, 1 µl of 0.1 M DTT, 1 µl of RNase inhibitor and 1 µl Super RT MuLV. Reverse transcription was carried out for 65°C for 5 minutes, followed by incubation at 55°C for 1 hour and then heat inactivating the reaction at 70°C for 15 minutes (Abdelmohsen et al. 2012).

22. Quantitative real-time PCR:

Total RNA was extracted with Trizol reagent from snap frozen colon and RNA concentration was determined using a nanodrop. The miR-122, occludin, claudin1, claudin 2, claudin 4, ZO-1, GAPDH, and U6 levels were quantified with Applied BiosystemsTM StepOneTM 268 Real Time PCR System with RT2 SYBR® Green qPCR Mastermix following the manufacturer's instructions. Each 20 µl qPCR reaction contained an amount of cDNA equivalent to 5 ng of total RNA , 10 µl of RT2 SYBR® Green qPCR Mastermix, 1 µM of the forward and reverse primer (each) and nuclease free water. Real-time PCR was performed with the following conditions: 95°C for 10 min, 40 cycles of 95°C for 30 sec, 60°C for 1 min and 72°C for 1 min PCR product was calculated according to the $2^{-\Delta\Delta Ct}$ method described previously (Abdelmohsen et al. 2012).

23. Western Blot:

Colon tissue protein and cell lysate were extracted in RIPA Lysis buffer. Protein concentration was measured using Pierce TM BCA Protein Assay Kit. Proteins (50 µg/lane) were separated by using SDS PAGE on 10% gel under reducing condition and electro transferred to PVDF membrane in a transferred buffer (25mM Tris-HCl, 150mM Glycine, 20% Methanol). Membranes were blocked at room temperature with 5% non-fat skim milk in TBS for 2 hours, and then incubated with primary antibody

against specific protein. The membranes were incubated with the horseradish peroxidase (HRP)-conjugated secondary antibodies at 37° C for 1 h. SuperSignal West Pico chemiluminescent substrate kit (Thermo) was used to visualize the blotting results. The blots were imaged with Fluor Chem R system (ProteinSimple, San Jose, CA, USA) (Abdelmohsen et al. 2012).

24. Statistical analysis:

All data (apart from contour plots) are reported as means ± SE. All statistical analyses were performed using Prism-5 Software (GraphPad, San Diego, CA). Statistical significance between two groups were analyzed using a non-parametric t-test with 95% confidence intervals while statistical significance between more than two groups were determined by one way Analysis of Variance (ANOVA) followed by Tukey's post hoc test. A p-value < 0.05 was considered significant

Chapter-1

Sub Chronic Arsenic Exposure affects immune system and increases risk of gastrointestinal infection to enteric *Shigella* pathogen

Introduction

Arsenic is a toxic metalloid found in air, water and land. Since most Arsenic compounds are devoid of color and smell, it cannot be easily detected in food, water or air thus presenting a very serious health hazard given its toxic nature (Mandal & Suzuki, 2002). Exposure to Arsenic is a growing concern for global public health due to the widespread distribution of the metalloid and its association with numerous adverse effects. Arsenic has been well known to be associated with cancers (skin, lung and bladder), skin lesions, diabetes and cardiovascular diseases (Hughes, Beck, Chen, Lewis, & Thomas, 2011; Humans, 2012; J. Liu & Waalkes, 2008).

Growing evidences indicate that arsenic have a toxic effect on the immune system (Ramos Elizagaray & Soria, 2014). A microarray-based genome wide expression study of peripheral blood mononuclear cells (PBMC) conducted from 21 subjects showed significant difference in T-cell receptor signaling, cell cycle regulation and apoptosis in people exposed to arsenic (Andrew et al., 2008). Apart from epidemiological studies, various in vitro and in vivo studies have also demonstrated immunotoxic nature of arsenic. In vitro treatment with 0.5 to 2 μM arsenic trioxide (ATO) was shown to disrupt the differentiation of human peripheral blood monocytes into functional macrophages by repressing NF-kB-related survival pathways (Lemarie et al., 2006). In another study, monocyte derived macrophages from individuals exposed to arsenic showed reduced cell adhesion capacity, F-actin expression and nitric oxide production and phagocytic activity (N. Banerjee et al., 2009). Exposure to arsenic has also been known to cause suppressed immunoglobulin production (Selgrade, 2007), decreased cytokine production (Conde, Acosta-Saavedra, Goytia-Acevedo, & Calderon-Aranda, 2007) and defective antigen driven T-cell proliferation and reduced IL-2 secretion by lymphocytes (Galicia, Leyva, Tenorio, Ostrosky-Wegman, & Saavedra, 2003; Vega et al., 1999). Chronic arsenic exposure have also contributed to decrease CD4+ cells and decreased CD/CD8 ratio in children as well as adults and with decreased ex-vivo proliferation of activated T lymphocytes (R. Biswas et al., 2008; Liao et al., 2009; Soto-Pena et al., 2006).

The gastrointestinal tract is an important component of the immune system. It plays an active role in preventing pathogens from entering the blood and lymph circulatory systems and maintains intestinal homeostasis (Bauman, Rembert, & Greenfield, 1989; Flannigan, Geem, Harusato, & Denning, 2015). The mesenteric lymph nodes (MLN) forms an important component of the gastrointestinal immune system as they are key sites for induction of tolerance of food proteins while also acting as a firewall to prevent the entry of intestinal bacteria in the systemic immune system (Macpherson & Smith, 2006). Since contaminated drinking water and food are the major sources of arsenic exposure in both humans and animals, and the gastrointestinal tract is therefore nearly always the first location at which arsenic exposure and its biotransformation occur and hence they become susceptible to any changes induced by the metalloid. Despite this there are no reports stating how arsenic exposure modulates the immunity of the gut and whether it leads to increased susceptibility to any enteric infections.

In the present study, we exposed first evaluated toxicity of Arsenic Trioxide *in vitro* in a murine macrophage cell line RAW 264.7 in terms of cell death and hampering of immune response when stimulated with lipopolysaccharide (LPS). This was followed by *in vivo* experiments where we exposed Balb/c mice to 4 ppm and 10 ppm Arsenic Trioxide (As_2O_3) through drinking water for 30 days to mimic sub chronic arsenic exposure model in laboratory animals. We investigated the abundance of CD4 and CD8 T and CD19 B cells present in spleen and MLN and also studied the circulating levels of IgG1 and IgG2a in the serum. Additionally, we investigated whether exposure to arsenic and any changes that were induced would lead to an increased susceptibility to an enteric Shigella infection. Our study is the first evidence to establish an association of arsenic exposure to severity of enteric pathogenesis.

Results

1. **Arsenic Trioxide (As_2O_3) reduces cell viability in RAW 264.7 macrophages in dose dependent manner:**

RAW 264.7 macrophages were incubated with varying concentrations of Arsenic Trioxide (0, 2, 4, 6, 8, 10 μg/ml) for 24 hours. There was a marked decrease in cell viability in RAW 264.7 cells in a dose dependent manner with the maximum cytotoxicity occurring at cells that were treated with 8 ug/ml (61% of viable cells) and 10 ug/ml (47% of viable cells) Arsenic Trioxide (As_2O_3). (Figure 1.1)

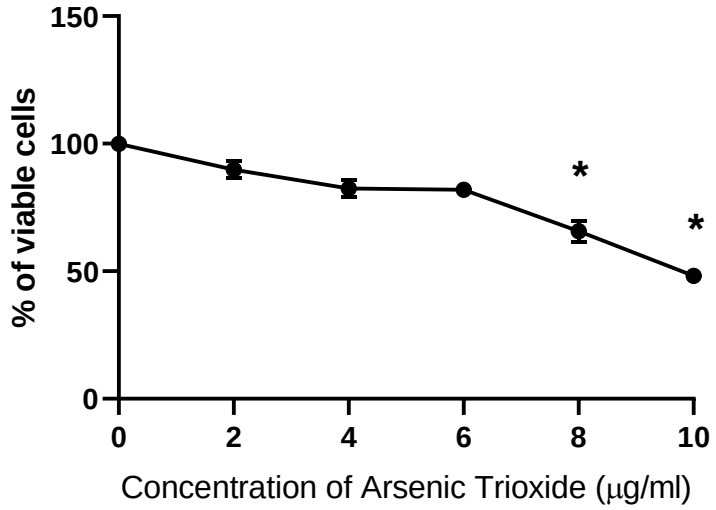


Figure 1.1: Effect of Arsenic Trioxide on cell viability of RAW 264.7 macrophages. RAW 264.7 cells were treated with varying concentrations of Arsenic Trioxide (As_2O_3) for 24 hours. The viability was then checked by MTT assay. Each treatment and the entire experiment were conducted in replicates. Values are mean± SEM. Values significantly different from control at *$p < 0.05$.

2. **Arsenic Trioxide reduces expression of inducible Nitric Oxide Synthase (iNOS) expression in RAW 267.4 macrophages:**

To determine whether Arsenic Trioxide (As_2O_3) inhibits NO production at the level of transcription, we used RT–PCR to examine the expression of the iNOS gene in activated macrophages. RAW 264.7 cells were treated with varying concentrations of

Arsenic Trioxide for 24 hours which were then followed by 0.5 ug/ml LPS treatment for another 24 hours. Following Arsenic Trioxide treatment, RAW 264.7 cells showed a significant decrease in iNOS expression upon LPS stimulation when compared to that of control in a dose dependent manner (Figure 1.2).

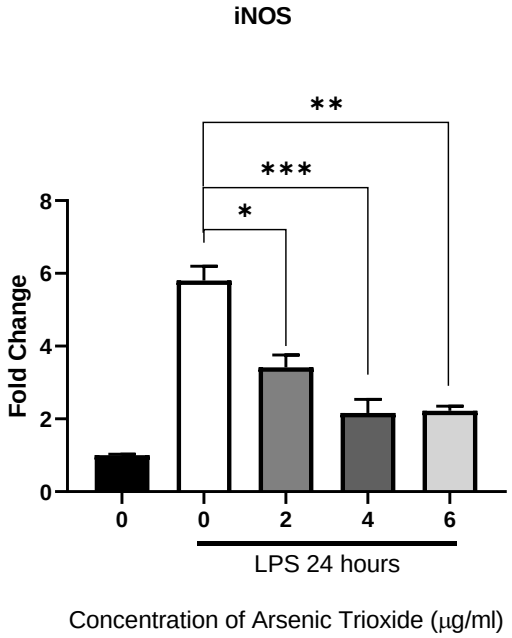


Figure 1.2: Effect of Arsenic Trioxide on iNOS expression in RAW 264.7 macrophages. iNOS expression determined quantitative Real Time PCR. All experiments were done in triplicates and three times. Values expressed as mean ± SEM. Values significantly different from control at *p<0.05, **p<0.01 and ***p<0.001.

3. Arsenic Trioxide (As_2O_3) inhibits the expression of proinflammatory cytokines in LPS-Stimulated RAW 264.7 Cells:

TNF-α, IL-12 and IL-4 are some of the proinflammatory cytokines that are key mediators of inflammation. The level of the cytokine production in LPS-induced macrophages was evaluated using ELISA. Pre-treatment with Arsenic Trioxide significantly suppressed the production of TNF-α in LPS stimulated RAW 264.7 cells in a dose dependent manner (Figure 1.3A). However, arsenic trioxide did not have any effect on the secretion of IL-12 and IL-4 in LPS stimulated RAW 264.7 cells (Figure 1.3B and 1.3C).

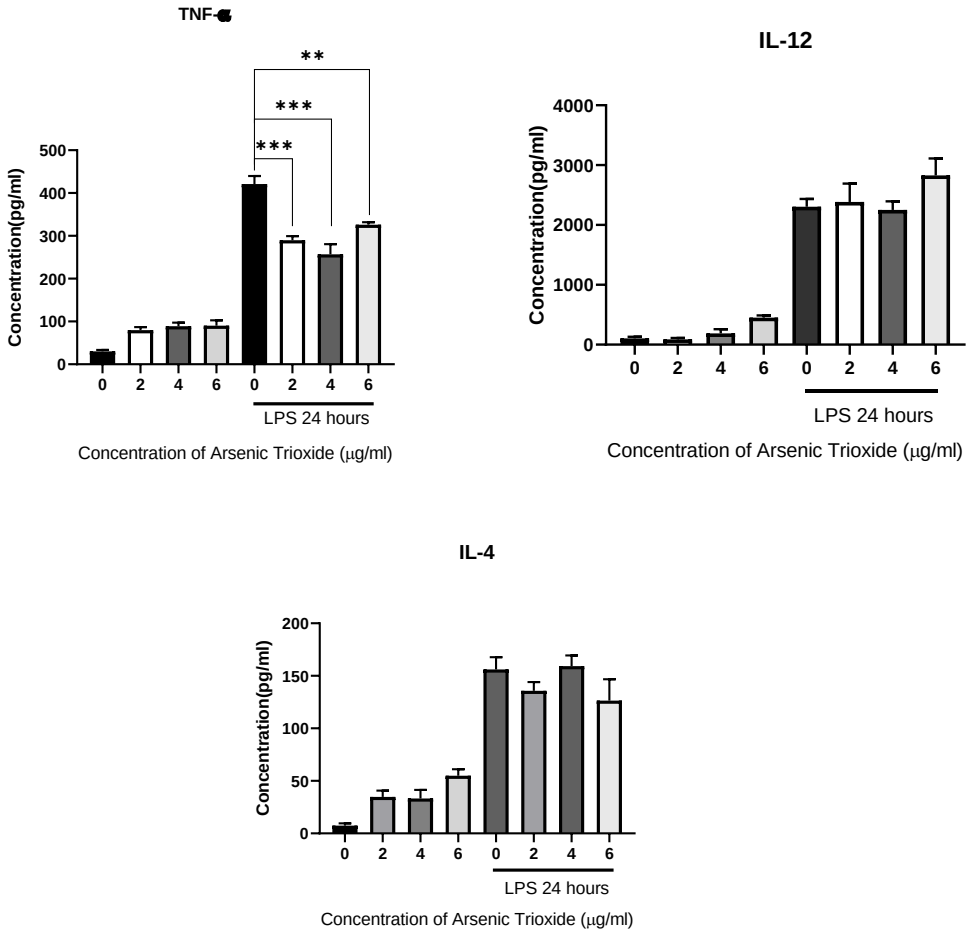


Figure 1.3: Effect of Arsenic Trioxide on expression of proinflammatory cytokines in RAW 264.7 cells. Evaluation of expression of **(A)** TNF-α **(B)** IL-12 and **(C)** IL-4 in RAW 264.7 cells. All experiments were done in triplicates and three times. Values expressed as mean ± SEM. Values significantly different from control at **p<0.01 and ***p<0.001.

4. Arsenic exposure effects on body weight, splenic index and tissue histology in BALB/C mice.

Balb/c mice were exposed to 4 ppm and 10 ppm arsenic trioxide (arsenic) through drinking water for 30 days. On day 30 the body weight was measured and it was observed that both arsenic exposed groups did not differ significantly compared to control (Figure 1.4A). But there was a significant decrease in splenic index in 10 ppm arsenic but not in 4 ppm arsenic fed mice compared to control (Fig 1.4B).

The spleens and mesenteric lymph nodes (MLNs) were dissected and histological sections were analysed after H&E staining. There was no change detected in the MLN

in both 4 ppm and 10 ppm arsenic fed mice compared to control (Fig 1.4C). But there was decrease in number and diameter of white pulp in the spleen of 10 ppm arsenic treated mice compared to control.

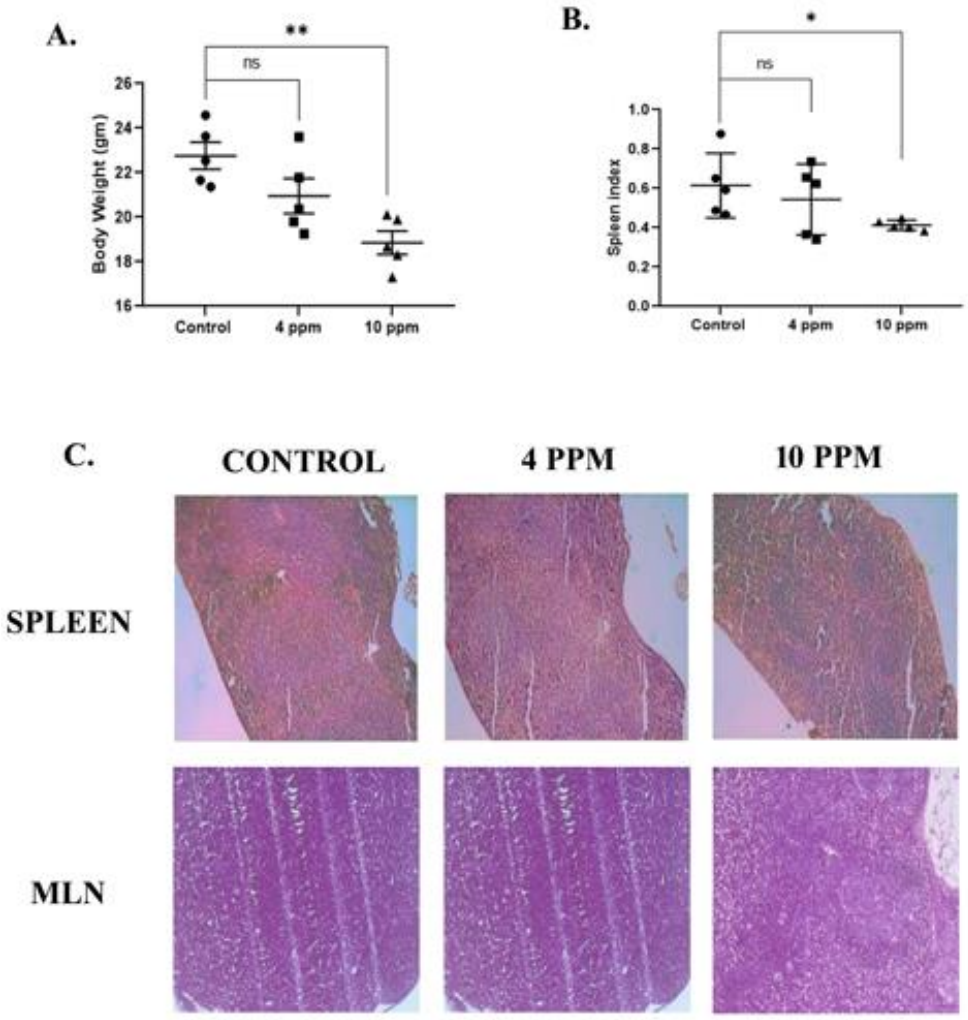
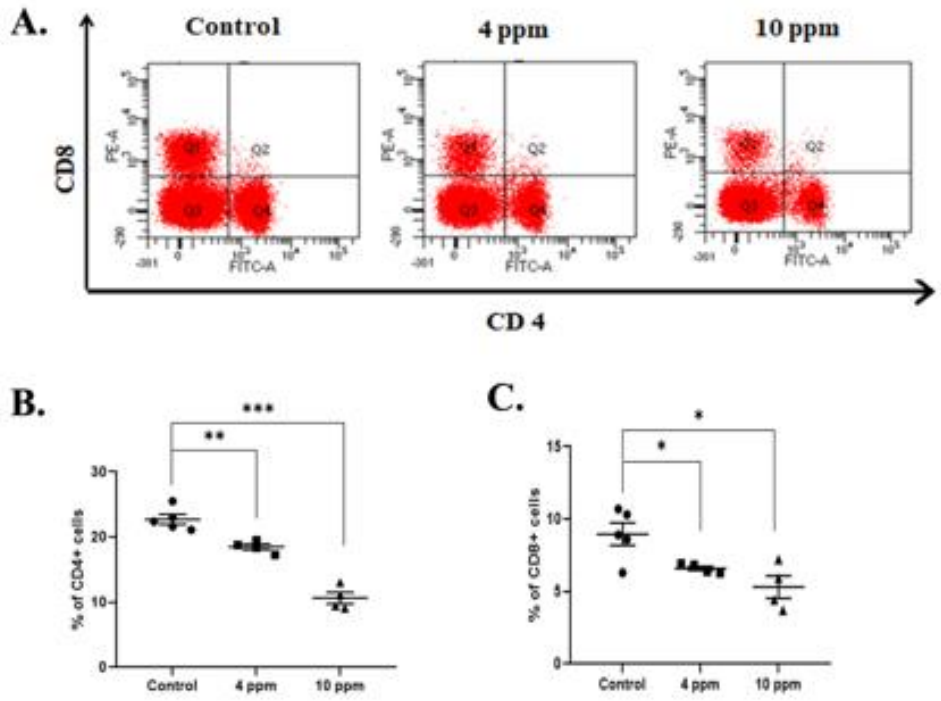


Figure 1.4: Effect of sub chronic arsenic exposure on body weight, spleen index and histoarchitecture of spleen and Mesenteric Lymph Nodes (MLN). Adult Balb/c mice were divided into three groups and were given ad libitum access to normal drinking water and water containing 4 ppm and 10 ppm arsenic trioxide (As) for 30 days. **(A)** Body weights **(B)** Splenic index and **(C)** representative micrographs of spleen and MLN (H&E; Spleen: 10X magnification and MLN: 20X magnification) taken from control, 4 ppm and 10 ppm Arsenic exposed mice. Data is represented as mean ±SEM. N= 5/group. Values significantly different from control at *$p<0.05$ and **$p<0.01$.

5. Arsenic exposure reduces T-cells in spleen and MLN in BALB/C mice:

The frequencies of CD4+ and CD8+ T-cells in spleen and mesenteric lymph nodes (MLN) were analyzed from arsenic exposed and control mice. There was a significant decrease in the frequencies of both CD4+ and CD8+ T-cells in spleen in 4 ppm and 10 ppm arsenic exposed mice compared to control (Figure 1.5A). We report 19 % and 26% decrease in CD4 and CD8 Tcells respectively in 4 ppm arsenic exposed mice compared to control. Similarly 53% and 41% CD4 and CD8 T cells respectively were reduced in the spleen of 10 ppm arsenic exposed mice compared to control (Figure 1.5B & Figure 1.5C). However, the percentages of double positive cells ($CD4^+CD8^+$) T cells remained unchanged in all three groups. Further analysis of T cells from MLN also showed similar decrease in CD4 and CD8 T cells upon arsenic exposure compared to control (Figure 1.5D). The percentages of CD4 T-cells were decreased by ≈ 34% and ≈ 19% whereas the percentages of CD8+ cells decreased by ≈ 79% and ≈ 81% in 4 ppm and 10 ppm exposed groups respectively when compared to that of control (Figure 1.5E and Figure 1.5F).



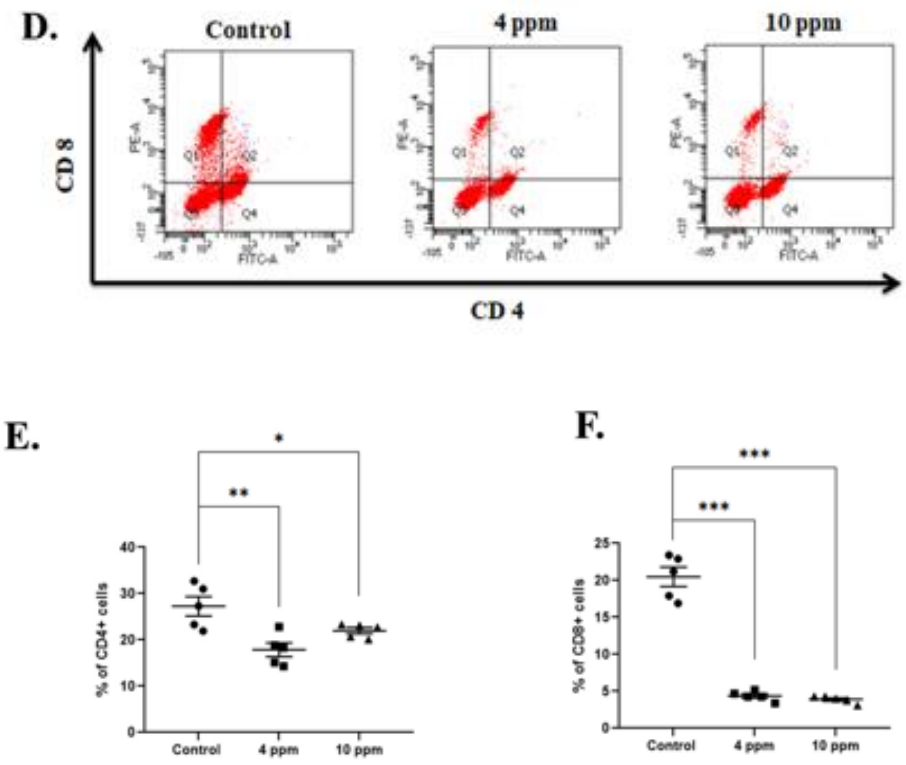


Figure 1.5: Effect of sub chronic arsenic exposure on CD4 and CD8 T-cells in spleen and MLN. Cells from spleen and MLN from individual mice of each group were collected, stained with anti CD4 and anti CD8 antibodies and the populations were then evaluated by flow cytometry. (A) Representative Dot plots of CD4 and CD8 T-cells in spleen. (B) Percentage of CD4 T-cells in spleen (C) Percentage of CD8 T-cells in spleen (D) Representative Dot plots of CD4 and CD8 T-cells in MLN (E) Percentages of CD4 T-cells in MLN (F) Percentages of CD8 T-cells in MLN from control and 4 ppm and 10 ppm Arsenic exposed groups. Data represented as mean ± SEM. N=5/group. Values significantly different from control at *p<0.05, **p<0.01 and ***p<0.001.

6. Arsenic Trioxide increases FoxP3 expression in cells from both spleen and MLN in BALB/C mice:

To investigate whether sub chronic arsenic exposure had any effect on Treg cells from spleen and MLN, mRNA expression of FoxP3 was evaluated. FoxP3 being a transcription factor and marker of regulatory T-cells (Tregs) we analyzed its expression by quantitative PCR from spleen and MLN. We observed a significant increase in FoxP3 expression in splenocytes (≈ 2.6 fold) (Figure 1.6A) and MLN cells

(≈ 2.7 fold and ≈ 3 fold) of mice exposed to 4 ppm and 10 ppm of Arsenic respectively compared to control (Figure 1.6B).

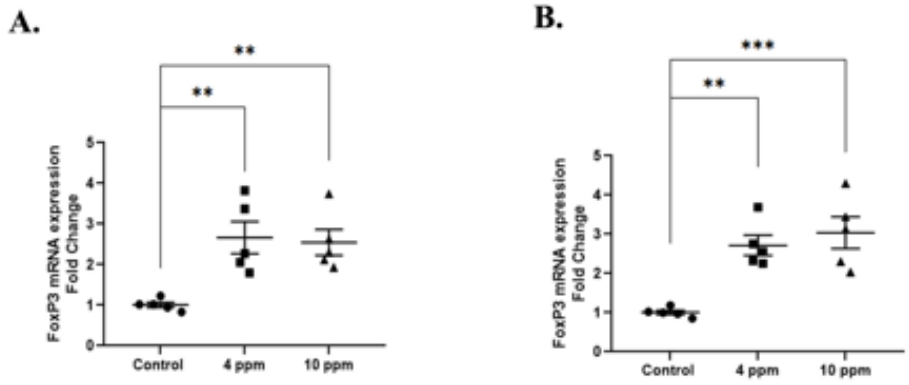
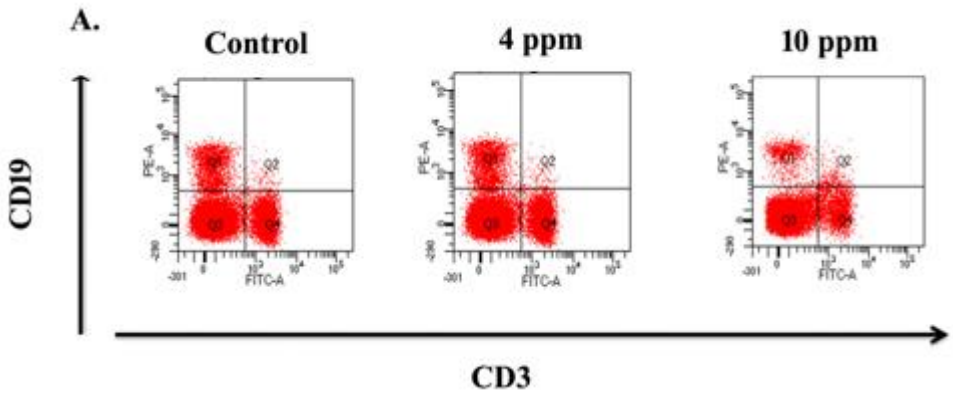


Figure 1.6: Effect of sub chronic arsenic exposure on expression of FoxP3 in cells from spleen and MLN. Expression of FoxP3 mRNA quantified by quantitative real time PCR in cells from (A) Spleen and (B) MLN from control and 4 ppm and 10 ppm arsenic exposed groups. Fold change was calculated using $2^{-\Delta\Delta Ct}$ method. Data is represented as mean ± SEM. N=5/group. Values significantly different from control at **p<0.01 and ***p<0.001.

7. Arsenic Exposure decreases CD19+ B cells in spleen and MLN

We also analysed the frequency of CD19+ B cells in spleen and MLN and found that there was a significant decrease in B cells in both spleen (Figure 1.7A and Figure 1.7C) and MLN (Figure 1.7B and Figure 1.7D) in arsenic exposed mice compared to control.



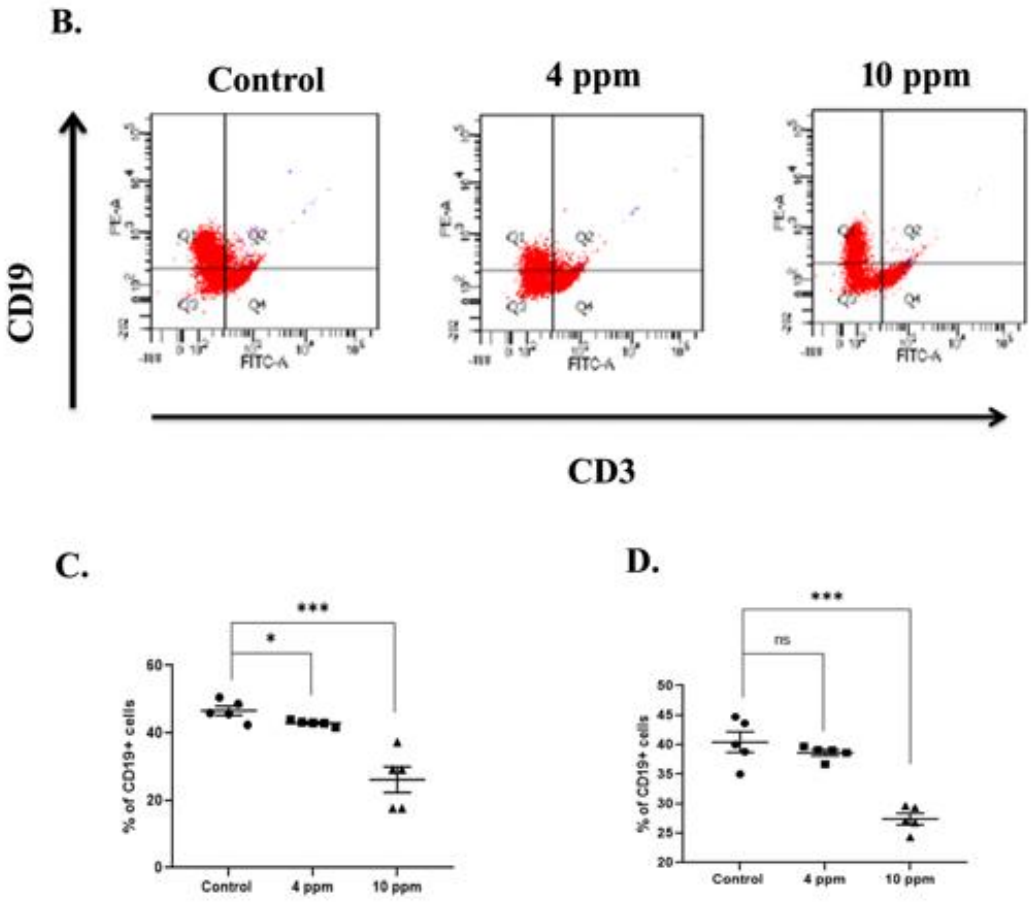


Figure 1.7: Effect of sub chronic arsenic exposure on B-cells in spleen and MLN. Cells from spleen and MLN from individual mice of each group were collected, stained with anti CD3 and anti CD19 antibodies and then B-cell populations were then evaluated by flow cytometry. (A) Representative dot plot of $CD19^+$ B-cells in spleen. (B) Representative dot plot of CD19+ B-cells in MLN. (C) Percentage of $CD19^+$ B-cells in spleen (D) Percentage of $CD19^+$ B-cells in MLN of control and 4 ppm and 10 ppm arsenic exposed groups. Data is represented as mean ± SEM. N=5/group. Values significantly different from control at *p<0.05 and ***p<0.001.

8. Arsenic exposure affects circulating levels of IgG1, IgG2a and IgG3

To investigate whether arsenic exposure had any potential effects on humoral immunity, we assessed circulating levels of IgG1, IgG2a and IgG3 in control and arsenic treated groups. Both IgG1 and IgG2a levels were significantly decreased in arsenic exposed groups (4 ppm and 10 ppm) when compared to that of control. The levels of total IgG1 were 34% lower in both the 4 ppm and 10 ppm exposed groups (Figure 1.8A) whereas IgG2a levels were reduced by 53% and 68% in 4 ppm and 10

ppm arsenic exposed groups respectively compared to control (Figure 1.8B). However, there were no changes in the levels of IgG3 in arsenic exposed groups when compared with that of control (Figure 1.8C). Since we observed an overall decrease in immune cell repertoire and IgG isotypes we wanted to evaluate whether the immunocompromised state leads to susceptibility to enteric infection.

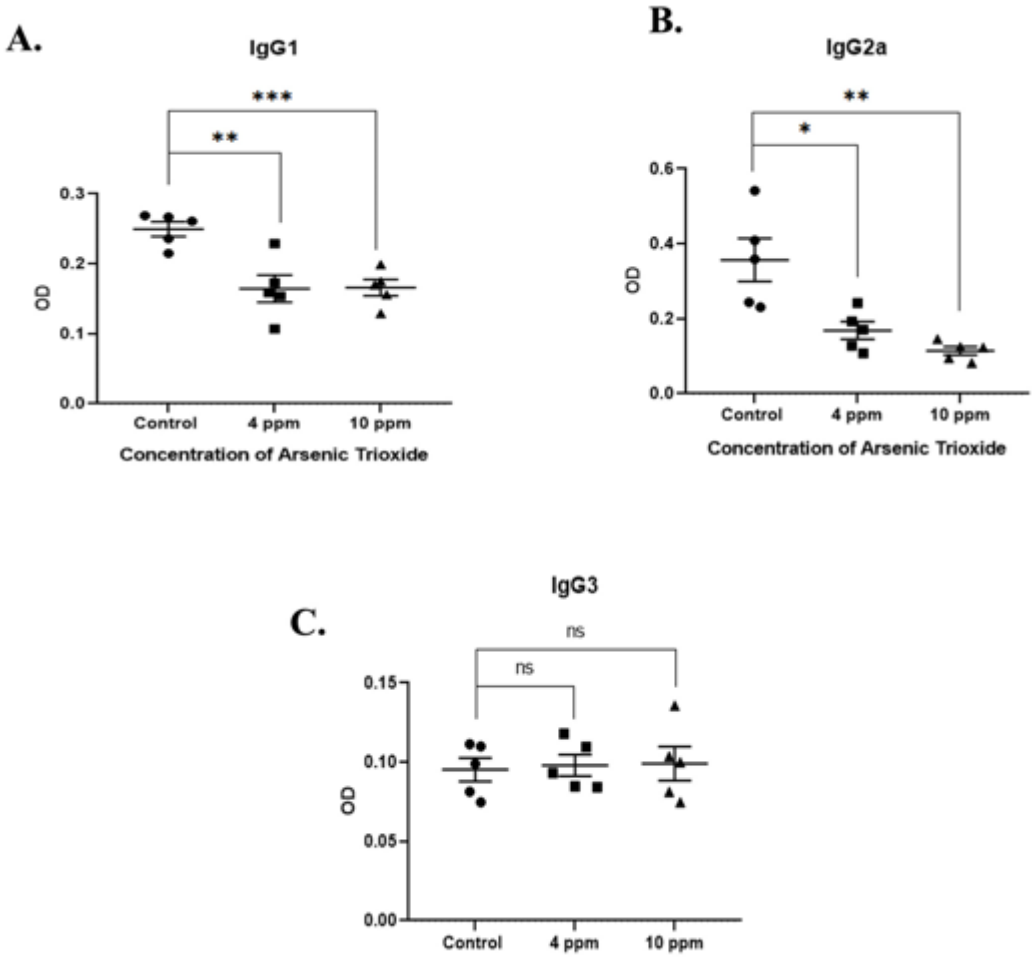


Figure 1.8: Effect of sub chronic arsenic exposure on circulating IgG levels.

Levels of circulating (A) IgG1 (B) IgG2a and (C) IgG3 in sera (diluted 1:1000) collected from mice of control and 4ppm and 10 ppm arsenic exposed groups. Data represented as mean ± SEM. N=5/group. Values significantly different from control at *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

9. Arsenic exposure increases susceptibility to enteric Shigella infection in mice

To determine the effects of arsenic on enteric infection in mice, we studied Shigella infection in arsenic exposed and control mice. Mice were infected intraperitoneally with 10^8 viable *Shigella flexneri* 2a strain (2457T) and bacterial load from colon was enumerated 24 h post infection. Bacterial load was expressed as CFU/gm colon tissue. We observed significant increase of ≈ 52% and ≈ 50% in CFU of enteric Shigella pathogen in colon of mice exposed to 4ppm and 10ppm of Arsenic respectively compared to control (Figure 1.9A). Increased pathogenic load also lead to aggravating inflammation in the colon as evidenced by increased expression of mucin-2 (4 ppm≈ 4.5 fold, 10 ppm ≈ 4.9 fold) (Figure 1.9B), iNOS (4 ppm≈ 2.8 fold, 10 ppm ≈ 2 fold), and proinflammatory cytokines like IL-6 (4 ppm ≈ 6 fold, 10 ppm ≈ 5.1 fold) and TNF-α (4 ppm ≈ 4.1 fold, 10 ppm ≈ 3.8 fold) (Figure 1.9C).

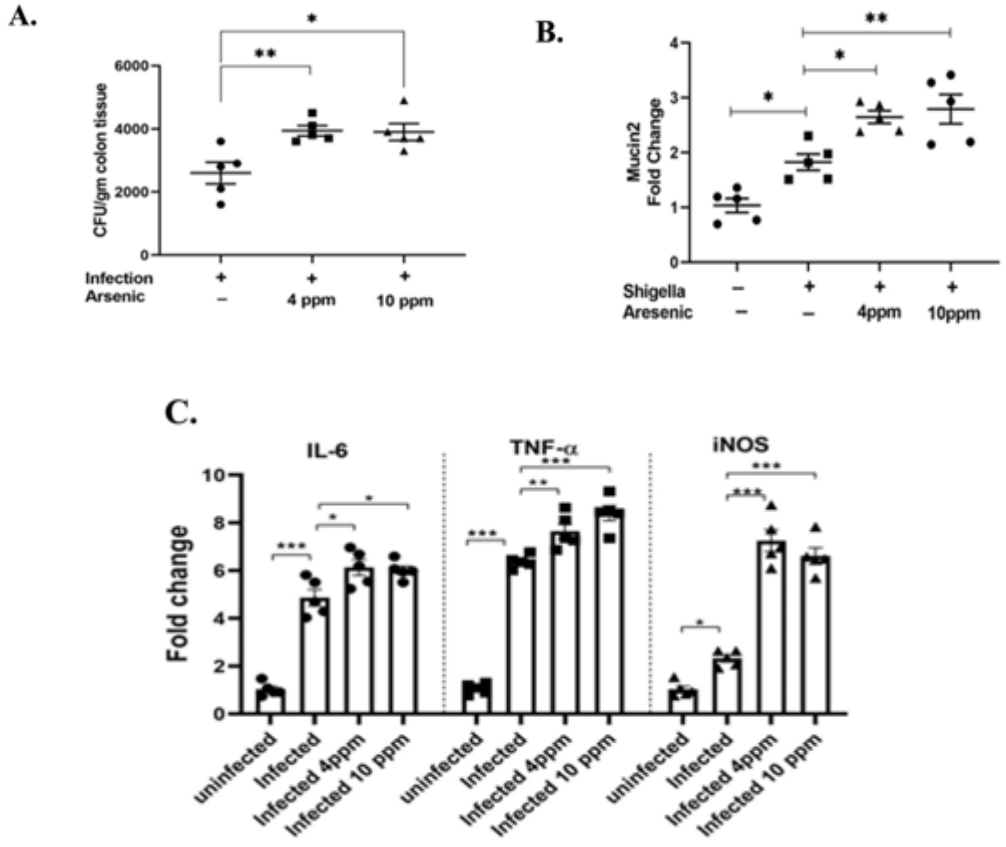


Figure 1.9: Effect of sub chronic arsenic exposure on susceptibility to enteric *Shigella* infection. Mice from control and arsenic exposed (4 ppm and 10 ppm) were infected with 10^8 CFU of *Shigella Flexneri* 2a strain (2457T) and were evaluated 24 hours after infection. (A) Colon sections were isolated, homogenized and plated to enumerate CFU's. (B) Mucin-2 mRNA expressions as quantified by real time PCR in colon tissues (C) Expression of TNF-α, IL-6 and iNOS as quantified by real time PCR

from colonic tissues of control and arsenic exposed groups. Data represented as mean ± SEM. N=5/group. Values significantly different from control at *p<0.05, **p<0.01 and ***p<0.001.

Discussion

Recent studies indicate that exposure to arsenic leads to detrimental effects on health in both animal models and humans (Abdul, Jayasinghe, Chandana, Jayasumana, & De Silva, 2015; Santra et al., 2000). In humans the extent of the detrimental effects is often determined by the amount of arsenic that they were exposed to and the duration of the exposure. To determine whether Arsenic interfered in proper immune responses, we conducted *in vitro* studies with RAW 264.7 cells where we found that Arsenic causes toxicity to RAW 264.7 cells in a dose dependent manner and also interfere in expression of inducible Nitric oxide syanthase release of crucial cytokine TNF-α when RAW 264.7 cells were stimulated with lipopolysaccharide (LPS) thereby exerting a suppressive effect on the immune system.

To determine whether sub chronic-dose of Arsenic contaminated drinking water compromises the overall immune response that may increase susceptibility to enteric infection; we analysed the outcome of *Shigella* infection in mice fed with drinking water containing with or without arsenic. Our studies demonstrated that Arsenic exposure had a significant impact on the immune system, resulting in a severely compromised response to Shigella infection. We also observed increased bacterial load and increased inflammatory cytokine production in the gut of As-exposed mice relative to controls. We expected the initial attenuated immune response in the As-exposed mice based on our previous study in prenatal arsenic exposed mice (Chakraborty & Bhaumik, 2020). Arsenic exposure affects a variety of aspects of host defence, including reduction of lymphocytes and IgG isotypes. An appropriate and sufficient response to an infectious challenge requires integration of the cell mediated and humoral response. Given that Arsenic exposure has important impacts on both components, it is not unexpected that the effects of Arsenic exposure on the immune response can ordeal enteric infection.

We have also shown that Arsenic decreased CD4, CD8 T cells and Cd19 B cells in spleen and MLN. With respect to enteric infection, decrease in immune cells in MLN

is a critical component for initiating an appropriate immune defense against *Shigella* infection. The relative decrease in this population of CD4 T cells in the lymph nodes may have contributed to the initial attenuated response of As-exposed mice and suggests that T cell response to presented antigen within the lymph node may have been compromised. The increase in FoxP3 expression in lymphocytes isolated from MLN and spleen indicating that arsenic exposure leads to a rise in Treg populations in MLN as well as spleen. The IgG isotypes have been widely accepted as markers for T_H1 and T_H2 responses respectively (Mountford, Fisher, & Wilson, 1994). Decrease in both IgG1 and IgG2a levels in mice exposed arsenic suggests that sub chronic exposure to arsenic hampers both T_H1 and T_H2 responses.

Having established arsenic exposure reduces the immune components in MLN we determined the susceptibility to enteric infection in arsenic exposed and control mice. Along with increased bacterial load in gut, we also observed increase in mucin 2 expression, inflammatory cytokines. Previous studies suggest that mucin in the colon can be subject to direct colonic infection and inflammation. For instance, Muc2−/− mice exhibited rapid weight loss, high mortality, and greater bacterial burden when infected with *Citrobacter rodentium* (Bergstrom et al., 2010). Similarly, we find increase in mucin 2 expression in infected mice which was further increased significantly in arsenic exposed mice. In order to further address whether arsenic treatment increases the inflammatory responses in the colon elicited by *Shigella* infection, we determined secretion levels of cytokines expression in colon tissue. We found that proinflammatory cytokines such as IL-6 and TNF-α were significantly higher in arsenic treated mice compared to control. Collectively, these data indicate that the increase in bacterial burden in arsenic exposed mice resident colon, *Shigella* organisms provoke predominant cytokines, which contribute to increased recruitment of polymononuclear cells into the colon.

Thus this study provides evidence that sub chronic levels of arsenic in drinking water results in immunosuppression in the gut by decreasing $CD4^+$ $CD8^+$ T-cells and B cells in MLN as well as circulating IgG1 and IgG2a levels. The compromised frequency of immune cells increases susceptibility to enteric *Shigella* infection and inflammation in colon.

Chapter-2

Prenatal Arsenic Exposure interferes in postnatal immunocompetence even in the absence of continued Arsenic Exposure

INTRODUCTION

Arsenic is a toxic metalloid and can induce a host of detrimental health effects making it a major concern all over the globe. Arsenic has been known to cross placenta thus making the fetus particularly vulnerable to its toxic effects (Concha et al., 1998; Davis et al., 2015). Toxicity to the immune system due to early life arsenic exposure has been studied extensively in humans as well as animal models. Studies have identified that arsenic exposure can lead to immunosuppression in the form of poor cellular immune function in cord blood cells in humans which is also accompanied by improper function of subsets of T-cells (Nadeau et al., 2014).

Prenatal arsenic exposure has been shown to reduce CD4/CD8 ratios in children along with poor cell mediated immunity (Soto-Pena et al., 2006). Arsenic exposure has also been shown to alter thymic function of children that were induced by apoptosis and generation of oxidative stress (Ahmed et al., 2012). These changes thus have the potential to make children, who have been exposed to arsenic *in utero*, increasingly susceptible to infections, autoimmune diseases and other chronic diseases in childhood and also later in life. Arsenic has been shown to affect fetal development that includes epigenetic reprograming of the fetus (Attreed et al., 2017; Bailey & Fry, 2014; Kippler et al., 2016; M. Vahter, 2008). Thus if these changes lead to any deficiency in immunocompetency in the children exposed, they will be impacted even if they are not exposed to arsenic anymore. There have been increasing evidences of increased susceptibility to infections due to prenatal arsenic exposure (Farzan et al., 2016; A. Rahman et al., 2011; Raqib et al., 2009).

While it is has become increasingly clearer from literature that arsenic exposure can affect all the three arms of the immune system innate, humoral and cellular immunity, most of these are the result of direct exposure to the metalloid. It is still unclear what are the effects of prenatal Arsenic exposure are. In this investigation, we have studied all the three arms of the immune system to better understand how prenatal arsenic exposure affects the immune competence of mice that were exposed to arsenic in utero and how it impacts their ability to mount an effective resistance to a pathogenic challenge.

RESULTS

1. **Prenatal Arsenic Exposure did not have any effect on body weight, thymic/ splenic weight and histological architecture:**

Balb/c mice were given 4 ppm of Arsenic Trioxide (As_2O_3) through drinking water during the entire length of the pregnancy. The water was changed twice weekly. After the birth of the pups, mothers were given access to clean Arsenic Free water. After 4 weeks the pups were chosen without any sex bias for evaluation. There was no change in body weights (Fig. 2.1A) of pups that were prenatally exposed to Arsenic when compared to that of control pups. Also, there was no difference in splenic (Fig. 2.1B) or thymic (Fig. 2.1C) weights. Histological examinations also revealed no changes in the spleen or thymus of prenatally Arsenic exposed pups when compared to that of control (Fig 2.1D).

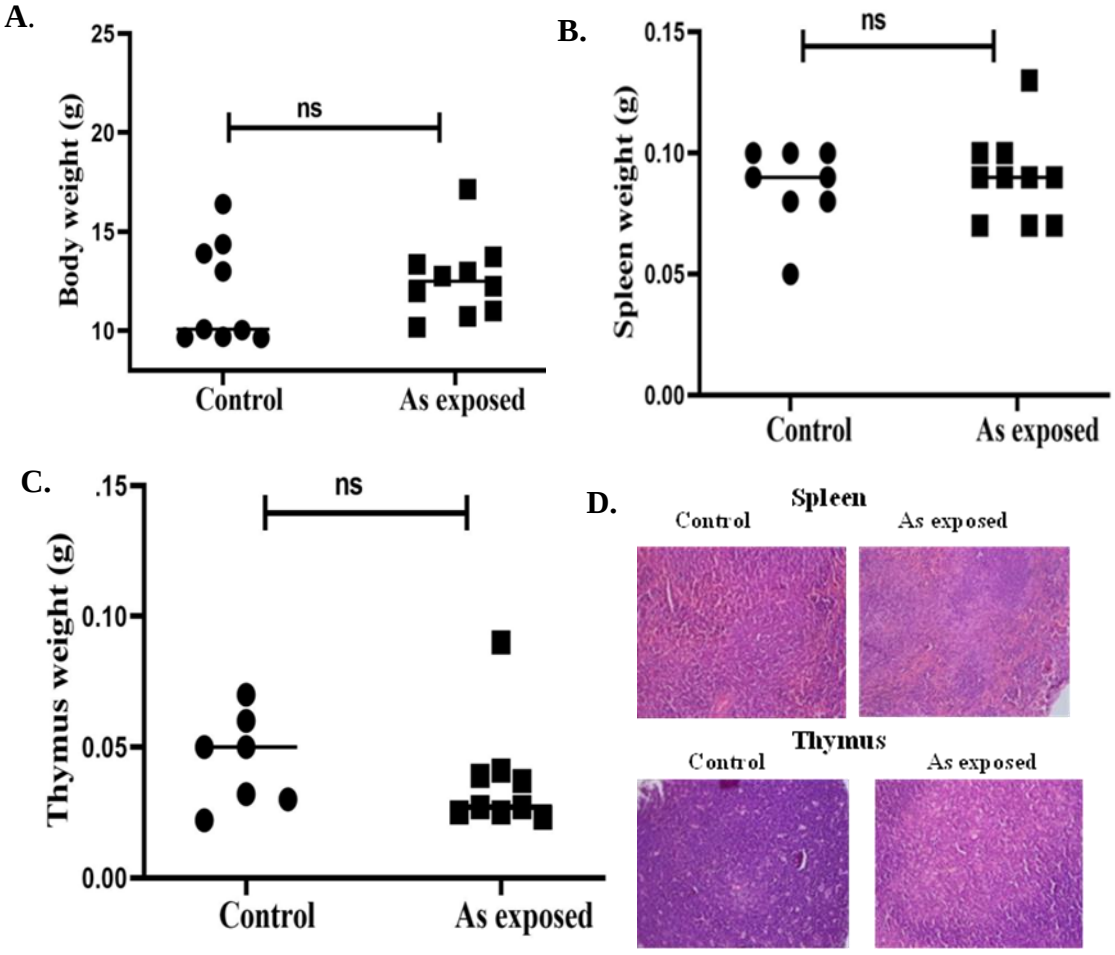


Figure 2.1: Effect of prenatal Arsenic exposure on pup body weight, spleen and thymus weight and histology. **(A)** Body weight **(B)** Spleen Weights **(C)** Thymus weights **(D)** Histological micrographs of spleen and thymus (H&E, 20 X

magnifications). All samples were collected from mice at 28 days age. (n≥7 per group).

2. **Prenatal Arsenic Exposure reduces IgG2a levels but not IgG1 levels in the serum:**

To assess whether prenatal Arsenic exposure leads to any detrimental effects or changes to the humoral immune response, circulating levels of IgG1 and IgG2a in the serum of As-exposed and unexposed individuals were evaluated. There were no differences in IgG1 levels (Fig 2.2A) in the serum between control and prenatally Arsenic exposed groups. However, serum IgG2a levels were decreased significantly (almost 30%) (Fig 2.2B) in prenatally Arsenic exposed Balb/c pups when compared to that of control pups.

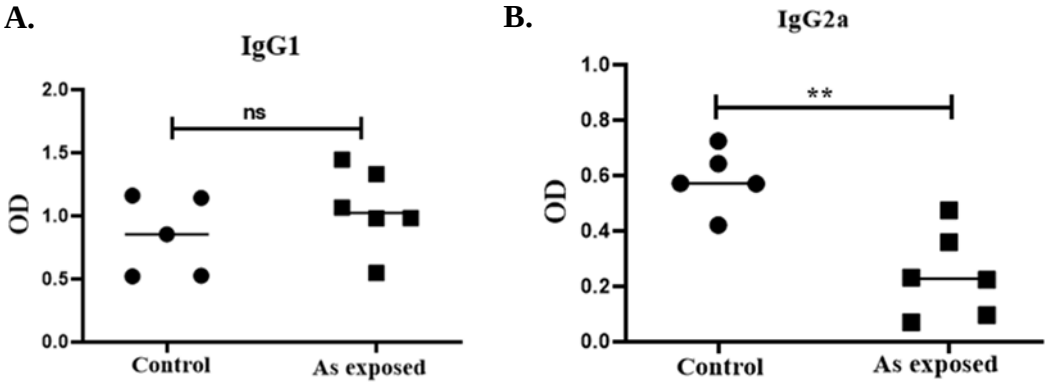


Figure 2.2: Effect of prenatal Arsenic Trioxide exposure on circulating levels of IgG in serum. (A) IgG1 **(B)** IgG2a. Sera was collected at 28- day age and diluted (10-3). Values shown are mean ± SEM. Data representative of 5 mice per group (n=5/group). Significant different values from control at **p< 0.01.

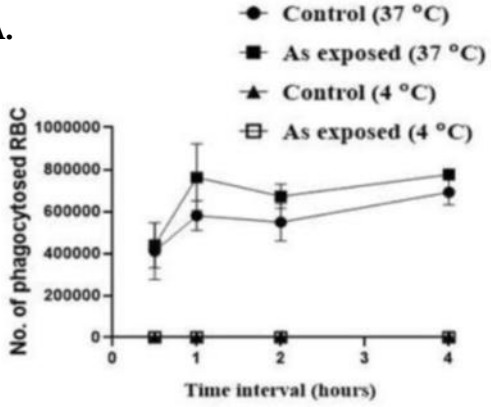
3. **Prenatal Arsenic exposure did not induce generalized defects in macrophage (Mϕ) function.**

Since the phagocytic cell or macrophages (Mϕ) play an important role in the innate immunity and also act as an antigen presenting cell (APC), we have considered four parameters of immunological importance to assess Mϕ function: phagocyte ability, cytokine production in response to LPS stimulation, expression of cell surface Class II MHC molecule and their T-cell stimulating ability in an MHC II-restricted manner. The Mϕ were harvested from the peritoneal cavity of exposed and age matched normal. The phagocytic ability of Mϕ of exposed and normal groups were studied in

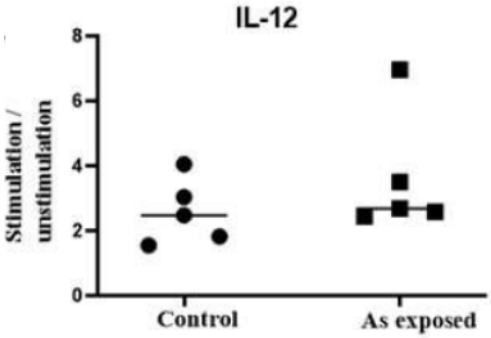
terms of the uptake of opsonized RBC as a function of time up to 4 hours at 37ºC and also 4ºC. It was observed that RBC uptake was increased by 1 hour and remained unaltered thereafter.

There was no significant difference observed between the exposed group and normal counterpart. As a control, the identical experiment was carried out at 4ºC and there was no uptake of RBC by Mϕ (Fig 2.3A). Mϕ is a major source of wide variety of cytokines to steer immune parameters. Here we studied two important cytokines which are immunologically relevant like IL-12 and TNF-α production from Mϕ upon stimulation with LPS. There was no significant difference in cytokine production between the two groups (Fig 2.3B & Fig 2.3C). Mϕ plays an important role as an antigen presenting cell (APC). The Class II MHC expression in the Mϕ was studied in the two groups and there was no significant difference in the expression of class II MHC transplantation antigen was observed and the frequency of Ad positive cells was compared (Fig 2.3D). The T-cell stimulating ability of the Mϕ was studied in terms of their ability to present LACK protein to anti-LACK specific T-cell Hybridoma and resulting IL-2 production. There was no significant difference in IL-2 production was observed from T-cell hybridomas regardless of the source of Mϕ either from normal or exposed mice as APCs (Fig 2.3E).

A.



B.



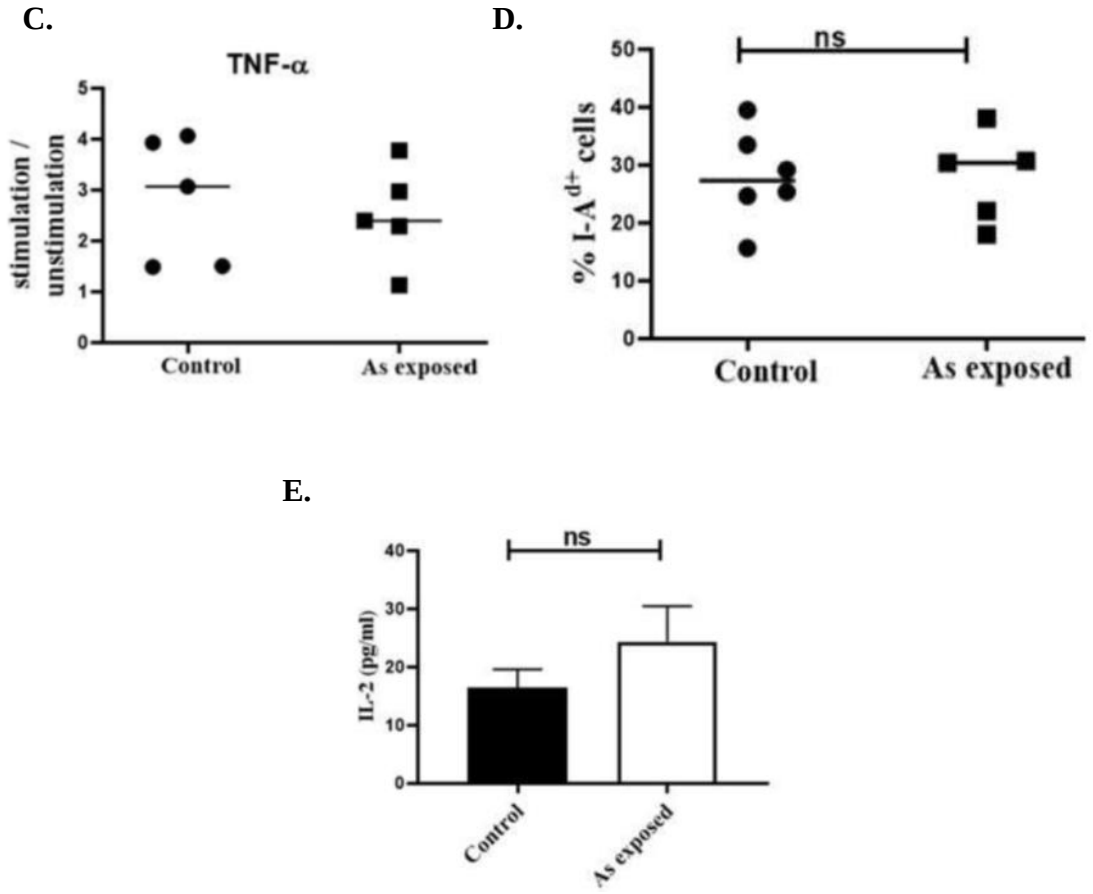


Figure 2.3: Effect of prenatal Arsenic Exposure on macrophage function. (A) Mϕ (2.5 x 10^5 cells/ml) were incubated with opsonized human RBC (5X) at 37ºC and 4ºC respectively. The cells were lysed and phagocytosed RBC then measured by a colorimetric assay. Data presented as number of RBC phagocytosed/50,000 Mϕ. (B) IL-12 and (C) TNF-α production by Mϕ after treatment with LPS (10 μg/ml) . Data shown is ratio of cytokine production in absence and presence of LPS (represented as ratio between stimulated/unstimulated). (D) Mϕ cell surface expression of I-Ad as measured by flowcytometry. (E) T-cell stimulating ability of Mϕ , i.e., ability to stimulate T cell hybridoma LMR7.5 in presence of LACK (Leishmania-activated C-kinase receptor) antigen. Extent of stimulation presented in terms of hybridoma IL-2 production.

4. **Prenatal Arsenic exposure reduces levels of CD4+ and CD8+ T-cells in spleen:**

The absolute numbers of CD4+ and CD8+ T-cells in spleen were evaluated. There was a significant decrease in the frequencies of splenic CD4+ and CD8+ T-cells in pups that were exposed to Arsenic prenatally (Fig 2.4A). There was almost 20% reduction in CD4+ T-cells (Fig 2.4B) and almost 5% reduction in CD8+ T-cells (Fig

2.4C) when compared with splenic cells of control pups.

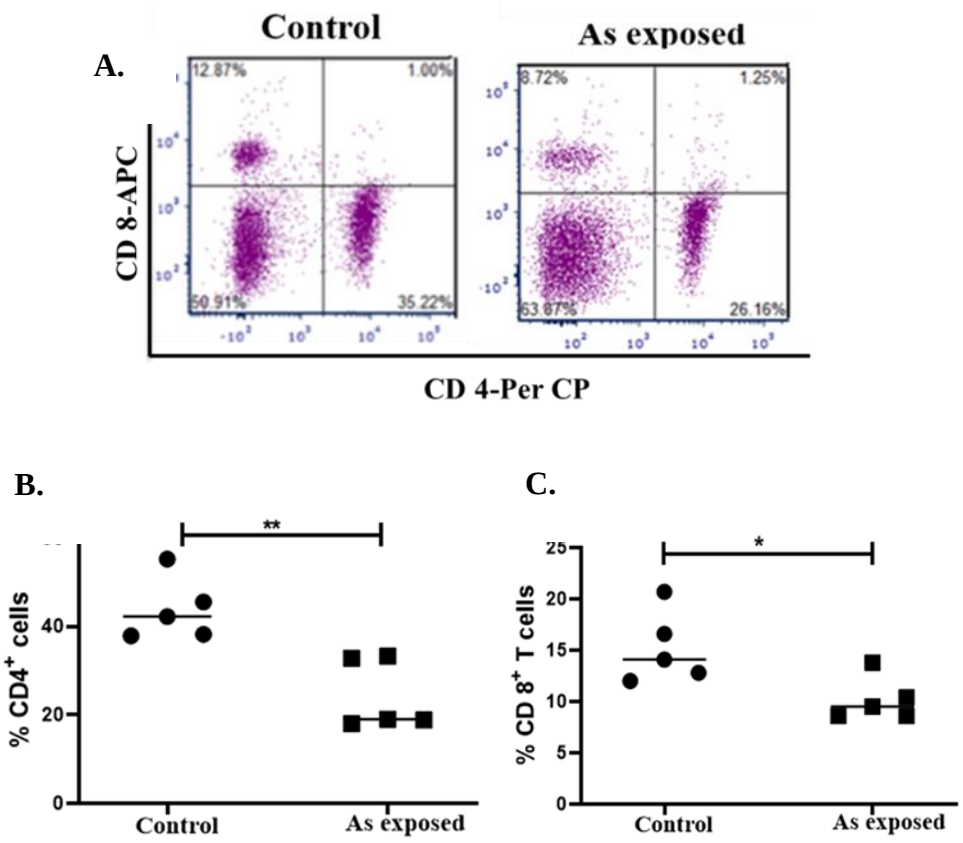


Figure 2.4: Effect of prenatal Arsenic exposure on CD4+ and CD8+ T-cell frequencies in the spleen. Splenocytes were collected from mice (28 days age) and were stained with antibodies and the populations were evaluated by flow cytometry. **(A)** Dot plot representation of splenic CD4+ and CD8+ T-cells in control and prenatally Arsenic exposed pups. Percentage of **(B)** CD4+ T-cells and **(C)** CD8+ T-cells (n=5/group). Significantly different values from control at *p< 0.05 and **p< 0.01.

5. **Prenatal Arsenic exposure affects Splenic CD4+T-cell responses to allo-stimulation in Mixed Leukocyte Reaction (MLR):**

A one-way mixed-lymphocyte reaction (MLR) was carried out wherein splenic CD4+ T-cells were derived from prenatally As exposed or unexposed of BALB/c pups were incubated for 48 hours with peritoneal Mϕ from allogenic normal C57BL/6 mice, and then levels of select T-cell-derived cytokines Il-2 and IFN-γ were evaluated. The results show that allogenic stimulation caused significant production of IL-2 and IFN-γ compared to syngenic coculture. With allogenic stimulation the T-cells isolated

from prenatal Arsenic exposed pups showed almost 50% less IL-2 production compared to the T cells isolated from the control counterparts (Fig 2.5A). Furthermore, there was also almost 30% reduction in IFN-γ production from T-cells of offspring that were exposed to Arsenic prenatally when compared to those of control pups (Fig 2.5B).

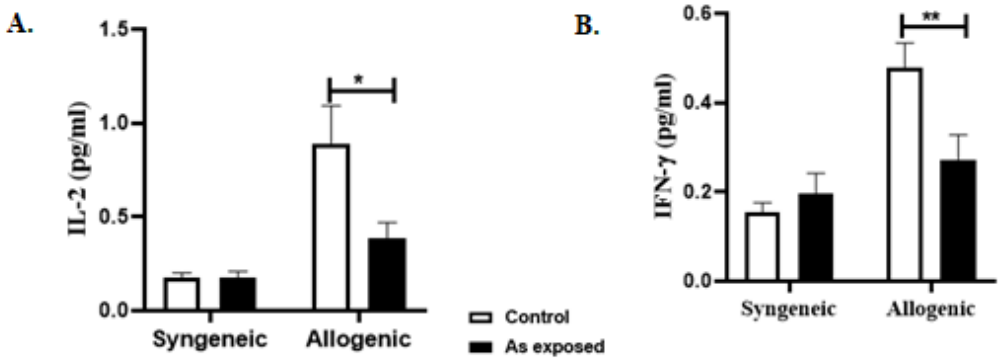


Figure 2.5: Production of cytokines from one way MLR. T-cells from control and prenatally Arsenic exposed pups were used as responder cells and peritoneal macrophages (PEM) from C57BL/6 were used as stimulators. The peritoneal macrophages (PEM) were isolated from C57BL/6 mice were treated with mitomycin C (10 µg/ml) for 6 hours, then purified CD4+ T-cells from control or prenatal Arsenic exposed Balb/c pups were added in a l: 5 (PEM: T-cell) ratio. A control assay was performed where splenic T-cells were mixed with PEM of Balb/c (syngenic) mice. After 48 hours the supernatant was collected and analyzed for levels of IL-2 and IFN-γ. Results indicate both syngenic and allogenic cultues of **(A)** IL-2 and **(B)** IFN-γ. Values represented are mean ± SD. Significantly different values from control at *p< 0.05 and **p< 0.01.

6. Prenatal Arsenic exposure affects splenic T-cell marker activation:

Splenic cells were harvested from control and prenatally Arsenic exposed pups to evaluate the expression of T-cell activation markers like CD25, CD44 and CD69. Expression of the aforementioned markers was evaluated in context of syngenic and allogenic co-cultures. The T-cells isolated from control pups showed significant increase in CD44 and CD69 expression when mixed with allogenic peritoneal macrophages (PEMϕ) whereas this increase was significantly reduced in T-cells from prenatally Arsenic pups (Fig 2.6A & Fig 2.6B). Consequently, the percentage of

double positive CD44+CD69+ cells were also significantly reduced in prenatally Arsenic exposed pups (Fig 2.6C). However, there was no difference observed in CD25 expression between the two groups (Fig 2.6D & Fig 2.6E).

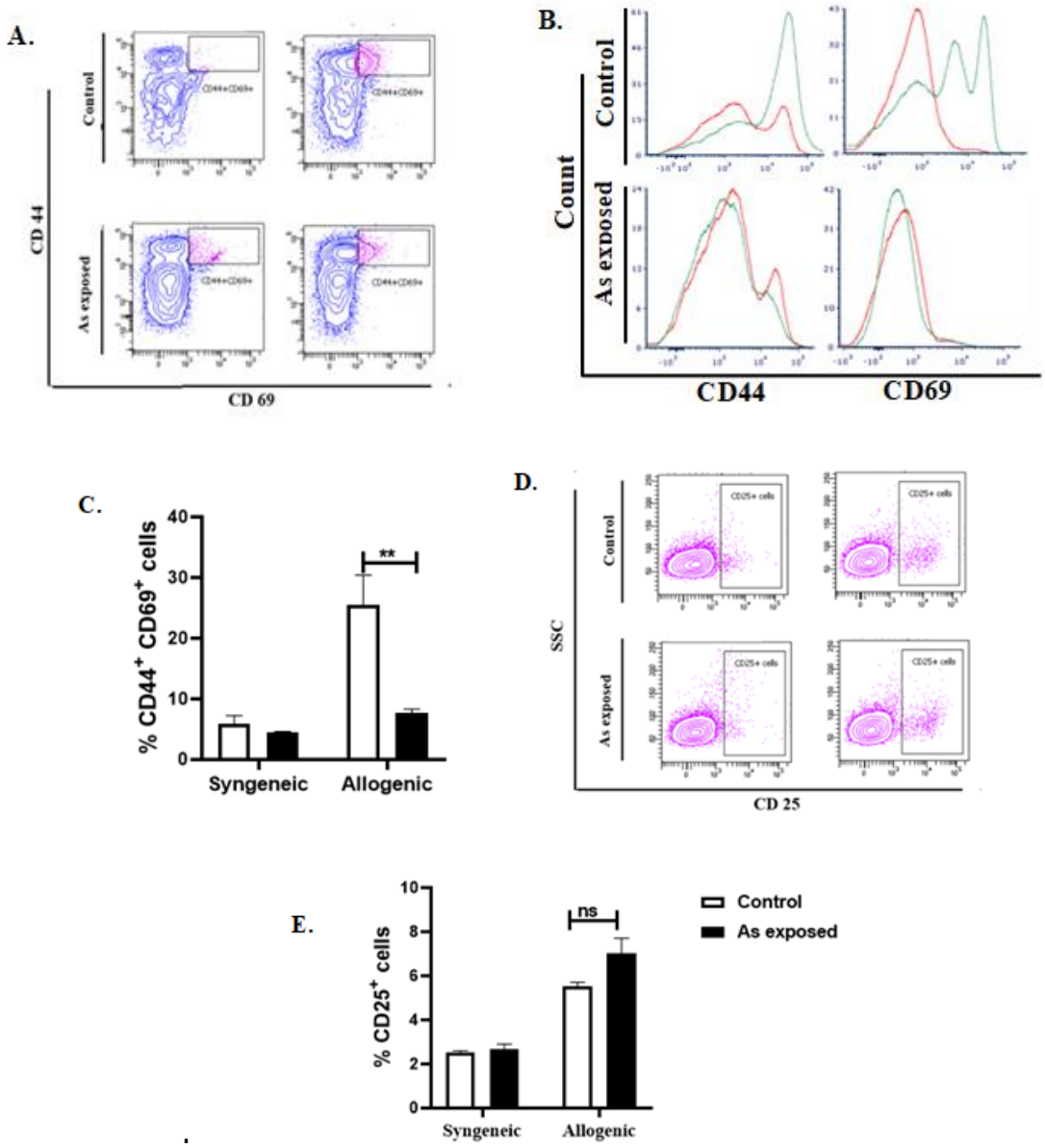


Figure 2.6: Effect on T-cell activation markers. Splenic T-cells were isolated from both control and prenatally Arsenic exposed groups and co-cultured with allogenic PEMϕ. After 24 hours of incubation, CD44 and CD69 expression was evaluated while CD25 expression was assessed after 72 hours. **(A)** Cd44+ and CD69+ expressions in syngenic and allogenic reactions (contour plot) **(B)** Histograms of stimulated CD44 and CD69 expressions. Red= syngeneic reactions Green= allogeneic reactions. **(C)** Percentages of CD44+CD69+ T-cells in syngeneic and allogeneic co-culture **(D)** CD25 expressions in syngeneic and allogeneic co-cultures (contour plot). **(E)** Percentages of CD25+ T-cells in syngeneic and allogeneic co-cultures. Values shown

are mean ± SE (n=5/group). Significant different values from control at **p< 0.01.

7. Prenatal Arsenic exposure reduces host response to infection:

To obtain a more complete evaluation of the effect on immunocompetency a host resistance to a pathogen challenge was carried out in pups from both control and prenatally Arsenic exposed pups. Mice from both groups were infected with 10^8 viable *E.coli* (strain E14) intraperitoneally and bacterial load was evaluated 24 hours later. Bacterial load was expressed as CFU/gram liver (Fig 2.7A), CFU/gram spleen (Fig 2.7B) and CFU/ml peritoneal wash (Fig 2.7C). It was observed that bacterial loads were significantly higher (almost 20%) in the pups that were exposed to Arsenic prenatally in all the sites that were evaluated when compared to their normal counterparts.

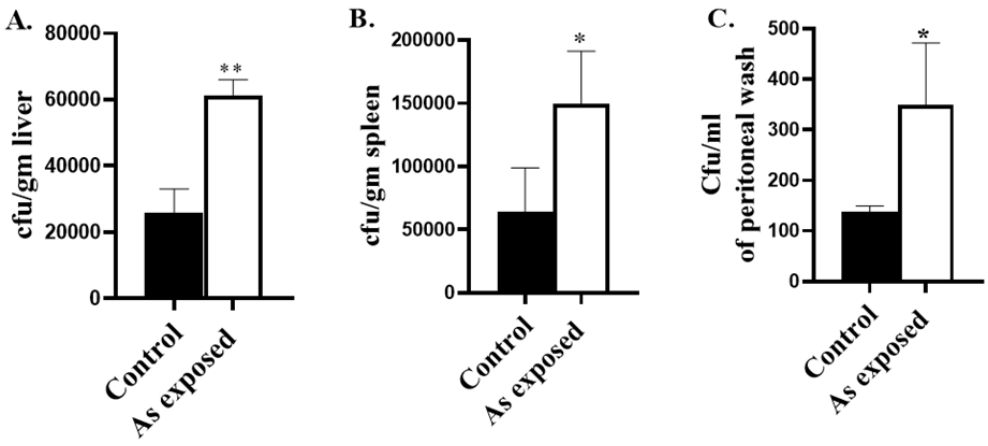


Figure 2.7: Effect of prenatal Arsenic exposure on host susceptibility to septicemic *E.coli* infection. Mice in both groups were infected intraperitoneally with 10^8 septicemic E. coli strain E14. The mice were then evaluated 24 hours post-infection. (A) Liver and (B) Spleen were isolated and homogenized and plated to evaluate Colony Forming Unit (CFU) per gram. (C) Peritoneal cavity lavage was also plated. *E. coli* cfu in each organ or cavity lavage are reported as mean ± SE. Significantly different values from control at *p< 0.05 and **p< 0.01.

Discussion

There are ample experimental and epidemiological evidences to suggest that exposure to Arsenic have detrimental consequences to human and animal health (Abdul et al., 2015; Santra et al., 2000). The duration of exposure and the levels of Arsenic in contaminated food or water often dictate the severity of the ill-effects. Evidences have indicated that children who have been exposed to Arsenic in utero had significantly low White Blood Cell (WBC) count (A. Saha et al., 2013) and animals with early life Arsenic exposure had significantly low resistance to influenza infections (Ramsey, Foong, Sly, Larcombe, & Zosky, 2013).

Gestation is a critical period for the development of the immune system (Dietert & Piepenbrink, 2006) hence a developing fetus becomes very vulnerable to toxic insult which could result in improper development and lead to various diseases later in life. We wanted to address a specific point i.e. how prenatal exposure to Arsenic affect the general immune repertoire (innate, cellular and humoral immunity) of an offspring and whether any developed immunomodulation persisted in post natal health and whether that lead to changes in resistance to pathogenic challenge.

We studied any potential immunotoxic effects that may arise from prenatal exposure to Arsenic in immune system organs spleen and thymus. There were no changes observed in their weight or histological architecture. There were also no changes observed in the structure or organization of the splenic white pulp nor did the overall frequencies of nucleated cells change in pups that were exposed to Arsenic prenatally. (Fig 2.1B, Fig 2.1C and Fig 2.1D). Prenatal Arsenic exposure also did not affect body weight of the pups (Fig 2.1A). These observations do not concur with studies in preschool children where Arsenic exposure has led to changes in the thymus which were the result of oxidative stress and apoptosis (Ahmed et al., 2012). This discrepancy can be explained that the study mentioned was monitoring the effects of ongoing Arsenic exposure, while in our study the pups were exposed to Arsenic well before they were studied for endpoint analysis.

However, a significant decrease in the frequency of splenic CD4+ and CD8+ T-cells were observed (Fig 2.4A & Fig 2.4B). The reduction in frequencies of CD4+ cells

was more pronounced than reduction in CD8+ cells. It is possible that any degree of change in the architecture of the white pulp may not reflect any change until there is a dramatic fall in T-cell levels. We also studied whether prenatal Arsenic exposure had any potential harmful effects on B-cell related outcomes in the offspring. Circulating levels of IgG1 and IgG2a levels in the serum were measured in both control and prenatally Arsenic exposed pups as these isotypes have been established and accepted as markers for T_H1 and T_H2 responses. Prenatal arsenic exposure had no effects on circulating IgG1 levels (Fig 2.2A), however, there was a significant decrease in circulating IgG2a levels in the serum of prenatally Arsenic exposed pups suggesting detrimental consequences to T_H1 responses.

Macrophages (MΦ) are the major cells in innate immunity that play an important role in many functions, including antigen presentation and activation of T-cells. The innate function of Mϕ of prenatally arsenic exposed mice was evaluated by phagocytosis of opsonized RBC as a function of time. It was observed that there was no difference in capability of phagocytosis between control and prenatally Arsenic exposed pups (Fig 2.3A). There was also no difference observed in release of cytokines TNF-α and IFN-γ from macrophages in between two groups in response to LPS stimulation (Fig 2.3B & Fig 2.3C) or expression of MHC Class II molecules on the surface of peritoneal macrophages (Fig 2.3D). MΦ isolated from prenatally Arsenic exposed pups also showed no difference in T-cell stimulating ability as measured by IL-2 production when stimulated by anti-LACK specific T-cell Hybridoma when compared to that of control pups (Fig 2.3E). Thus these results indicate that prenatal Arsenic exposure does not induce any defects in the function peritoneal macrophages (PEMΦ) with respect to their T-cell stimulating ability.

In contrast, when peritoneal macrophages (PEMΦ) from allogenic C57BL/6 mice were used to stimulate T-cells from prenatally Arsenic exposed pups; they failed to produce IL-2 and IFN-γ. (Fig 2.5A & Fig 2.5B). There was also a considerably significant reduction in inducible T-cell markers CD44 and CD69 (Fig 2.6A, Fig 2.6B &Fig 2.6C). Curiously, there was no change observed in CD25 expression between two groups. Thus it is evident that not all cell surface molecules are affected by prenatal Arsenic exposure. This can be explained by the difference of assembly of molecules in the lipid bilayer. Although we have not studied the role of prenatal

arsenic exposure on modulating cell membrane fluidity, there are reports that state that arsenic induced stress can change the fluidics of the cell membrane (Ghosh, Bhadury, & Routh, 2018). We also evaluated whether prenatal arsenic exposure had any overall effect on immunocompetency of the pups in response to any pathogenic challenge. Control and prenatally Arsenic exposed pups were infected with 10^8 septicemic E.coli (strain E14). Prenatal arsenic exposure resulted in increased bacterial burden in spleen, liver and peritoneal wash (Fig 2.7A, Fig 2.7B & Fig 2.7C). Thus we conclude that prenatal Arsenic exposure hampers the development of T_H1 responses which is essential for cell mediated immunity and ultimately resistance against any pathogens. Thus, a defect in T-cell repertoire development due to prenatal Arsenic exposure results in increased bacterial burdens in several organs/sites in Arsenic-exposed mice, even when the exposure had only been prenatally. This clearly indicates to us that Arsenic induced a generalized defect in immune function that persisted – even in the absence of any further Arsenic exposures.

Chapter-3

Prenatal Arsenic Exposure reduces gut butyrate production and enhances gut permeability by miR122-Occludin pathway

Introduction

Arsenic is a very highly toxic environmental pollutant that arises out of both natural and anthropogenic causes. It affects almost 200 million people worldwide, especially in south east Asian countries like India, Bangladesh, Nepal, Pakistan and China (Mukherjee et al., 2006). Arsenic has known to been readily absorbed through the placenta, thus it increases the risk of mortality in infants or it may lead to various health risks or impaired intellectual development leading to associated impacts later in life. Since contaminated food and drinking water are the major sources of intake of arsenic in humans and animals, the gastrointestinal tract therefore becomes the first location of exposure to the metalloid and its effects. Many studies have reported that exposure to arsenic causes alteration of gut microbial composition (Chi et al., 2017; K. Lu et al., 2014). Children in arsenic affected areas have been also known to have altered gut microbial composition (Dong et al., 2017).

The gut microbiome consists of trillions of symbiotic bacteria in the gastrointestinal tract where they play a major role in metabolism (Tremaroli & Backhed, 2012; Zhang et al., 2010). The gut microbiota has many faceted utilities that it carries out for the host like digesting food fibers like cellulose and starch that cannot be directly utilized by the host and producing Short Chain Fatty Acids (SCFA's) as a result of fermentation mainly butyrate, acetate and propionate. These metabolites provide energy to the colonic epithelial cells thus cementing the important role of gut microbiome in homeostasis and development of the gut immune system (den Besten et al., 2013; P. M. Smith et al., 2013). However, the gut microbiome is highly susceptible to many xenobiotics such as heavy metals which affect its composition and ultimately its function. Perturbations of the gut microbiome from environmentally induced factors have been linked with multiple diseases such as obesity and diabetes. Thus the interaction of the xenobiotics and gut microbiome can play a substantial role in the metabolism and diseases that may arise from such changes.

Maternal-offspring exchange of microbiota play a very crucial role in development and function of the neonatal microbiome (Dong et al., 2015). There are epidemiological evidences that suggest that antibiotic usage during pregnancy can be linked with an increased incidence of juvenile obesity and asthma (Mor et al., 2015;

Stensballe, Simonsen, Jensen, Bonnelykke, & Bisgaard, 2013). Studies also indicate that children born to mothers suffering from ulcerative colitis during pregnancy tend to develop a higher risk of childhood illnesses (Hashash & Kane, 2015). Thus these observations nudge us to investigate the potential impact of prenatal arsenic exposure on the gut microbial composition and gut physiology in post natal life. Given that the mother's microbiome plays an important role in the establishment of the neonatal microbiome, it is reasonable to assume that alterations, no matter how subtle it is, to the microbiome in early life may contribute to disturbances in the physiological functions in the gut and lead to many disorders in adult life.

Short Chain Fatty Acids (SCFA), as mentioned above, are an important metabolite that is secreted by the gut microbiome as a result of fermentation of dietary fibers. Secretion of SCFA is known be hampered by arsenic exposure (Chi et al., 2017). Butyrate, one of the most important SCFA plays vital roles in many bodily functions which includes development of the immune system (Schulthess et al., 2019; Yip et al., 2021). Butyrate has also been known to down-regulate inflammation by inhibiting growth of pathogenic bacteria and increasing barrier integrity of the mucosa (J. Chen & Vitetta, 2020; Okumura et al., 2021). Butyrate also encourages the growth of obligate anaerobic bacteria and decreases the availability of oxygen in the gut (J. Chen & Vitetta, 2020; Kelly et al., 2015). In vitro studies with butyrate also show that it enhances intestinal barrier function by increasing the transepithelial electrical resistance (TEER) while simultaneously decreasing insulin permeability in Caco-2 cells (Peng, He, Chen, Holzman, & Lin, 2007; Peng, Li, Green, Holzman, & Lin, 2009). Studies have shown that butyrate can correct defective intestinal tight junction barrier which is linked to many diseases like obesity, non- alcoholic steatohepatitis (NASH), non-alcoholic fatty liver disease (NAFLD) and Inflammatory Bowel Disease (IBD) (Coppola, Avagliano, Calignano, & Berni Canani, 2021; Endo, Niioka, Kobayashi, Tanaka, & Watanabe, 2013; Silva et al., 2018). Reports also show that butyrate enhances intestinal barrier by regulating the assembly of tight junction proteins like Occludin (Peng et al., 2009).

Occludin has four transmembrane domains and is highly expressed at cell-cell contact sites. It also plays an important role in the assembly and maintenance of tight junctions (Al-Sadi et al., 2011). Occludin knockout mice is shown to exhibit elevated inflammation, retardation of growth and hyperplasia (Saitou et al., 2000).

Dysregulation of tight junctions have been known to contribute to various diseases such as Inflammatory Bowel Disease (IBD) and colon cancer (Casalino et al., 2016). Expression of tight junction proteins at the cellular level has been known to be governed by microRNAs and small regulatory RNAs (Ye, Guo, Al-Sadi, & Ma, 2011). MiR122 is one of the microRNA that promotes the decay of Occludin mRNA by binding to its 3' UTR (Jingushi et al., 2017; W. Yang et al., 2022). Mir122 is found abundantly in the liver but also has been reported to be found in gut where it plays a vital role in colon cancer by targeting NOD2 (Li et al., 2019).

In this study we investigate the effect of prenatal arsenic exposure on the gut microbial composition, metabolite production and intestinal permeability and expression of tight junction proteins in post natal life, thus attempting to understand any changes that may arise from this exposure which could lead to any disease in adulthood.

Results

1. **Direct Arsenic Trioxide (As_2O_3) exposure in drinking water decreases abundance of *Firmicutes* while increasing the abundance of *Bacteroidetes* in female mice.**

Female Balb/c mice were fed water laced with 4 ppm Arsenic Trioxide (As2O3) since the time of their breeding and this treatment continued till parturition. Another set of mice were fed arsenic free clean drinking water and they served as control. We investigated whether arsenic would affect the abundance of the *Firmicutes* and *Bacteroidetes* and consequently F/B ratio in the gut microbiome of the dams. The abundance of the phyla was analyzed by quantitative real time PCR using phyla specific primers. The relative abundance was calculated by normalizing with bacterial pan specific primer. We observed 2.5 fold decrease (p=0.04) in *Firmicutes* and 7 fold increase (p=0.0008) in *Bacteroidetes* in As-exposed dams (Fig 3.1A & 3.1B) resulting to 18 fold decrease (p=0.005) in F/B ratio compared to control (Fig 3.1C). Next we asked whether arsenic exposure during breeding and gestation would perturb the compositional profile of the gut microbiome of the offspring.

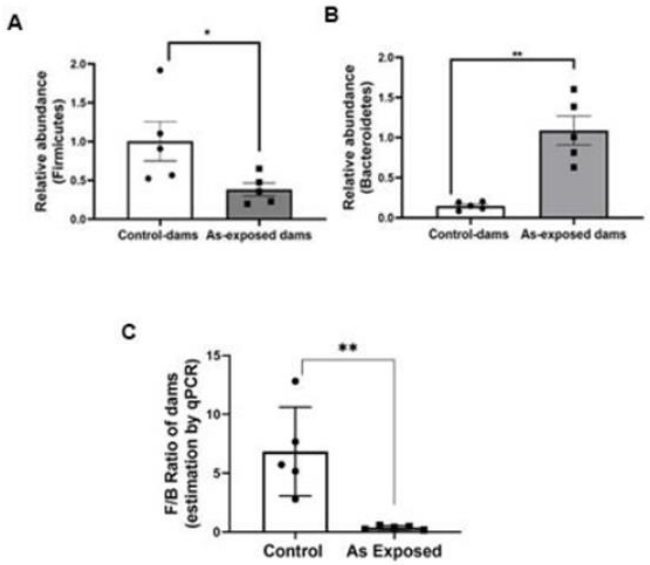
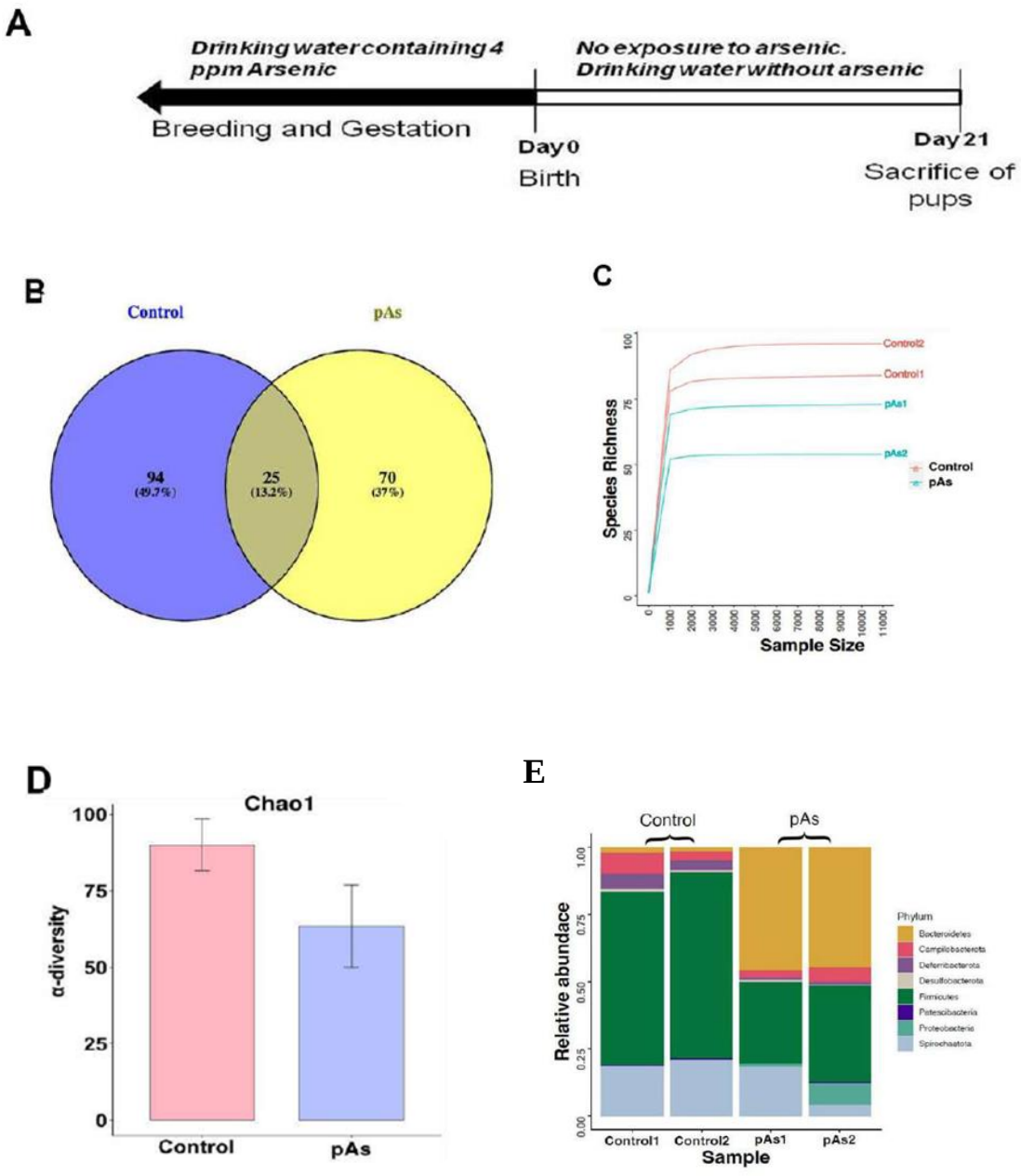


Figure 3.1: Effect of arsenic exposure on *Firmicutes* and *Bacteroidetes* abundance in female mice. (A) Relative Abundance of *Firmicutes* as quantified by qrtPCR **(B)** Relative abundance of *Bacteroidetes* as quantified by qrtPCR. **(C)** Firmicutes:Bacteroidetes (F/B) ratio. N=5 per group. Data represented as mean±SE. Values significantly different from control at *p<0.05, **p<0.01.

2. Prenatal Arsenic exposure alters normal composition of gut microbiome in mice:

The pups from arsenic exposed dams are henceforth referred to as pAs-exposed for convenience. The feces were collected from both the control and pAs exposed group on day 21 post birth as represented by the picture (Fig 3.2A). The Venn diagram (Fig 3.2B) represents the total number of Amplicon Sequence Variants (ASV's) that were identified after the analysis of the 16S-rRNA dataset that were sequenced from the feces of both the control and pAs exposed groups. Around 13.25 of ASV's were common in both groups whereas the control group and the pAs exposed group showed 49.7% and 37% of unique ASV's respectively. This observation indicates that pAs exposed mice have lost about 12.7% of ASV when compared to that of control mice. The predominant bacterial phyla in both the groups remained *Firmicutes* and *Bacteroidetes*. The microbial component of the gut microbiome was significantly lowered in pAs exposed mice when compared to that of control as it is reflected in the species rarefaction curve (blue-control; red-pAs exposed) (Fig 3.2C). The alpha diversity of the samples was measured by chao1 index. A decrease in alpha diversity

(chao1 index values for sample Control1=84, Control2=96, pAs1=73, pAs2=54) was observed in pAs mice when compared to that of control (Fig 3.2D). At the phylum level, the relative abundance of *Firmicutes* decreased significantly whereas the relative abundance of *Bacteroidetes* significantly increased in pAs exposed mice (Fig 3.2E & Fig 3.2F) resulting in a decreased *Firmicutes* (F) to *Bacteroidetes* (B) ratio (F/B ratio) in pAs exposed mice with respect to that of control (Fig 3.2G). The abundances of the phyla were then further validated by quantitative real time PCR by using phyla specific primers which corroborated our findings (Fig 3.2H & Fig 3.2I).



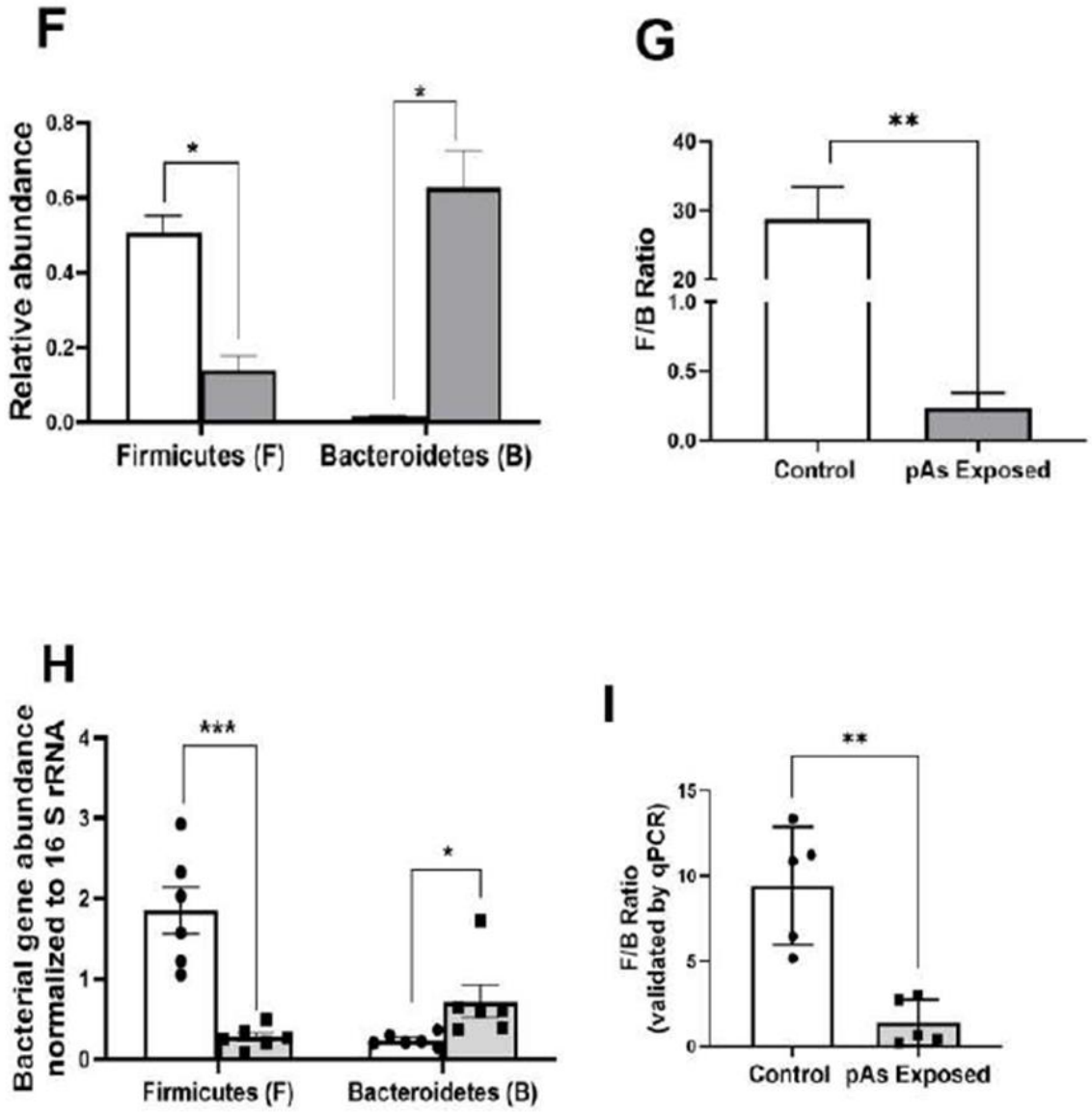


Figure 3.2: Effect of prenatal arsenic exposure on gut microbiome composition and diversity. (A) The experimental plan represented schematically. Adult Balb/c mice were bred in conventional manner by housing two females with one male and were given ad libitum access to water containing 4ppm Arsenic Trioxide (As_2O_3). After the birth of the pups, the mothers were then given arsenic free clean water. After the pups reached 4 weeks of age, their feces were collected at random. Feces from age matched control pups were also taken. **(B)** Venn- Diagram showing the number of microbial genera common and exclusive to control and prenatally As exposed mice (pAs-mice). **(C)** Species rarefaction curves presenting the number of bacterial species

in control and pAs mice. **(D)** The chao1 α-diversity index of control and pAs mice samples. **(E)** Major phyla of bacterial species in control and pAs exposed mice as represented by stacked histograms. **(F)** Relative Abundances of *Firmicutes* and *Bacteroidetes* in control and pAs exposed samples. **(G)** *Firmicutes: Bacteroidetes* ratio in control and pAs exposed samples. **(H)** Abundance of *Firmicutes* and *Bacteroidetes* as quantified by qPCR. (I) *Firmicutes: Bacteroidetes* ratio (F/B ratio) as measured from qPCR. (n=6/group for qPCR and n=2/ group for 16S rRNA sequencing). Data represented as mean±SE. Values significantly different from control at *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

3. Prenatal Arsenic Exposure results in reduced Short Chain Fatty Acid (SCFA) production:

From our aforementioned results it is evident that there has been a reduction in the amount of bacteria that produce Short Chain Fatty Acids (SCFA) in the gut of pAs exposed mice. Therefore, we resolved to estimate the amount of fecal SCFA in both control and pAs exposed mice by GC-MS. The decrease in relative abundance of Firmicutes correlated with a decrease in fecal SCFA in pAs exposed mice when compared to that of control. Important SCFAs like propionate, acetate and butyrate were decreased significantly in pAs exposed mice. There was a 4 fold ($p=0.0079$), 2 fold ($p=0.0049$) and1.7 fold ($p=0.025$) decrease in butyrate, aceatate and propionate respectively in pAs exposed group (Fig 3.3). Since SCFAs play vital roles in the gut which includes maintenance of intestinal barrier integrity, mucous production and protection against inflammation, it was imperative to study the gut physiology in pAs exposed mice.

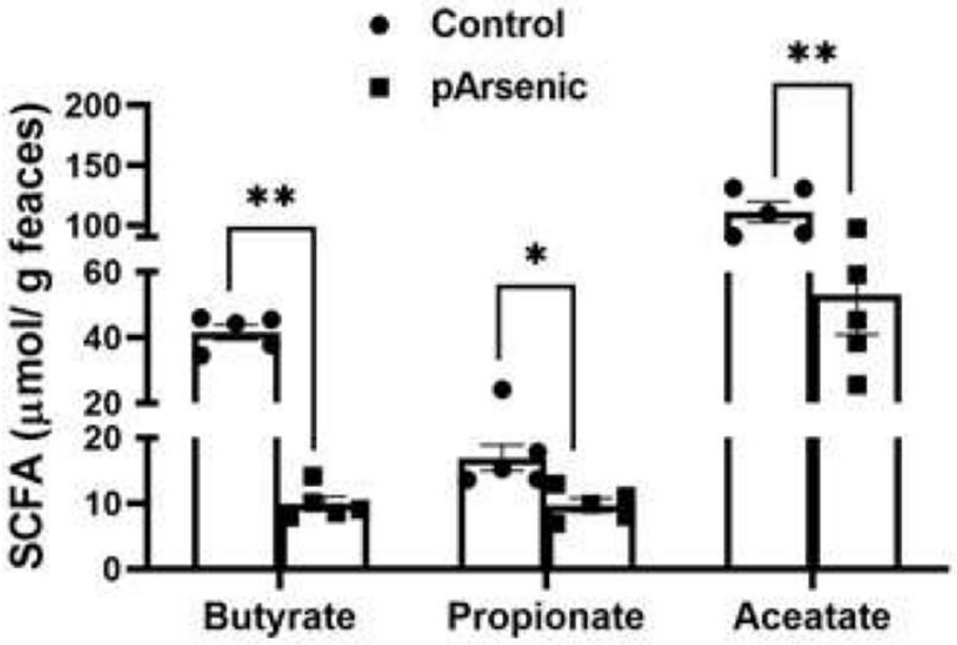


Figure 3.3: Effect of prenatal arsenic exposure on concentration of Short Chain Fatty Acids in feces. Concentration of butyrate, propionate and acetate was determined by GC-MS. Data represented as mean±SE (n=5/group) *p<0.05 and **p<0.01.

4. Prenatal Arsenic exposure results in increased intestinal permeability coupled with decreased expression of tight junction protein Occludin:

To assess gut physiology and barrier function histological slides and expression histological slides of colon and expression of tight junction proteins were studied. Gut barrier function was evaluated by permeability to FITC Dextran. There was a 3.9 fold increase (p=0.0079) in gut permeability in pAs-mice compared to the control-mice (Fig 3.4A). Evaluation of tight junction protein expression in the colon by quantitative real time PCR unearthed a 4 fold (p= 0.007) decrease in the expression of Occludin in pAs exposed mice whereas there was no change in expression of claudin-1, claudin-2, claudin-4, ZO-1 and JAM-A (Fig 3.4B). Histological evaluation of the colon revealed infiltration of inflammatory cells and goblet cells also showed hyperplasia in pAs exposed mice (Fig 3.4C).

Recalling our analysis of altered gut microbial composition and reduced SCFA production in gut, we sought to investigate whether this lack of SCFA production plays any important role in expression of Occludin. We also sought to investigate the

mechanism if any by which SCFAs influence the expression of occludin.

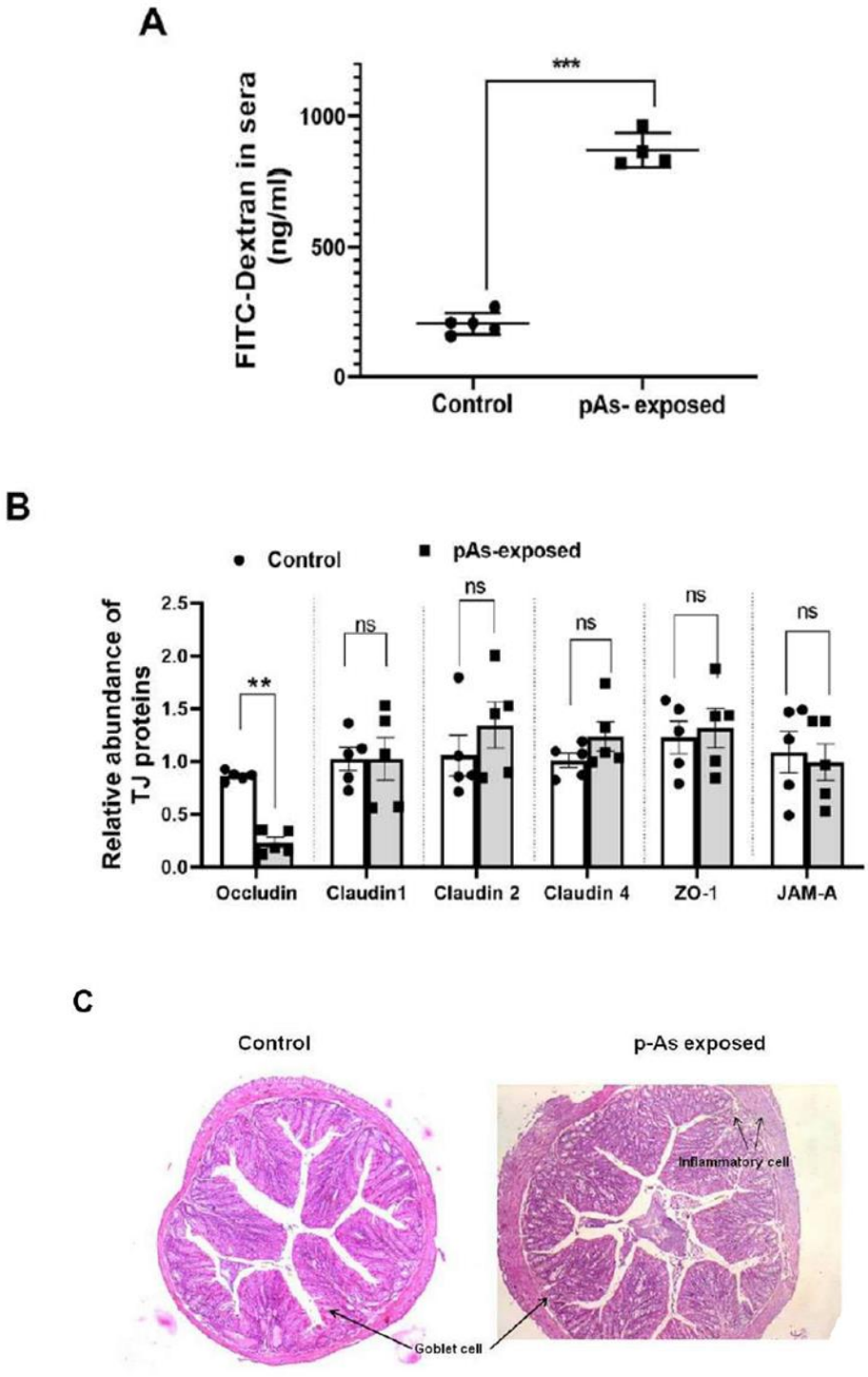


Figure 3.4: Effect of prenatal arsenic exposure on intestinal permeability and histoarchitecture. **(A)** Control-mice and pAs-mice were starved for 16 hours and then fed with FITC- Dextran 4000 (FD4) by oral gavage at a dose of 44 mg/100 g body weight. After 4 hours the blood was collected and serum was extracted to determine the fluorescence of Dextran in sera. **(B)** The expression of tight junction

proteins (Occludin, Claudin-1, Claudin-2, Claudin-4, ZO-1 and JAM-A) was determined by quantitative real time PCR. **(C)** Pictorial micrograph of H&E stained colon section (10X magnification). The arrows show neutrophil infiltration. Data represented as mean ±SE. N=5/ group. Values significantly different from control at **p< 0.01 and ***p<0.001.

5. *In vitro* treatment of Butyrate decreases permeability and increases Occludin expression in HT-29 cells:

HT-29, a colon carcinoma cell line was used for the in vitro experiments. HT-29 cells were treated with functional concentrations (0-20 mM) of acetate, propionate and butyrate. It was observed that treatment with 10 mM and 20 mM of butyrate increased expression of occludin 45% and 58 % respectively (Fig 3.5). Treatment with acetate and propionate did not have any effect on expression of occludin. This provided adequate evidence to state that butyrate but no propionate or acetate increase expression of occludin expression. To further decipher the mechanism of regulation of occludin expression we studied the effect of butyrate treatment on miR122 expression as it is known that miR122 binds to the 3'UTR region of the occludin mRNA, leading to its degradation (Jingushi et al., 2017).

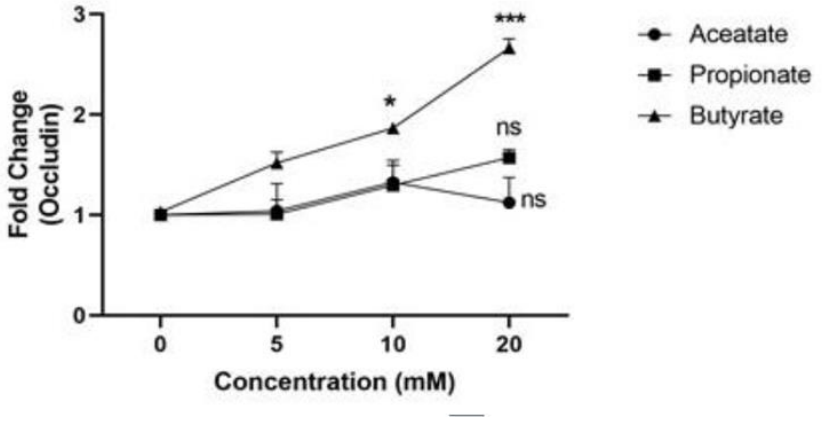


Figure 3.5: Expression of Occludin in HT29 cells treated with butyrate, propionate and acetate at varying concentrations (5mM, 10mM and 20mM) for 24 h as measured by quantitative real time PCR. Each treatment and the entire experiment were conducted in triplicates. Data represented as mean ± SE. Values significantly different from control at *p<0.05 and ***p<0.001.

6. ***In vitro*** **treatment with Butyrate reduced expression of miR122 in HT-29 cells:**

HT-29 cells were treated with butyrate and the expression of miR122 was evaluated by qrt PCR. It was observed that butyrate reduced expression of miR122 in a dose dependent manner (Fig 3.6). Treatment with acetate or propionate does not have any effect on the expression of miR122. Thus our results point towards an essential set of events which can be described as butyrate- miR122- occludin. This axis was further evaluated in our pAs exposed mice model.

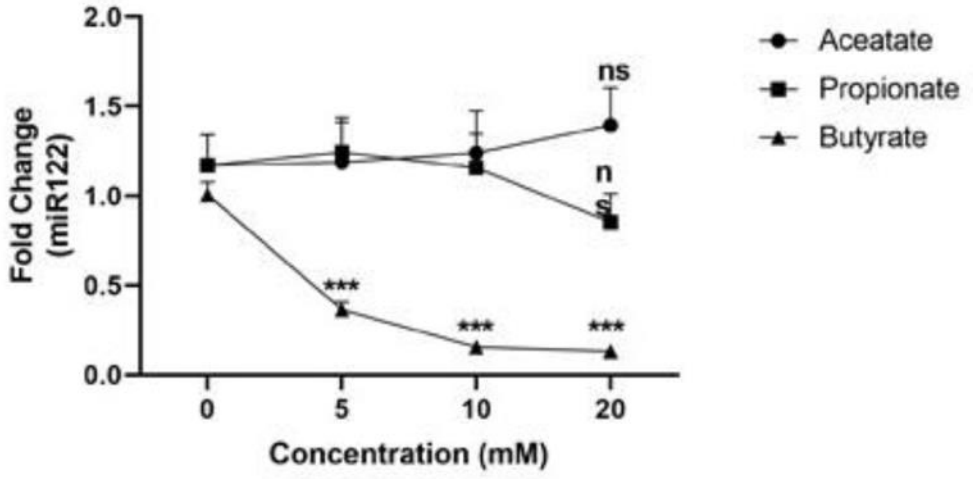
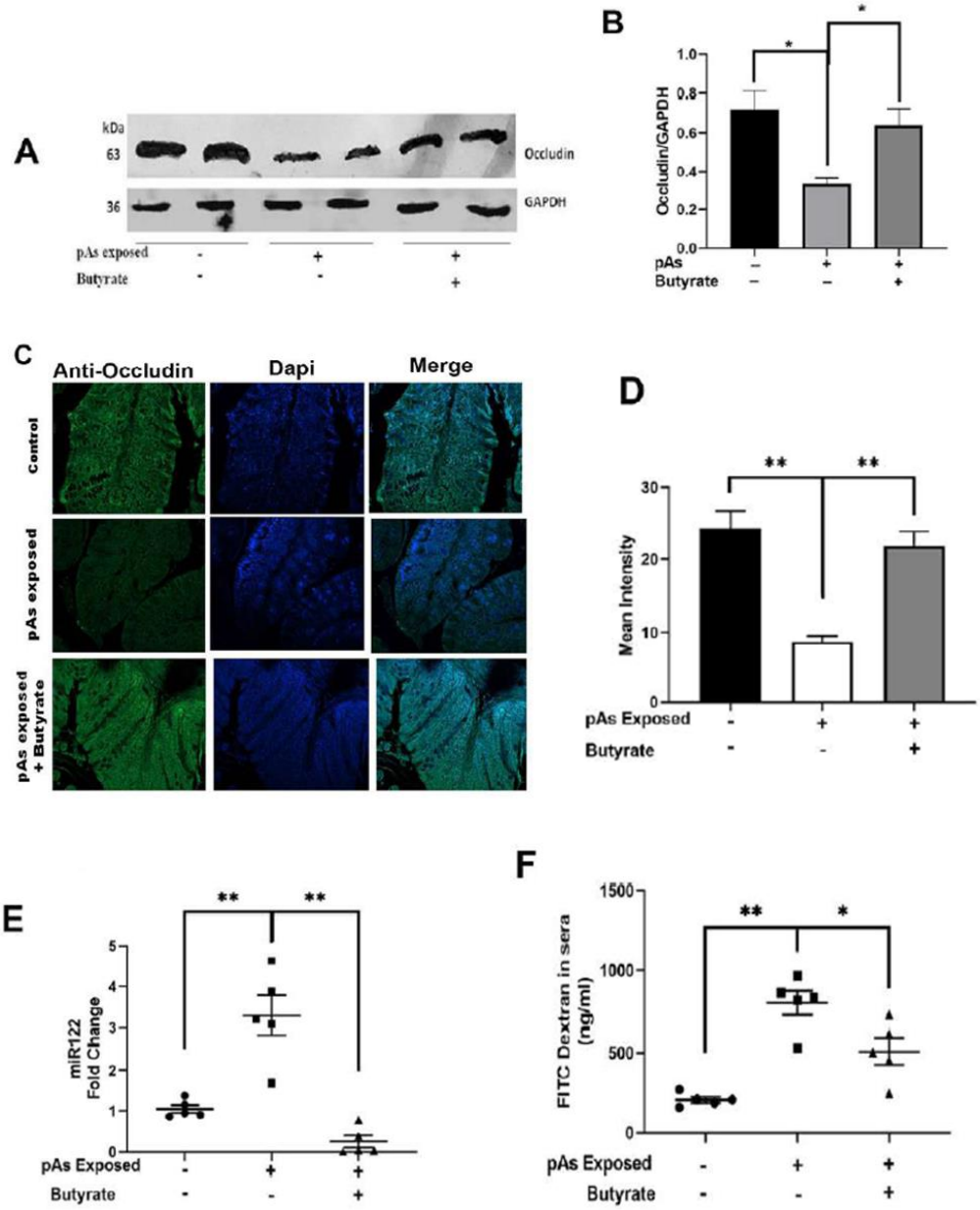


Figure 3.6: Expression of miR122 in HT29 cells treated with butyrate, propionate and acetate at varying concentrations (5mM, 10mM and 20mM) for 24 h as measured by quantitative real time PCR. Each treatment and the entire experiment were conducted in triplicates. Data represented as mean ± SE. Values significantly different from control at ***$p<0.001$.

7. **Oral supplementation with Butyrate recovers gut permeability by reducing expression of miR122 and increasing expression of occludin in mice exposed to arsenic prenatally:**

To investigate whether gut derived butyrate is intimately linked with the decrease in gut barrier function in pAs exposed mice we orally supplemented them with butyrate. The pAs exposed mice were fed 5% butyrate mixed with diet from day 21-28. The expression Occludin in the gut was evaluated in control, pAs exposed and pAs exposed+ oral butyrate supplemented mice. There was significant down regulation of occludin expression in the gut of pAs exposed mice which recovered with oral

supplementation of butyrate (Fig 3.7A& 3.7B). Immunohistochemical analysis also shows that expression of occludin was reduced 2.8 fold in pAs exposed mice and the expression was restored in pAs-butyrate mice (Fig 3.7C & 3.7D). The expression of miR122 showed a 3 fold increase in pAs exposed mice while it returned to the levels equivalent in control with oral supplementation of butyrate (Fig 3.7E). There was a significant increase in gut permeability in pAs exposed mice which was expected, while oral supplementation with butyrate decreased intestinal permeability (Fig 3.7F). Histological sections of the colon reveal that oral supplementation with butyrate inhibited the recruitment of inflammatory cells like neutrophils that were induced by pAs exposure (Fig 3.7G).



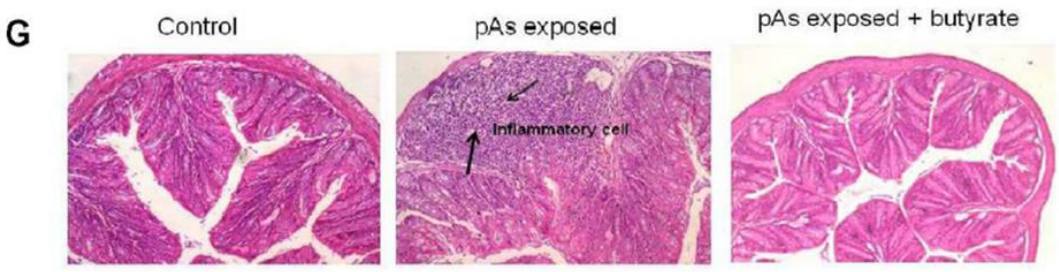


Figure 3.7: Effect of oral supplementation of butyrate on colon histoarchitecture and intestinal permeability of prenatally arsenic exposed mice. Expression of Occludin evaluated by **(A)** Western Blot and **(B)** corresponding densitometry **(C)** Immunofluorescence **(D)** Mean intensity. **(E)** Expression of miR122 was evaluated by qrt PCR. (F) Intestinal permeability as measured by the presence of FITC- Dextran 4000 (FD4) in serum. **(G)** Representative micrographs of colon sections (H&E; 20x magnification). Arrows indicating neutrophil infiltration. Data represented as mean ±SE. N=5/ group. Values significantly different from control at *$p<0.05$ and **$p<0.01$.

8. **Overexpression of miR122 reverses the effect of oral supplementation of butyrate in prenatally arsenic exposed mice:**

Previous results have indicated that butyrate downregulates the expression of miR122 while simultaneously increasing the expression of occludin. Thus it became imperative to confirm the hypothesis by over expressing miR122 in pAs exposed mice that were orally supplemented with butyrate. The miR122 expressing plasmid was injected through tail vein (25µg/mouse) in pAs exposed mice which were orally supplemented with butyrate while another group was injected with mock plasmid. The animals were sacrificed 4 days post injection. The expression of occludin and miR122 was evaluated in colon tissues of control-mice, pAs-mice and pAs-butyrate mice. Overexpression of miR122 in pAs-butyrate mice showed elevated levels of miR122 in colon; indicating successful overexpression (Fig 3.8A). Corresponding analysis of occludin expression in the colon of pAs-butyrate-mice showed significant decrease with miR122-plasmid injection compared to mock-plasmid injection as evaluated by qrt PCR (Fig 3.8B) and western blot (Fig 3.8C& 3.8D).

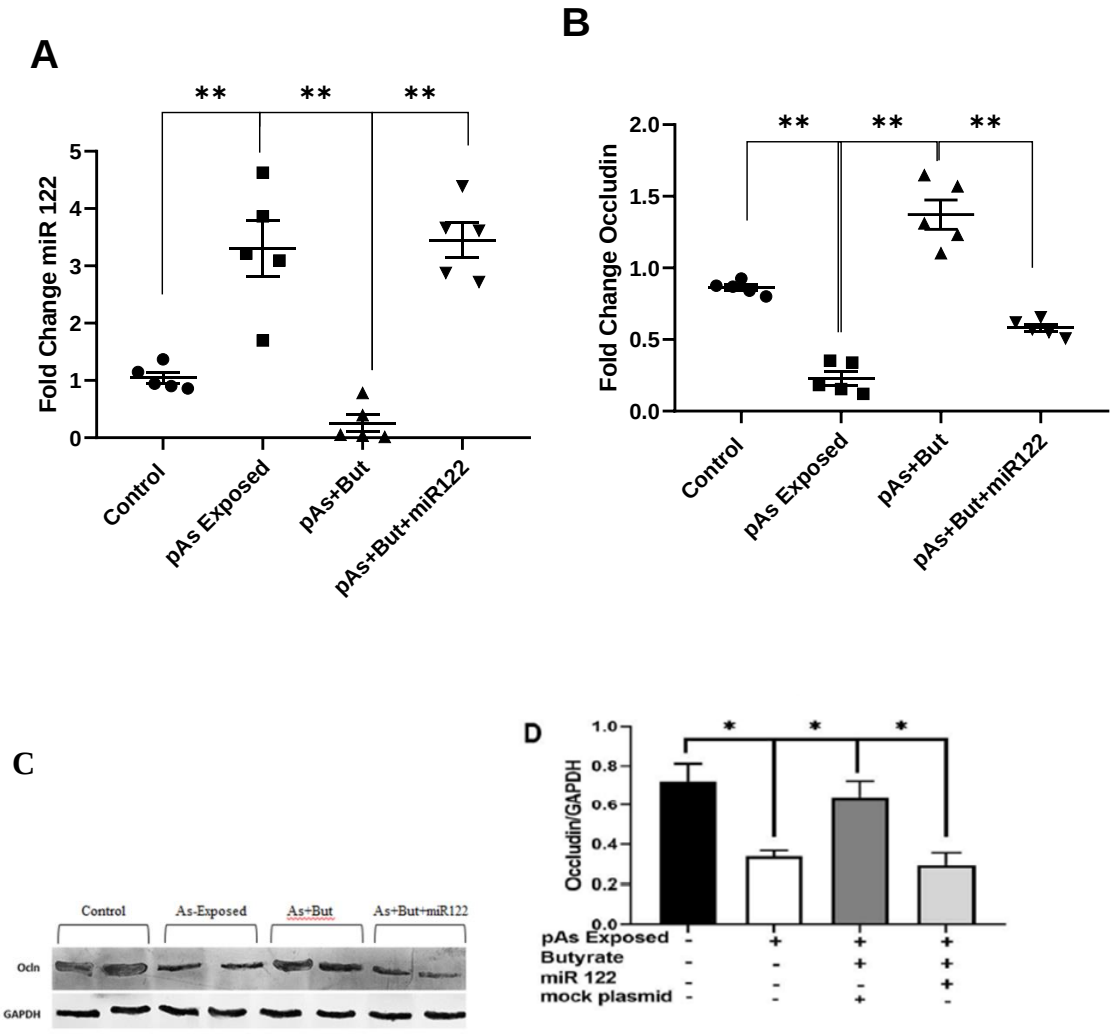


Figure 3.8: Effect of overexpression of miR-122 on Occludin expression in mice colon. Each butyrate treated pAs-mice were injected with either 25 µg in 100 µl of miR122 expressing plasmid or 25 µg in 100 µl of mock plasmid in tail vein. The mice were sacrificed 4 days post injection. Expression of **(A)** miR122 and **(B)** Occludin in colon tissues was evaluated by qrt PCR and **(C)** Western Blot and its **(D)** corresponding densitometry. N=5, the data is represented as mean ± SE. The experiment was repeated twice. Values significantly different from control at *p<0.05 and ** p<0.01.

Discussion

In this study we investigated whether prenatal arsenic exposure has any effect on the gut microbiome which in turn could lead to altered gut physiology. Studies have reported that levels of arsenic that are environmentally relevant indeed perturb the gut microbiome of mice decreasing the amount of *Firmicutes* and increasing the amount of *Bacteroidetes* while simultaneously also changing pattern and proportions of

metabolites particularly short chain fatty acids (SCFA) (Chi et al., 2017). We also validated this observation as our results show that oral treatment with arsenic did indeed decrease the proportion of *Firmicutes* and increase the proportion of *Bacteroidetes* in the dams. Since the transmission of the microbiome from mother to offspring plays a vital role, we compared the gut microbiome composition of mice that were exposed to arsenic prenatally with that of mice whose mothers were given clean arsenic free water. Overall, there was a loss of around 12.7% of ASV's (Amplicon Sequence Variant) in pAs exposed mice when it was compared to that of control mice. Arsenic has been known to prolong glycan residues of cell membrane glycoproteins in skin cancer cells (C. H. Lee et al., 2016). Glycosylation of the intestinal mucus and epithelium is a complex process that can change as a response to microbial colonization (Pickard, Zeng, Caruso, & Nunez, 2017). This was intriguing because host glycans have been known to serve as nutrient sources or adhesion receptors for microbes (Pickard et al., 2017). Therefore it leads us to speculate whether arsenic is able to reshape the gut microbiome composition of mice by changing the glycosylation pattern of receptor proteins in the epithelial cell membrane. The diversity of a community is dependent on the intensity of the sampling. Although the difference was not significant, Chao1 alpha diversity of the microbiome showed a decreasing trend in pAs exposed mice when compare with that of control. In some cases, microbial compositions rather than diversity can play a key role to determine the phenotype as it had been reported earlier (Worsley et al., 2021). Further analysis of the phylum composition of gut microbiota in pAs-mice and control-mice revealed a significant shift in the relative abundance of *Firmicutes* (F) and *Bacteriodetes* (B) as evidenced by metagenomic analysis and qrt PCR. We report a decrease in *Firmicutes* (F) and increase in *Bacteriodetes* (B) leading to consolidated decrease in F/B ratio in the gut communities of pAs-mice compared to control-mice mirroring the F/B ratio of the dams. A decrease in F/B ratio is a major marker for Inflammatory Bowel Disease (IBD) (Guo et al., 2021) therefore suggesting that there is a possibility that pAs exposed mice may suffer physiological dysfunction in the gut. The decrease in the abundance of *Firmicutes* has been known to be an indicator of a decrease in SCFA in the gut (den Besten et al., 2013). Likewise, we did indeed observed significant decrease in all SCFAs including acetate, propionate and butyrate in the feces of pAs exposed mice. Interestingly, the decrease in butyrate was more pronounced than the decrease in acetate and propionate. Disruption in bacterial

community and metabolite has a long lasting effect on gut physiological functions for example SCFAs such as butyrate play a vital role in many physiological processes in the host (Canani et al., 2011). Butyrate serves as an important energy source for the intestinal epithelial cells (B. Singh, Halestrap, & Paraskeva, 1997) while maintaining colon homeostasis (Gasaly, Hermoso, & Gotteland, 2021) and inhibiting inflammation (Segain et al., 2000). The gastrointestinal epithelium is the site of the body's largest interface with the external environment (Groschwitz & Hogan, 2009). It is a highly effective barrier that limits the permeation of luminal antigens and toxins through the mucosa (Suzuki, 2013). The location of the intestinal epithelium is such that a breach in the mucosal barrier would lead to mucosal inflammation (R. Ahmad, Sorrell, Batra, Dhawan, & Singh, 2017). Studies with knockout mice have revealed that tight junction proteins play a key role in permeability and contribute to the overall barrier function of the mucosa and ultimately maintenance of mucosal immune homeostasis (R. Ahmad et al., 2017; Z. Lu, Ding, Lu, & Chen, 2013).

With this in mind we evaluated gut physiological function which promptly revealed an increase in the intestinal permeability and down regulation of tight junction protein, occludin. Other tight junction proteins such as Claudin 1, claudin-2, claudin 4, ZO-1 and JaM-A remained unaffected. Tight junctions function as complex centers of signaling in a continuously changing mileu (Weber, 2012). They serve as a permeability barrier and prevent free passage of solutes through the intercellular space. Claudins along with cytoplasmic scaffold ZO create tight junction strands and perform critical roles in the assembly of epithelial barriers (Furuse, 2010). Apart from claudins, tight junctions also contain other integral membrane proteins such as occluding which is a tetra spanning membrane protein and immunoglobulin superfamily proteins, which includes junctional adhesion molecules (JAMs) (Furuse, 2010). These proteins also play vital roles in the maintenance and regulation of paracellular permeability. The coiled coil domain of occludin plays an important role in organizing the structural and functional elements of the tight junction (Nusrat et al., 2000). The tight junctions regulate paracellular permeability to a great extent and a disruption in their expression, integrity or assembly results in increased intestinal permeability (Liang & Weber, 2014). Occludin is an important component of tight junction, and any decrease in its expression or function leads to an increase in permeability. The entry of luminal content into the host could flare up both local and

systemic inflammatory pathways in case of enhanced intestinal permeability (Mu, Kirby, Reilly, & Luo, 2017). A study has shown that arsenic treated Caco-2 cells exhibited increased paracellular permeability coupled with reduced claudin-1 expression and redistribution of ZO-1 (Chiocchetti, Velez, et al., 2019). In contrast we observed that prenatal arsenic exposure did not change the expression of either claudin-1 or ZO-1. It is quite possible that pAs exposed mice did not experience toxic enough concentrations of arsenic to reduce the expression of claudin-1.

Recent reports have shown that treatment with arsenic impairs distinct population of intestinal stromal cells coupled with intraepithelial and innate immune cells (Kellett, Jatko, Darling, Ventrello, & Bain, 2022; Medina et al., 2020). The histology of the colon sections of pAs exposed mice shows infiltration of inflammatory cells and goblet cell hyperplasia. Gut barrier disruption and neutrophil infiltration are known to be closely associated phenomena (Lin et al., 2020). The precise mechanism of goblet cell hyperplasia observed is unclear, however, a previous study indicate that IL-13 which is a key regulator in type-2 mediated inflammation induces goblet cell hyperplasia to accelerate inflammation (Huang et al., 2020). Arsenic has been known to cause IL-13 induction (A. Rahman et al., 2021), which could possibly lead to hyperplasia of the goblet cells. Short Chain Fatty Acids (SCFAs) particularly butyrate has been known to strengthen barrier function and decrease intestinal permeability in several studies that includes cell culture and animal models (Peng et al., 2009). Recalling our previous observation of a decrease in SCFA producing bacteria and SCFA production in pAs exposed mice we investigated whether SCFAs have any effect on occludin expression. We undertook an in vitro study where we treated colon carcinoma cell line HT29 with various concentrations of butyrate, acetate and propionate. The concentrations of the SCFAs were effectively luminal concentration (H. Liu et al., 2018). Treatment with butyrate and not propionate or acetate resulted in an increase in the expression of occludin in HT29 cells. To further unearth the mechanism through which butyrate regulates occludin expression we investigated the role of miRNA that plays crucial roles in regulation of gene expression (Kaikkonen, Lam, & Glass, 2011). It has been reported that miR122, binds to the 3'UTR of Occludin mRNA and leads to its degradation (Jingushi et al., 2017). Although, miR122 is abundantly found in liver, it is known to be expressed sufficiently in intestinal tissue (Runtsch, Round, & O'Connell, 2014). To confirm that the inhibition

of miR122 is specifically brought on by butyrate and not other SCFAs we showed that neither propionate nor acetate changes either miR122 or occludin expression. In another study from our group we have shown that a RNA binding protein, AUF1 plays a fulcrum point in miR122 regulation by butyrate (data not shown). Thus our findings report that miR122 could be a key player in butyrate induced decrease in paracellular permeability. We further validated the interaction of these molecular players by orally supplementing butyrate in pAs exposed mice. We observed that oral supplementation with butyrate increased occludin expression in the gut coupled with decrease in intestinal permeability and miR122 expression. To further cement the role of miR122 in our experimental paradigm, we overexpressed miR122 which resulted in decreased expression of occludin in the gut coupled with increased intestinal permeability.

Overall our present study deals with an intriguing relationship between prenatal arsenic exposure and altered gut physiology in post natal life. We report that gut microbial dysbiosis in pAs exposed mice leads to a decrease in *Firmicutes* to *Bacteroidetes* ratio and ultimately result in decreased production of SCFAs. We also document that pAs exposed mice had a reduction in the expression of occludin coupled with an increase in intestinal permeability which was reversed by oral supplementation of butyrate. We employed in vitro and in vivo experiments to show that butyrate down regulates miR122 expression and is responsible for increase in Occludin expression leading to the decrease in permeability. By rescuing miR122 expression after butyrate treatment we further establish that miR122 and occludin plays a vital role in butyrate mediated increase in barrier function in prenatal arsenic exposed mice.

Summary

Many aquifers around the world have been discovered to contain arsenic at levels that are higher than the WHO-permitted limit of 0.01 mg/L. The telltale indications of chronic arsenic toxicity include distinctive skin lesions, including pigmentation, depigmentation, and keratosis. Emerging evidences show arsenic increases risk of infectious diseases that have significant health implications when exposed to subchronic level in adults and children. But its ability to impair critical functions of the immune system and gastrointestinal physiology is mostly obscured. Continuance of these gaps prevents us from fully comprehending important immunologic mechanisms, processes, and pathways. Due to the unknown optimal points for intervention to avoid arsenic-related immunotoxicity and morbidity in adults and early life, this poses a significant concern. In this thesis, we studied the mechanisms of arsenic-related immune response alterations that could underlie arsenic-associated increased risk of infection in adult and juveniles.

Our study demonstrated that sub-chronic exposure to arsenic had a significant impact on the immune system of mice by severely downregulating CD4+, CD8+ T-cells and CD19+ B cells in spleen and mesenteric lymph nodes combined with decreased circulating levels of IgG1 and IgG2a in the serum. These compromised immune responses eventually lead to an increased susceptibility to an enteric *Shigella* infection and inflammation in the colon.

Another area of our research was determining the effect of arsenic on most critical exposure windows during the prenatal periods. As arsenic is also readily absorbed through placenta, prenatal exposure to the metalloid also increases risk of health in children. However, little is known about this topic. We investigated the effects of prenatal arsenic exposure on immunity in postnatal life by studying the function of the immune cell functions in mice model. Our studies reveal that prenatal arsenic exposure led to defect in T-cell repertoire development as evidenced by decreased frequency of CD4+ and CD8+ T-cells in spleen coupled with reductions in inducible activation markers CD44 and CD69 which ultimately resulted in these T-cells failing to produce IL-2 and IFN-γ after allogeneic stimulation. Furthermore, there was decrease in circulating IgG2a levels in the serum thus suggesting that there is a

potential impact on TH1 responses. This immunotoxic effect resulted in increased susceptibility to septicemic *E.Coli* infection with increased bacterial burden observed in several organs.

Apart from immune function we also investigated any changes incurred on the gastrointestinal physiology as a result of prenatal arsenic exposure. We investigated the effects of prenatal arsenic exposure on functional gut phenotype in postnatal life by studying gut microbial composition, metabolite production and gut permeability in terms of tight junction proteins. We found that prenatal arsenic exposure results in gut microbial dysbiosis leading to decrease in *Firmicutes* to *Bacteroidetes* ratio and decrease in production of SCFAs (Butyrate, Acetate and Propionate). We also observed a decreased expression of tight junction protein occludin which was reversible by oral supplementation with Butyrate. Further investigations revealed that miR122 play a vital role in the regulation of occludin expression. Butyrate down regulates miR122 expression which is responsible for increase in occludin expression and ultimately leading to a decrease in gut permeability. Loss of gut barrier integrity can be a strong driver of susceptibility to numerous infectious diseases, particularly enteric infections.

All of these studies point to mechanisms of immune toxicity by arsenic, with the potential to lead to disrupt gut physiology and infection susceptibility in prenatal exposure to arsenic. Although future research needs to evaluate the association among immune system and gastrointestinal physiology, the robust findings from our study in mice model warrants the need for appropriate mitigation and policy efforts not only for the reduction of arsenic exposure during pregnancy but also on other factors including intake of food that enhances butyrate production within gut to reduce the morbidity caused by arsenic-related inflammatory problems in gut.

References

Abdul, K. S., Jayasinghe, S. S., Chandana, E. P., Jayasumana, C., & De Silva, P. M. (2015). Arsenic and human health effects: A review. *Environ Toxicol Pharmacol, 40*(3), 828-846. doi:10.1016/j.etap.2015.09.016

Aggarwal, M., Naraharisetti, S. B., Dandapat, S., Degen, G. H., & Malik, J. K. (2008). Perturbations in immune responses induced by concurrent subchronic exposure to arsenic and endosulfan. *Toxicology, 251*(1-3), 51-60. doi:10.1016/j.tox.2008.07.050

Ahamed, S., Sengupta, M. K., Mukherjee, S. C., Pati, S., Mukherjee, A., Rahman, M. M., . . . Pal, A. (2006). An eight-year study report on arsenic contamination in groundwater and health effects in Eruani village, Bangladesh and an approach for its mitigation. *Journal of Health, Population and Nutrition*, 129-141.

Ahmad, K. (2001). Report highlights widespread arsenic contamination in Bangladesh. *The Lancet, 358*(9276), 133.

Ahmad, R., Sorrell, M. F., Batra, S. K., Dhawan, P., & Singh, A. B. (2017). Gut permeability and mucosal inflammation: bad, good or context dependent. *Mucosal Immunol, 10*(2), 307-317. doi:10.1038/mi.2016.128

Ahmed, S., Ahsan, K. B., Kippler, M., Mily, A., Wagatsuma, Y., Hoque, A. M., . . . Vahter, M. (2012). In utero arsenic exposure is associated with impaired thymic function in newborns possibly via oxidative stress and apoptosis. *Toxicol Sci, 129*(2), 305-314. doi:10.1093/toxsci/kfs202

Al-Sadi, R., Khatib, K., Guo, S., Ye, D., Youssef, M., & Ma, T. (2011). Occludin regulates macromolecule flux across the intestinal epithelial tight junction barrier. *Am J Physiol Gastrointest Liver Physiol, 300*(6), G1054-1064. doi:10.1152/ajpgi.00055.2011

Anderson, J. M., & Van Itallie, C. M. (2009). Physiology and function of the tight junction. *Cold Spring Harb Perspect Biol, 1*(2), a002584. doi:10.1101/cshperspect.a002584

Andrew, A. S., Bernardo, V., Warnke, L. A., Davey, J. C., Hampton, T., Mason, R. A., . . . Hamilton, J. W. (2007). Exposure to arsenic at levels found inU.S. drinking water modifies expression in the mouse lung. *Toxicol Sci, 100*(1), 75-87. doi:10.1093/toxsci/kfm200

Andrew, A. S., Jewell, D. A., Mason, R. A., Whitfield, M. L., Moore, J. H., & Karagas, M. R. (2008). Drinking-water arsenic exposure modulates gene expression in human lymphocytes from a U.S. population. *Environ Health Perspect, 116*(4), 524-531. doi:10.1289/ehp.10861

Aposhian, H. V., Gurzau, E. S., Le, X. C., Gurzau, A., Healy, S. M., Lu, X., . . . Aposhian, M. M. (2000). Occurrence of monomethylarsonous acid in urine of humans exposed to inorganic arsenic. *Chem Res Toxicol, 13*(8), 693-697. doi:10.1021/tx000114o

Argos, M., Chen, L., Jasmine, F., Tong, L., Pierce, B. L., Roy, S., . . . Ahsan, H. (2015). Gene-specific differential DNA methylation and chronic arsenic exposure in an epigenome-wide association study of adults in Bangladesh. *Environ Health Perspect, 123*(1), 64-71. doi:10.1289/ehp.1307884

Argos, M., Kibriya, M. G., Parvez, F., Jasmine, F., Rakibuz-Zaman, M., & Ahsan, H. (2006). Gene expression profiles in peripheral lymphocytes by arsenic exposure and skin lesion status in a Bangladeshi population. *Cancer Epidemiol Biomarkers Prev, 15*(7), 1367-1375. doi:10.1158/1055-9965.EPI-06-0106

Assimakopoulos, S. F., Triantos, C., Maroulis, I., & Gogos, C. (2018). The Role of the Gut Barrier Function in Health and Disease. *Gastroenterology Res, 11*(4), 261-263. doi:10.14740/gr1053w

Atkinson, H. J., & Babbitt, P. C. (2009). Glutathione transferases are structural and functional outliers in the thioredoxin fold. *Biochemistry, 48*(46), 11108-11116. doi:10.1021/bi901180v

ATSDR. (2005). ATSDR CERCLA Priority List of Hazardous Substances. *Department of Health and Human Services*. Retrieved from Available at: URL: http://www.atsdr.cdc.gov/

Attreed, S. E., Navas-Acien, A., & Heaney, C. D. (2017). Arsenic and Immune Response to Infection During Pregnancy and Early Life. *Curr Environ Health Rep, 4*(2), 229-243. doi:10.1007/s40572-017-0141-4

Bagla, P., & Kaiser, J. (1996). India's spreading health crisis draws global arsenic experts. *Science, 274*(5285), 174-175.

Bahari, A., & Salmani, V. (2017). Environmentally relevant dose of arsenic interferes in functions of human monocytes derived dendritic cells. *Toxicol Lett, 275*, 118-122. doi:10.1016/j.toxlet.2017.05.005

Bailey, K., & Fry, R. C. (2014). Long-term health consequences of prenatal arsenic exposure: links to the genome and the epigenome. *Rev Environ Health, 29*(1-2), 9-12. doi:10.1515/reveh-2014-0006

Banerjee, N., Banerjee, S., Sen, R., Bandyopadhyay, A., Sarma, N., Majumder, P., . . . Giri, A. K. (2009). Chronic arsenic exposure impairs macrophage functions in the exposed individuals. *J Clin Immunol, 29*(5), 582-594. doi:10.1007/s10875-009-9304-x

Banerjee, S., Sousa, A. D., & Bhat, M. A. (2006). Organization and function of septate junctions: an evolutionary perspective. *Cell Biochem Biophys, 46*(1), 65-77. doi:10.1385/CBB:46:1:65

Basu, A., Mahata, J., Roy, A. K., Sarkar, J. N., Poddar, G., Nandy, A. K., . . . Giri, A. K. (2002). Enhanced frequency of micronuclei in individuals exposed to arsenic through drinking water in West Bengal, India. *Mutat Res, 516*(1-2), 29-40. doi:10.1016/s1383-5718(02)00014-1

Bauman, R. P., Rembert, J. C., & Greenfield, J. C., Jr. (1989). Regional atrial blood flow in dogs. Effect of hypertrophy on coronary flow reserve. *J Clin Invest, 83*(5), 1563-1569. doi:10.1172/JCI114053

Beaugerie, L., & Petit, J. C. (2004). Microbial-gut interactions in health and disease. Antibiotic-associated diarrhoea. *Best Pract Res Clin Gastroenterol, 18*(2), 337-352. doi:10.1016/j.bpg.2003.10.002

Bellamri, N., Morzadec, C., Fardel, O., & Vernhet, L. (2018). Arsenic and the immune system. *Current Opinion in Toxicology, 10*, 60-68.

Bencko, V., Wagner, V., Wagnerova, M., & Batora, J. (1988). Immunological profiles in workers of a power plant burning coal rich in arsenic content. *J Hyg Epidemiol Microbiol Immunol, 32*(2), 137-146. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/2457611

Bentley, R., & Chasteen, T. G. (2002). Arsenic curiosa and humanity. *The Chemical Educator, 7*(2), 51-60.

Bergstrom, K. S., Kissoon-Singh, V., Gibson, D. L., Ma, C., Montero, M., Sham, H. P., . . . Vallance, B. A. (2010). Muc2 protects against lethal infectious colitis by disassociating pathogenic and commensal bacteria from the colonic mucosa. *PLoS Pathog, 6*(5), e1000902. doi:10.1371/journal.ppat.1000902

Biggs, M. L., Kalman, D. A., Moore, L. E., Hopenhayn-Rich, C., Smith, M. T., & Smith, A. H. (1997). Relationship of urinary arsenic to intake estimates and a

biomarker of effect, bladder cell micronuclei. *Mutat Res, 386*(3), 185-195. doi:10.1016/s1383-5742(97)00012-4

Biswas, J., Sinha, D., Mukherjee, S., Roy, S., Siddiqi, M., & Roy, M. (2010). Curcumin protects DNA damage in a chronically arsenic-exposed population of West Bengal. *Hum Exp Toxicol, 29*(6), 513-524. doi:10.1177/0960327109359020

Biswas, R., Ghosh, P., Banerjee, N., Das, J. K., Sau, T., Banerjee, A., . . . Giri, A. K. (2008). Analysis of T-cell proliferation and cytokine secretion in the individuals exposed to arsenic. *Hum Exp Toxicol, 27*(5), 381-386. doi:10.1177/0960327108094607

Borgono, J. M., Vicent, P., Venturino, H., & Infante, A. (1977). Arsenic in the drinking water of the city of Antofagasta: epidemiological and clinical study before and after the installation of a treatment plant. *Environ Health Perspect, 19*, 103-105. doi:10.1289/ehp.19-1637404

Bourdonnay, E., Morzadec, C., Sparfel, L., Galibert, M. D., Jouneau, S., Martin-Chouly, C., . . . Vernhet, L. (2009). Global effects of inorganic arsenic on gene expression profile in human macrophages. *Mol Immunol, 46*(4), 649-656. doi:10.1016/j.molimm.2008.08.268

Brabec, J. L., Wright, J., Ly, T., Wong, H. T., McClimans, C. J., Tokarev, V., . . . Sherchan, S. P. (2020). Arsenic disturbs the gut microbiome of individuals in a disadvantaged community in Nepal. *Heliyon, 6*(1), e03313. doi:10.1016/j.heliyon.2020.e03313

Bu, N., Wang, H. Y., Hao, W. H., Liu, X., Xu, S., Wu, B., . . . Naranmandura, H. (2011). Generation of thioarsenicals is dependent on the enterohepatic circulation in rats. *Metallomics, 3*(10), 1064-1073. doi:10.1039/c1mt00036e

Buchanan, W. D. (1962). Toxicity of arsenic compounds. *Toxicity of Arsenic Compounds.*

Buchet, J. P., Lauwerys, R., & Roels, H. (1981). Comparison of the urinary excretion of arsenic metabolites after a single oral dose of sodium arsenite, monomethylarsonate, or dimethylarsinate in man. *Int Arch Occup Environ Health, 48*(1), 71-79. doi:10.1007/BF00405933

Burchiel, S. W., Lauer, F. T., Beswick, E. J., Gandolfi, A. J., Parvez, F., Liu, K. J., & Hudson, L. G. (2014). Differential susceptibility of human peripheral blood T cells to suppression by environmental levels of sodium arsenite and

monomethylarsonous acid. *PLoS One, 9*(10), e109192. doi:10.1371/journal.pone.0109192

Calatayud, M., Devesa, V., & Velez, D. (2013). Differential toxicity and gene expression in Caco-2 cells exposed to arsenic species. *Toxicol Lett, 218*(1), 70-80. doi:10.1016/j.toxlet.2013.01.013

Calatayud, M., Gimeno-Alcaniz, J. V., Velez, D., & Devesa, V. (2014). Trivalent arsenic species induce changes in expression and levels of proinflammatory cytokines in intestinal epithelial cells. *Toxicol Lett, 224*(1), 40-46. doi:10.1016/j.toxlet.2013.09.016

Canani, R. B., Costanzo, M. D., Leone, L., Pedata, M., Meli, R., & Calignano, A. (2011). Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. *World J Gastroenterol, 17*(12), 1519-1528. doi:10.3748/wjg.v17.i12.1519

Casalino, G., Del Turco, C., Corvi, F., Rafaeli, O., Bandello, F., & Querques, G. (2016). Short-Term Retinal Sensitivity and Metamorphopsia Changes following Half-Fluence Photodynamic Therapy in Central Serous Chorioretinopathy. *Ophthalmic Res, 56*(1), 23-29. doi:10.1159/000444328

Cebrian, M. E., Albores, A., Aguilar, M., & Blakely, E. (1983). Chronic arsenic poisoning in the north of Mexico. *Hum Toxicol, 2*(1), 121-133. doi:10.1177/096032718300200110

Chakraborti, D., Das, B., Rahman, M. M., Chowdhury, U. K., Biswas, B., Goswami, A. B., . . . Ahamed, S. (2009). Status of groundwater arsenic contamination in the state of West Bengal, India: A 20-year study report. *Molecular nutrition & food research, 53*(5), 542-551.

Chakraborty, M., & Bhaumik, M. (2020). Prenatal arsenic exposure interferes in postnatal immunocompetence despite an absence of ongoing arsenic exposure. *J Immunotoxicol, 17*(1), 135-143. doi:10.1080/1547691X.2020.1767238

Challenger, F. (1945). Biological methylation. *Chemical reviews, 36*(3), 315-361.

Chappell, W. R., Abernathy, C. O., & Calderon, R. L. (2001). *Arsenic exposure and health effects IV*: Elsevier.

Chattopadhyay, S., Ghosh, S., Debnath, J., & Ghosh, D. (2001). Protection of sodium arsenite-induced ovarian toxicity by coadministration of L-ascorbate (vitamin C) in mature wistar strain rat. *Arch Environ Contam Toxicol, 41*(1), 83-89. doi:10.1007/s002440010223

Chattopadhyay, S., Pal Ghosh, S., Ghosh, D., & Debnath, J. (2003). Effect of dietary co-administration of sodium selenite on sodium arsenite-induced ovarian and uterine disorders in mature albino rats. *Toxicol Sci, 75*(2), 412-422. doi:10.1093/toxsci/kfg194

Chen, C. J., Hsu, L. I., Wang, C. H., Shih, W. L., Hsu, Y. H., Tseng, M. P., . . . Wu, M. M. (2005). Biomarkers of exposure, effect, and susceptibility of arsenic-induced health hazards in Taiwan. *Toxicol Appl Pharmacol, 206*(2), 198-206. doi:10.1016/j.taap.2004.10.023

Chen, G., Mao, J., Zhao, J., Zhang, Y., Li, T., Wang, C., . . . Wu, Q. (2016). Arsenic trioxide mediates HAPI microglia inflammatory response and the secretion of inflammatory cytokine IL-6 via Akt/NF-kappaB signaling pathway. *Regul Toxicol Pharmacol, 81*, 480-488. doi:10.1016/j.yrtph.2016.09.027

Chen, J., & Vitetta, L. (2020). The Role of Butyrate in Attenuating Pathobiont-Induced Hyperinflammation. *Immune Netw, 20*(2), e15. doi:10.4110/in.2020.20.e15

Cherry, N., Shaikh, K., McDonald, C., & Chowdhury, Z. (2008). Stillbirth in rural Bangladesh: arsenic exposure and other etiological factors: a report from Gonoshasthaya Kendra. *Bull World Health Organ, 86*(3), 172-177. doi:10.2471/blt.07.043083

Chi, L., Bian, X., Gao, B., Tu, P., Ru, H., & Lu, K. (2017). The Effects of an Environmentally Relevant Level of Arsenic on the Gut Microbiome and Its Functional Metagenome. *Toxicol Sci, 160*(2), 193-204. doi:10.1093/toxsci/kfx174

Chilvers, D., & Peterson, P. (1987). Global cycling of arsenic. *Lead, mercury, cadmium and arsenic in the environment, 31*, 279-301.

Chiocchetti, G. M., Domene, A., Kuhl, A. A., Zuniga, M., Velez, D., Devesa, V., & Monedero, V. (2019). In vivo evaluation of the effect of arsenite on the intestinal epithelium and associated microbiota in mice. *Arch Toxicol, 93*(8), 2127-2139. doi:10.1007/s00204-019-02510-w

Chiocchetti, G. M., Velez, D., & Devesa, V. (2019). Inorganic arsenic causes intestinal barrier disruption. *Metallomics, 11*(8), 1411-1418. doi:10.1039/c9mt00144a

Choudhury, A. R., Das, T., & Sharma, A. (1997). Mustard oil and garlic extract as inhibitors of sodium arsenite-induced chromosomal breaks in vivo. *Cancer*

Lett, 121(1), 45-52. doi:10.1016/s0304-3835(97)00334-0

Choudhury, S., Gupta, P., Ghosh, S., Mukherjee, S., Chakraborty, P., Chatterji, U., & Chattopadhyay, S. (2016). Arsenic-induced dose-dependent modulation of the NF-kappaB/IL-6 axis in thymocytes triggers differential immune responses. *Toxicology, 357-358*, 85-96. doi:10.1016/j.tox.2016.06.005

Chowdhury, U. K., Biswas, B. K., Chowdhury, T. R., Samanta, G., Mandal, B. K., Basu, G. C., . . . Mukherjee, S. K. (2000). Groundwater arsenic contamination in Bangladesh and West Bengal, India. *Environmental health perspectives, 108*(5), 393-397.

Clarke, G., Stilling, R. M., Kennedy, P. J., Stanton, C., Cryan, J. F., & Dinan, T. G. (2014). Minireview: Gut microbiota: the neglected endocrine organ. *Mol Endocrinol, 28*(8), 1221-1238. doi:10.1210/me.2014-1108

Cohen, S. M., Yamamoto, S., Cano, M., & Arnold, L. L. (2001). Urothelial cytotoxicity and regeneration induced by dimethylarsinic acid in rats. *Toxicol Sci, 59*(1), 68-74. doi:10.1093/toxsci/59.1.68

Concha, G., Vogler, G., Lezcano, D., Nermell, B., & Vahter, M. (1998). Exposure to inorganic arsenic metabolites during early human development. *Toxicol Sci, 44*(2), 185-190. doi:10.1006/toxs.1998.2486

Conde, P., Acosta-Saavedra, L. C., Goytia-Acevedo, R. C., & Calderon-Aranda, E. S. (2007). Sodium arsenite-induced inhibition of cell proliferation is related to inhibition of IL-2 mRNA expression in mouse activated T cells. *Arch Toxicol, 81*(4), 251-259. doi:10.1007/s00204-006-0152-7

Coppola, S., Avagliano, C., Calignano, A., & Berni Canani, R. (2021). The Protective Role of Butyrate against Obesity and Obesity-Related Diseases. *Molecules, 26*(3). doi:10.3390/molecules26030682

Coryell, M., Roggenbeck, B. A., & Walk, S. T. (2019). The Human Gut Microbiome's Influence on Arsenic Toxicity. *Curr Pharmacol Rep, 5*(6), 491-504. doi:10.1007/s40495-019-00206-4

Cullen, N. M., Wolf, L. R., & St Clair, D. (1995). Pediatric arsenic ingestion. *Am J Emerg Med, 13*(4), 432-435. doi:10.1016/0735-6757(95)90133-7

Cullen, W. R., & Reimer, K. J. (1989). Arsenic speciation in the environment. *Chemical reviews, 89*(4), 713-764.

Dangleben, N. L., Skibola, C. F., & Smith, M. T. (2013). Arsenic immunotoxicity: a review. *Environ Health, 12*(1), 73. doi:10.1186/1476-069X-12-73

Das, D., Samanta, G., Mandal, B. K., Roy Chowdhury, T., Chanda, C. R., Chowdhury, P. P., . . . Chakraborti, D. (1996). Arsenic in groundwater in six districts of West Bengal, India. *Environmental Geochemistry and Health, 18*(1), 5-15.

Das, T. K. (2019). Arsenic menace in West Bengal (India) and its mitigation through toolbox intervention: an experience to share. In *Ground Water Development-Issues and Sustainable Solutions* (pp. 305-314): Springer.

Datta, D., & Kaul, M. (1976). Arsenic content of drinking water in villages in northern India. A concept of arsenicosis. *The Journal of the Association of Physicians of India, 24*(9), 599-604.

Davis, M. A., Higgins, J., Li, Z., Gilbert-Diamond, D., Baker, E. R., Das, A., & Karagas, M. R. (2015). Preliminary analysis of in utero low-level arsenic exposure and fetal growth using biometric measurements extracted from fetal ultrasound reports. *Environ Health, 14*, 12. doi:10.1186/1476-069X-14-12

De Santis, S., Cavalcanti, E., Mastronardi, M., Jirillo, E., & Chieppa, M. (2015). Nutritional Keys for Intestinal Barrier Modulation. *Front Immunol, 6*, 612. doi:10.3389/fimmu.2015.00612

Del Razo, L. M., Quintanilla-Vega, B., Brambila-Colombres, E., Calderon-Aranda, E. S., Manno, M., & Albores, A. (2001). Stress proteins induced by arsenic. *Toxicol Appl Pharmacol, 177*(2), 132-148. doi:10.1006/taap.2001.9291

Del Razo, L. M., Styblo, M., Cullen, W. R., & Thomas, D. J. (2001). Determination of trivalent methylated arsenicals in biological matrices. *Toxicol Appl Pharmacol, 174*(3), 282-293. doi:10.1006/taap.2001.9226

den Besten, G., van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D. J., & Bakker, B. M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res, 54*(9), 2325-2340. doi:10.1194/jlr.R036012

Dheeman, D. S., Packianathan, C., Pillai, J. K., & Rosen, B. P. (2014). Pathway of human AS3MT arsenic methylation. *Chemical research in toxicology, 27*(11), 1979-1989.

Dietert, R. R., & Piepenbrink, M. S. (2006). Perinatal immunotoxicity: why adult exposure assessment fails to predict risk. *Environ Health Perspect, 114*(4), 477-483. doi:10.1289/ehp.8566

Dogra, S., Sakwinska, O., Soh, S. E., Ngom-Bru, C., Bruck, W. M., Berger, B., . . .

Group, G. S. (2015). Dynamics of infant gut microbiota are influenced by delivery mode and gestational duration and are associated with subsequent adiposity. *mBio, 6*(1). doi:10.1128/mBio.02419-14

Dong, X., Gao, Y., Chen, W., Wang, W., Gong, L., Liu, X., & Luo, J. (2015). Spatiotemporal distribution of phenolamides and the genetics of natural variation of hydroxycinnamoyl spermidine in rice. *Mol Plant, 8*(1), 111-121. doi:10.1016/j.molp.2014.11.003

Dong, X., Shulzhenko, N., Lemaitre, J., Greer, R. L., Peremyslova, K., Quamruzzaman, Q., . . . Kile, M. L. (2017). Arsenic exposure and intestinal microbiota in children from Sirajdikhan, Bangladesh. *PLoS One, 12*(12), e0188487. doi:10.1371/journal.pone.0188487

Drobna, Z., Del Razo, L. M., Garcia-Vargas, G. G., Sanchez-Pena, L. C., Barrera-Hernandez, A., Styblo, M., & Loomis, D. (2013). Environmental exposure to arsenic, AS3MT polymorphism and prevalence of diabetes in Mexico. *J Expo Sci Environ Epidemiol, 23*(2), 151-155. doi:10.1038/jes.2012.103

Drobna, Z., Xing, W., Thomas, D. J., & Styblo, M. (2006). shRNA silencing of AS3MT expression minimizes arsenic methylation capacity of HepG2 cells. *Chem Res Toxicol, 19*(7), 894-898. doi:10.1021/tx060076u

Duan, J., & Tan, J. (2013). Atmospheric heavy metals and arsenic in China: situation, sources and control policies. *Atmospheric Environment, 74*, 93-101.

Duker, A. A., Carranza, E., & Hale, M. (2005). Arsenic geochemistry and health. *Environment international, 31*(5), 631-641.

Dutta, K., Prasad, P., & Sinha, D. (2015). Chronic low level arsenic exposure evokes inflammatory responses and DNA damage. *Int J Hyg Environ Health, 218*(6), 564-574. doi:10.1016/j.ijheh.2015.06.003

Endo, H., Niioka, M., Kobayashi, N., Tanaka, M., & Watanabe, T. (2013). Butyrate-producing probiotics reduce nonalcoholic fatty liver disease progression in rats: new insight into the probiotics for the gut-liver axis. *PLoS One, 8*(5), e63388. doi:10.1371/journal.pone.0063388

Engel, P., & Moran, N. A. (2013). The gut microbiota of insects - diversity in structure and function. *FEMS Microbiol Rev, 37*(5), 699-735. doi:10.1111/1574-6976.12025

Engel, R. R., & Smith, A. H. (1994). Arsenic in drinking water and mortality from vascular disease: an ecologic analysis in 30 counties in the United States. *Arch*

Environ Health, 49(5), 418-427. doi:10.1080/00039896.1994.9954996

Enterline, P. E., Day, R., & Marsh, G. M. (1995). Cancers related to exposure to arsenic at a copper smelter. *Occup Environ Med, 52*(1), 28-32. doi:10.1136/oem.52.1.28

Ezeh, P. C., Lauer, F. T., MacKenzie, D., McClain, S., Liu, K. J., Hudson, L. G., . . . Burchiel, S. W. (2014). Arsenite selectively inhibits mouse bone marrow lymphoid progenitor cell development in vivo and in vitro and suppresses humoral immunity in vivo. *PLoS One, 9*(4), e93920. doi:10.1371/journal.pone.0093920

Ezeh, P. C., Xu, H., Lauer, F. T., Liu, K. J., Hudson, L. G., & Burchiel, S. W. (2016). Monomethylarsonous acid (MMA+3) Inhibits IL-7 Signaling in Mouse Pre-B Cells. *Toxicol Sci, 149*(2), 289-299. doi:10.1093/toxsci/kfv233

Faderl, M., Noti, M., Corazza, N., & Mueller, C. (2015). Keeping bugs in check: The mucus layer as a critical component in maintaining intestinal homeostasis. *IUBMB Life, 67*(4), 275-285. doi:10.1002/iub.1374

Farzan, S. F., Li, Z., Korrick, S. A., Spiegelman, D., Enelow, R., Nadeau, K., . . . Karagas, M. R. (2016). Infant Infections and Respiratory Symptoms in Relation to in Utero Arsenic Exposure in a U.S. Cohort. *Environ Health Perspect, 124*(6), 840-847. doi:10.1289/ehp.1409282

Ferrario, D., Gribaldo, L., & Hartung, T. (2016). Arsenic Exposure and Immunotoxicity: a Review Including the Possible Influence of Age and Sex. *Curr Environ Health Rep, 3*(1), 1-12. doi:10.1007/s40572-016-0082-3

Flannigan, K. L., Geem, D., Harusato, A., & Denning, T. L. (2015). Intestinal Antigen-Presenting Cells: Key Regulators of Immune Homeostasis and Inflammation. *Am J Pathol, 185*(7), 1809-1819. doi:10.1016/j.ajpath.2015.02.024

Furuse, M. (2010). Molecular basis of the core structure of tight junctions. *Cold Spring Harb Perspect Biol, 2*(1), a002907. doi:10.1101/cshperspect.a002907

Galanis, A., Karapetsas, A., & Sandaltzopoulos, R. (2009). Metal-induced carcinogenesis, oxidative stress and hypoxia signalling. *Mutat Res, 674*(1-2), 31-35. doi:10.1016/j.mrgentox.2008.10.008

Galicia, G., Leyva, R., Tenorio, E. P., Ostrosky-Wegman, P., & Saavedra, R. (2003). Sodium arsenite retards proliferation of PHA-activated T cells by delaying the production and secretion of IL-2. *Int Immunopharmacol, 3*(5), 671-682.

doi:10.1016/S1567-5769(03)00049-3

Garcia-Manyes, S., Jimenez, G., Padro, A., Rubio, R., & Rauret, G. (2002). Arsenic speciation in contaminated soils. *Talanta, 58*(1), 97-109.

Gasaly, N., Hermoso, M. A., & Gotteland, M. (2021). Butyrate and the Fine-Tuning of Colonic Homeostasis: Implication for Inflammatory Bowel Diseases. *Int J Mol Sci, 22*(6). doi:10.3390/ijms22063061

Gebel, T. W. (2002). Arsenic methylation is a process of detoxification through accelerated excretion. *Int J Hyg Environ Health, 205*(6), 505-508. doi:10.1078/1438-4639-00177

Gemert M, E. M. (1998). Chronic carcinogenicity assessment of cacodylic acid. *Society for Environmental Geochemistry and Health (SEGH) Third International Conference on Arsenic Exposure and Health Effects, Book of Abstracts,*, 113.

Gera, R., Singh, V., Mitra, S., Sharma, A. K., Singh, A., Dasgupta, A., . . . Ghosh, D. (2017). Arsenic exposure impels CD4 commitment in thymus and suppress T cell cytokine secretion by increasing regulatory T cells. *Sci Rep, 7*(1), 7140. doi:10.1038/s41598-017-07271-z

Germolec, D. R., Spalding, J., Boorman, G. A., Wilmer, J. L., Yoshida, T., Simeonova, P. P., . . . Luster, M. I. (1997). Arsenic can mediate skin neoplasia by chronic stimulation of keratinocyte-derived growth factors. *Mutat Res, 386*(3), 209-218. doi:10.1016/s1383-5742(97)00006-9

Gerova, V. (2016). Chapter 1 Intestinal Barrier Dysfunction in Inflammatory Bowel Disease: Cause or Consequence? In.

Ghosh, D., Bhadury, P., & Routh, J. (2018). Coping with arsenic stress: Adaptations of arsenite-oxidizing bacterial membrane lipids to increasing arsenic levels. *Microbiologyopen, 7*(5), e00594. doi:10.1002/mbo3.594

Gonsebatt, M. E., Vega, L., Montero, R., Garcia-Vargas, G., Del Razo, L. M., Albores, A., . . . Ostrosky-Wegman, P. (1994). Lymphocyte replicating ability in individuals exposed to arsenic via drinking water. *Mutat Res, 313*(2-3), 293-299. doi:10.1016/0165-1161(94)90059-0

Groschwitz, K. R., & Hogan, S. P. (2009). Intestinal barrier function: molecular regulation and disease pathogenesis. *J Allergy Clin Immunol, 124*(1), 3-20; quiz 21-22. doi:10.1016/j.jaci.2009.05.038

Guarner, F., & Malagelada, J. R. (2003). Gut flora in health and disease. *Lancet,*

361(9356), 512-519. doi:10.1016/S0140-6736(03)12489-0

Guha Mazumder, D. N. (2007). Arsenic and non-malignant lung disease. *J Environ Sci Health A Tox Hazard Subst Environ Eng, 42*(12), 1859-1867. doi:10.1080/10934520701566926

Guha Mazumder, D. N. (2008). Chronic arsenic toxicity & human health. *Indian J Med Res, 128*(4), 436-447. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/19106439

Guha Mazumder, D. N., Chakraborty, A. K., Ghose, A., Gupta, J. D., Chakraborty, D. P., Dey, S. B., & Chattopadhyay, N. (1988). Chronic arsenic toxicity from drinking tubewell water in rural West Bengal. *Bull World Health Organ, 66*(4), 499-506. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/3262450

Guo, X., Huang, C., Xu, J., Xu, H., Liu, L., Zhao, H., . . . Zhou, Y. (2021). Gut Microbiota Is a Potential Biomarker in Inflammatory Bowel Disease. *Front Nutr, 8*, 818902. doi:10.3389/fnut.2021.818902

Hashash, J. G., & Kane, S. (2015). Pregnancy and Inflammatory Bowel Disease. *Gastroenterol Hepatol (N Y), 11*(2), 96-102. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/27099578

Hayakawa, T., Kobayashi, Y., Cui, X., & Hirano, S. (2005). A new metabolic pathway of arsenite: arsenic-glutathione complexes are substrates for human arsenic methyltransferase Cyt19. *Arch Toxicol, 79*(4), 183-191. doi:10.1007/s00204-004-0620-x

Hayes, J. D., Flanagan, J. U., & Jowsey, I. R. (2005). Glutathione transferases. *Annu Rev Pharmacol Toxicol, 45*, 51-88. doi:10.1146/annurev.pharmtox.45.120403.095857

He, J., Wang, M., Jiang, Y., Chen, Q., Xu, S., Xu, Q., . . . Liu, L. Z. (2014). Chronic arsenic exposure and angiogenesis in human bronchial epithelial cells via the ROS/miR-199a-5p/HIF-1alpha/COX-2 pathway. *Environ Health Perspect, 122*(3), 255-261. doi:10.1289/ehp.1307545

Hermann, A. C., & Kim, C. H. (2005). Effects of arsenic on zebrafish innate immune system. *Mar Biotechnol (NY), 7*(5), 494-505. doi:10.1007/s10126-004-4109-7

Hinwood, A. L., Sim, M. R., Jolley, D., de Klerk, N., Bastone, E. B., Gerostamoulos, J., & Drummer, O. H. (2003). Hair and toenail arsenic concentrations of residents living in areas with high environmental arsenic concentrations.

Environ Health Perspect, 111(2), 187-193. doi:10.1289/ehp.5455

Holson, J. F., Stump, D. G., Ulrich, C. E., & Farr, C. H. (1999). Absence of prenatal developmental toxicity from inhaled arsenic trioxide in rats. *Toxicol Sci, 51*(1), 87-97. doi:10.1093/toxsci/51.1.87

Howrylak, J. A., & Nakahira, K. (2017). Inflammasomes: Key Mediators of Lung Immunity. *Annu Rev Physiol, 79*, 471-494. doi:10.1146/annurev-physiol-021115-105229

Huang, Z. Q., Liu, J., Ong, H. H., Yuan, T., Zhou, X. M., Wang, J., . . . Wang, D. Y. (2020). Interleukin-13 Alters Tight Junction Proteins Expression Thereby Compromising Barrier Function and Dampens Rhinovirus Induced Immune Responses in Nasal Epithelium. *Front Cell Dev Biol, 8*, 572749. doi:10.3389/fcell.2020.572749

Hughes, M. F. (2002). Arsenic toxicity and potential mechanisms of action. *Toxicology letters, 133*(1), 1-16.

Hughes, M. F. (2006). Biomarkers of exposure: a case study with inorganic arsenic. *Environmental health perspectives, 114*(11), 1790-1796.

Hughes, M. F., Beck, B. D., Chen, Y., Lewis, A. S., & Thomas, D. J. (2011). Arsenic exposure and toxicology: a historical perspective. *Toxicol Sci, 123*(2), 305-332. doi:10.1093/toxsci/kfr184

Hughes, M. F., & Kenyon, E. M. (1998). Dose-dependent effects on the disposition of monomethylarsonic acid and dimethylarsinic acid in the mouse after intravenous administration. *J Toxicol Environ Health A, 53*(2), 95-112. doi:10.1080/009841098159385

Humans, I. W. G. o. t. E. o. C. R. t. (2012). Arsenic, metals, fibres, and dusts. *IARC Monogr Eval Carcinog Risks Hum, 100*(Pt C), 11-465. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/23189751

IARC (International Agency for Research on Cancer). (2004). IARC monographs on the evaluation of carcinogenic risks to humans: Some drinking-water disinfectants and contaminants, including arsenic. *IARC Press, Lyon, France, 84*, 1-256.

Islam, L. N., Nabi, A. H., Rahman, M. M., & Zahid, M. S. (2007). Association of respiratory complications and elevated serum immunoglobulins with drinking water arsenic toxicity in human. *J Environ Sci Health A Tox Hazard Subst Environ Eng, 42*(12), 1807-1814. doi:10.1080/10934520701566777

Jager, S., Stange, E. F., & Wehkamp, J. (2013). Inflammatory bowel disease: an impaired barrier disease. *Langenbecks Arch Surg, 398*(1), 1-12. doi:10.1007/s00423-012-1030-9

Jeong, C. H., Seok, J. S., Petriello, M. C., & Han, S. G. (2017). Arsenic downregulates tight junction claudin proteins through p38 and NF-kappaB in intestinal epithelial cell line, HT-29. *Toxicology, 379*, 31-39. doi:10.1016/j.tox.2017.01.011

Jingushi, K., Kashiwagi, Y., Ueda, Y., Kitae, K., Hase, H., Nakata, W., . . . Tsujikawa, K. (2017). High miR-122 expression promotes malignant phenotypes in ccRCC by targeting occludin. *Int J Oncol, 51*(1), 289-297. doi:10.3892/ijo.2017.4016

Kaikkonen, M. U., Lam, M. T., & Glass, C. K. (2011). Non-coding RNAs as regulators of gene expression and epigenetics. *Cardiovasc Res, 90*(3), 430-440. doi:10.1093/cvr/cvr097

Kannan, G. M., Tripathi, N., Dube, S. N., Gupta, M., & Flora, S. J. (2001). Toxic effects of arsenic (III) on some hematopoietic and central nervous system variables in rats and guinea pigs. *J Toxicol Clin Toxicol, 39*(7), 675-682. doi:10.1081/clt-100108508

Kapaj, S., Peterson, H., Liber, K., & Bhattacharya, P. (2006). Human health effects from chronic arsenic poisoning--a review. *J Environ Sci Health A Tox Hazard Subst Environ Eng, 41*(10), 2399-2428. doi:10.1080/10934520600873571

Kellett, M. P., Jatko, J. T., Darling, C. L., Ventrello, S. W., & Bain, L. J. (2022). Arsenic exposure impairs intestinal stromal cells. *Toxicol Lett, 361*, 54-63. doi:10.1016/j.toxlet.2022.03.006

Kelly, C. J., Zheng, L., Campbell, E. L., Saeedi, B., Scholz, C. C., Bayless, A. J., . . . Colgan, S. P. (2015). Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function. *Cell Host Microbe, 17*(5), 662-671. doi:10.1016/j.chom.2015.03.005

Kerr, C. A., Grice, D. M., Tran, C. D., Bauer, D. C., Li, D., Hendry, P., & Hannan, G. N. (2015). Early life events influence whole-of-life metabolic health via gut microflora and gut permeability. *Crit Rev Microbiol, 41*(3), 326-340. doi:10.3109/1040841X.2013.837863

Khairul, I., Wang, Q. Q., Jiang, Y. H., Wang, C., & Naranmandura, H. (2017). Metabolism, toxicity and anticancer activities of arsenic compounds.

Oncotarget, *8*(14), 23905.

Khanna, S., & Tosh, P. K. (2014). A clinician's primer on the role of the microbiome in human health and disease. *Mayo Clin Proc*, *89*(1), 107-114. doi:10.1016/j.mayocp.2013.10.011

Kippler, M., Skroder, H., Rahman, S. M., Tofail, F., & Vahter, M. (2016). Elevated childhood exposure to arsenic despite reduced drinking water concentrations--A longitudinal cohort study in rural Bangladesh. *Environ Int*, *86*, 119-125. doi:10.1016/j.envint.2015.10.017

Kitchin, K. T. (2001). Recent advances in arsenic carcinogenesis: modes of action, animal model systems, and methylated arsenic metabolites. *Toxicol Appl Pharmacol*, *172*(3), 249-261. doi:10.1006/taap.2001.9157

Kozul, C. D., Hampton, T. H., Davey, J. C., Gosse, J. A., Nomikos, A. P., Eisenhauer, P. L., . . . Hamilton, J. W. (2009). Chronic exposure to arsenic in the drinking water alters the expression of immune response genes in mouse lung. *Environ Health Perspect*, *117*(7), 1108-1115. doi:10.1289/ehp.0800199

Kwok, R. K., Kaufmann, R. B., & Jakariya, M. (2006). Arsenic in drinking-water and reproductive health outcomes: a study of participants in the Bangladesh Integrated Nutrition Programme. *J Health Popul Nutr*, *24*(2), 190-205. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/17195560

Laird, B. D., Van de Wiele, T. R., Corriveau, M. C., Jamieson, H. E., Parsons, M. B., Verstraete, W., & Siciliano, S. D. (2007). Gastrointestinal microbes increase arsenic bioaccessibility of ingested mine tailings using the simulator of the human intestinal microbial ecosystem. *Environ Sci Technol*, *41*(15), 5542-5547. doi:10.1021/es062410e

Laukens, D., Brinkman, B. M., Raes, J., De Vos, M., & Vandenabeele, P. (2016). Heterogeneity of the gut microbiome in mice: guidelines for optimizing experimental design. *FEMS Microbiol Rev*, *40*(1), 117-132. doi:10.1093/femsre/fuv036

Le, X. C., Lu, X., Ma, M., Cullen, W. R., Aposhian, H. V., & Zheng, B. (2000). Speciation of key arsenic metabolic intermediates in human urine. *Anal Chem*, *72*(21), 5172-5177. doi:10.1021/ac000527u

Le, X. C., Ma, M., Cullen, W. R., Aposhian, H. V., Lu, X., & Zheng, B. (2000). Determination of monomethylarsonous acid, a key arsenic methylation intermediate, in human urine. *Environ Health Perspect*, *108*(11), 1015-1018.

doi:10.1289/ehp.001081015

Lee, C. H., Hong, C. H., Yu, C. L., Wang, L. F., Clausen, B. E., Liao, W. T., . . . Yu, H. S. (2012). Arsenic mobilizes Langerhans cell migration and induces Th1 response in epicutaneous protein sensitization via CCL21: a plausible cause of decreased Langerhans cells in arsenic-induced intraepithelial carcinoma. *Biochem Pharmacol, 83*(9), 1290-1299. doi:10.1016/j.bcp.2012.01.028

Lee, C. H., Hsu, C. Y., Huang, P. Y., Chen, C. I., Lee, Y. C., & Yu, H. S. (2016). Arsenite Regulates Prolongation of Glycan Residues of Membrane Glycoprotein: A Pivotal Study via Wax Physisorption Kinetics and FTIR Imaging. *Int J Mol Sci, 17*(3), 427. doi:10.3390/ijms17030427

Lee, S. H. (2015). Intestinal permeability regulation by tight junction: implication on inflammatory bowel diseases. *Intest Res, 13*(1), 11-18. doi:10.5217/ir.2015.13.1.11

Lee, T. C., & Ho, I. C. (1994). Differential cytotoxic effects of arsenic on human and animal cells. *Environ Health Perspect, 102 Suppl 3*(Suppl 3), 101-105. doi:10.1289/ehp.94102s3101

Lemarie, A., Bourdonnay, E., Morzadec, C., Fardel, O., & Vernhet, L. (2008). Inorganic arsenic activates reduced NADPH oxidase in human primary macrophages through a Rho kinase/p38 kinase pathway. *J Immunol, 180*(9), 6010-6017. doi:10.4049/jimmunol.180.9.6010

Lemarie, A., Morzadec, C., Merino, D., Micheau, O., Fardel, O., & Vernhet, L. (2006). Arsenic trioxide induces apoptosis of human monocytes during macrophagic differentiation through nuclear factor-kappaB-related survival pathway down-regulation. *J Pharmacol Exp Ther, 316*(1), 304-314. doi:10.1124/jpet.105.092874

Li, H., Zhang, X., Jin, Z., Yin, T., Duan, C., Sun, J., . . . Li, Z. (2019). MiR-122 Promotes the Development of Colon Cancer by Targeting ALDOA In Vitro. *Technol Cancer Res Treat, 18*, 1533033819871300. doi:10.1177/1533033819871300

Liang, G. H., & Weber, C. R. (2014). Molecular aspects of tight junction barrier function. *Curr Opin Pharmacol, 19*, 84-89. doi:10.1016/j.coph.2014.07.017

Liao, W. T., Yu, C. L., Lan, C. C., Lee, C. H., Chang, C. H., Chang, L. W., . . . Yu, H. S. (2009). Differential effects of arsenic on cutaneous and systemic immunity: focusing on CD4+ cell apoptosis in patients with arsenic-induced Bowen's

disease. *Carcinogenesis, 30*(6), 1064-1072. doi:10.1093/carcin/bgp095

Lin, E. Y., Lai, H. J., Cheng, Y. K., Leong, K. Q., Cheng, L. C., Chou, Y. C., . . . Chiang, H. S. (2020). Neutrophil Extracellular Traps Impair Intestinal Barrier Function during Experimental Colitis. *Biomedicines, 8*(8). doi:10.3390/biomedicines8080275

Lindberg, A. L., Rahman, M., Persson, L. A., & Vahter, M. (2008). The risk of arsenic induced skin lesions in Bangladeshi men and women is affected by arsenic metabolism and the age at first exposure. *Toxicol Appl Pharmacol, 230*(1), 9-16. doi:10.1016/j.taap.2008.02.001

Liu, H., Wang, J., He, T., Becker, S., Zhang, G., Li, D., & Ma, X. (2018). Butyrate: A Double-Edged Sword for Health? *Adv Nutr, 9*(1), 21-29. doi:10.1093/advances/nmx009

Liu, J., & Waalkes, M. P. (2008). Liver is a target of arsenic carcinogenesis. *Toxicol Sci, 105*(1), 24-32. doi:10.1093/toxsci/kfn120

Liu, J., Zheng, B., Aposhian, H. V., Zhou, Y., Chen, M. L., Zhang, A., & Waalkes, M. P. (2002). Chronic arsenic poisoning from burning high-arsenic-containing coal in Guizhou, China. *Environ Health Perspect, 110*(2), 119-122. doi:10.1289/ehp.02110119

Liu, S. X., Athar, M., Lippai, I., Waldren, C., & Hei, T. K. (2001). Induction of oxyradicals by arsenic: implication for mechanism of genotoxicity. *Proc Natl Acad Sci U S A, 98*(4), 1643-1648. doi:10.1073/pnas.98.4.1643

Liu, W. Y., Wang, Z. B., Zhang, L. C., Wei, X., & Li, L. (2012). Tight junction in blood-brain barrier: an overview of structure, regulation, and regulator substances. *CNS Neurosci Ther, 18*(8), 609-615. doi:10.1111/j.1755-5949.2012.00340.x

Lu, C. Y., & Ni, Y. H. (2015). Gut microbiota and the development of pediatric diseases. *J Gastroenterol, 50*(7), 720-726. doi:10.1007/s00535-015-1082-z

Lu, H., Crawford, R. B., North, C. M., Kaplan, B. L., & Kaminski, N. E. (2009). Establishment of an immunoglobulin m antibody-forming cell response model for characterizing immunotoxicity in primary human B cells. *Toxicol Sci, 112*(2), 363-373. doi:10.1093/toxsci/kfp224

Lu, K., Abo, R. P., Schlieper, K. A., Graffam, M. E., Levine, S., Wishnok, J. S., . . . Fox, J. G. (2014). Arsenic exposure perturbs the gut microbiome and its metabolic profile in mice: an integrated metagenomics and metabolomics

analysis. *Environ Health Perspect, 122*(3), 284-291. doi:10.1289/ehp.1307429

Lu, Z., Ding, L., Lu, Q., & Chen, Y. H. (2013). Claudins in intestines: Distribution and functional significance in health and diseases. *Tissue Barriers, 1*(3), e24978. doi:10.4161/tisb.24978

Luissint, A. C., Artus, C., Glacial, F., Ganeshamoorthy, K., & Couraud, P. O. (2012). Tight junctions at the blood brain barrier: physiological architecture and disease-associated dysregulation. *Fluids Barriers CNS, 9*(1), 23. doi:10.1186/2045-8118-9-23

Macoch, M., Morzadec, C., Fardel, O., & Vernhet, L. (2013). Inorganic arsenic impairs differentiation and functions of human dendritic cells. *Toxicol Appl Pharmacol, 266*(2), 204-213. doi:10.1016/j.taap.2012.11.008

Macpherson, A. J., & Smith, K. (2006). Mesenteric lymph nodes at the center of immune anatomy. *J Exp Med, 203*(3), 497-500. doi:10.1084/jem.20060227

Madden, E. F., & Fowler, B. A. (2000). Mechanisms of nephrotoxicity from metal combinations: a review. *Drug Chem Toxicol, 23*(1), 1-12. doi:10.1081/dct-100100098

Maier, N. K., Crown, D., Liu, J., Leppla, S. H., & Moayeri, M. (2014). Arsenic trioxide and other arsenical compounds inhibit the NLRP1, NLRP3, and NAIP5/NLRC4 inflammasomes. *J Immunol, 192*(2), 763-770. doi:10.4049/jimmunol.1301434

Maiti, S., Chattopadhyay, S., Deb, B., Samanta, T., Maji, G., Pan, B., . . . Ghosh, D. (2012). Antioxidant and metabolic impairment result in DNA damage in arsenic-exposed individuals with severe dermatological manifestations in Eastern India. *Environ Toxicol, 27*(6), 342-350. doi:10.1002/tox.20647

Majumdar, K. K., Guha Mazumder, D. N., Ghose, N., Ghose, A., & Lahiri, S. (2009). Systemic manifestations in chronic arsenic toxicity in absence of skin lesions in West Bengal. *Indian J Med Res, 129*(1), 75-82. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/19287062

Maki-Paakkanen, J., Kurttio, P., Paldy, A., & Pekkanen, J. (1998). Association between the clastogenic effect in peripheral lymphocytes and human exposure to arsenic through drinking water. *Environ Mol Mutagen, 32*(4), 301-313. doi:10.1002/(sici)1098-2280(1998)32:4<301::aid-em3>3.0.co;2-i

Mandal, B. K., Ogra, Y., & Suzuki, K. T. (2001). Identification of dimethylarsinous and monomethylarsonous acids in human urine of the arsenic-affected areas in

West Bengal, India. *Chem Res Toxicol, 14*(4), 371-378. doi:10.1021/tx000246h

Mandal, B. K., & Suzuki, K. T. (2002). Arsenic round the world: a review. *Talanta, 58*(1), 201-235. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/18968746

Marquez, M., Fernandez Gutierrez del Alamo, C., & Giron-Gonzalez, J. A. (2016). Gut epithelial barrier dysfunction in human immunodeficiency virus-hepatitis C virus coinfected patients: Influence on innate and acquired immunity. *World J Gastroenterol, 22*(4), 1433-1448. doi:10.3748/wjg.v22.i4.1433

Martinez, V. D., & Lam, W. L. (2021). Health effects associated with pre-and perinatal exposure to arsenic. *Frontiers in Genetics, 12*, 664717.

Mathew, L., Vale, A., & Adcock, J. E. (2010). Arsenical peripheral neuropathy. *Pract Neurol, 10*(1), 34-38. doi:10.1136/jnnp.2009.201830

Matsui, M., Nishigori, C., Toyokuni, S., Takada, J., Akaboshi, M., Ishikawa, M., . . . Miyachi, Y. (1999). The role of oxidative DNA damage in human arsenic carcinogenesis: detection of 8-hydroxy-2'-deoxyguanosine in arsenic-related Bowen's disease. *J Invest Dermatol, 113*(1), 26-31. doi:10.1046/j.1523-1747.1999.00630.x

Mazumder, D. N., Steinmaus, C., Bhattacharya, P., von Ehrenstein, O. S., Ghosh, N., Gotway, M., . . . Smith, A. H. (2005). Bronchiectasis in persons with skin lesions resulting from arsenic in drinking water. *Epidemiology, 16*(6), 760-765. doi:10.1097/01.ede.0000181637.10978.e6

McCarty, K. M., Chen, Y.-C., Quamruzzaman, Q., Rahman, M., Mahiuddin, G., Hsueh, Y.-M., . . . Christiani, D. C. (2007). Arsenic methylation, GSTT1, GSTM1, GSTP1 polymorphisms, and skin lesions. *Environmental health perspectives, 115*(3), 341-345.

Medina, S., Lauer, F. T., Castillo, E. F., Bolt, A. M., Ali, A. S., Liu, K. J., & Burchiel, S. W. (2020). Exposures to uranium and arsenic alter intraepithelial and innate immune cells in the small intestine of male and female mice. *Toxicol Appl Pharmacol, 403*, 115155. doi:10.1016/j.taap.2020.115155

Meropol, S. B., & Edwards, A. (2015). Development of the infant intestinal microbiome: A bird's eye view of a complex process. *Birth Defects Res C Embryo Today, 105*(4), 228-239. doi:10.1002/bdrc.21114

Milton, A. H., Smith, W., Rahman, B., Hasan, Z., Kulsum, U., Dear, K., . . . Ali, A.

(2005). Chronic arsenic exposure and adverse pregnancy outcomes in bangladesh. *Epidemiology, 16*(1), 82-86. doi:10.1097/01.ede.0000147105.94041.e6

Mitic, L. L., Van Itallie, C. M., & Anderson, J. M. (2000). Molecular physiology and pathophysiology of tight junctions I. Tight junction structure and function: lessons from mutant animals and proteins. *Am J Physiol Gastrointest Liver Physiol, 279*(2), G250-254. doi:10.1152/ajpgi.2000.279.2.G250

Moore, M. M., Harrington-Brock, K., & Doerr, C. L. (1997). Relative genotoxic potency of arsenic and its methylated metabolites. *Mutat Res, 386*(3), 279-290. doi:10.1016/s1383-5742(97)00003-3

Mor, A., Antonsen, S., Kahlert, J., Holsteen, V., Jorgensen, S., Holm-Pedersen, J., . . . Ehrenstein, V. (2015). Prenatal exposure to systemic antibacterials and overweight and obesity in Danish schoolchildren: a prevalence study. *Int J Obes (Lond), 39*(10), 1450-1455. doi:10.1038/ijo.2015.129

Morzadec, C., Bouezzedine, F., Macoch, M., Fardel, O., & Vernhet, L. (2012). Inorganic arsenic impairs proliferation and cytokine expression in human primary T lymphocytes. *Toxicology, 300*(1-2), 46-56. doi:10.1016/j.tox.2012.05.025

Morzadec, C., Macoch, M., Robineau, M., Sparfel, L., Fardel, O., & Vernhet, L. (2012). Inorganic arsenic represses interleukin-17A expression in human activated Th17 lymphocytes. *Toxicol Appl Pharmacol, 262*(3), 217-222. doi:10.1016/j.taap.2012.05.004

Moszak, M., Szulinska, M., & Bogdanski, P. (2020). You Are What You Eat-The Relationship between Diet, Microbiota, and Metabolic Disorders-A Review. *Nutrients, 12*(4). doi:10.3390/nu12041096

Mountford, A. P., Fisher, A., & Wilson, R. A. (1994). The profile of IgG1 and IgG2a antibody responses in mice exposed to Schistosoma mansoni. *Parasite Immunol, 16*(10), 521-527. doi:10.1111/j.1365-3024.1994.tb00306.x

Mu, Q., Kirby, J., Reilly, C. M., & Luo, X. M. (2017). Leaky Gut As a Danger Signal for Autoimmune Diseases. *Front Immunol, 8*, 598. doi:10.3389/fimmu.2017.00598

Mukherjee, A., Sengupta, M. K., Hossain, M. A., Ahamed, S., Das, B., Nayak, B., . . . Chakraborti, D. (2006). Arsenic contamination in groundwater: a global perspective with emphasis on the Asian scenario. *Journal of Health,*

Population and Nutrition, 142-163.

Mumford, J. L., Wu, K., Xia, Y., Kwok, R., Yang, Z., Foster, J., & Sanders, W. E. (2007). Chronic arsenic exposure and cardiac repolarization abnormalities with QT interval prolongation in a population-based study. *Environ Health Perspect, 115*(5), 690-694. doi:10.1289/ehp.9686

Myers, S. L., Lobdell, D. T., Liu, Z., Xia, Y., Ren, H., Li, Y., . . . Mendola, P. (2010). Maternal drinking water arsenic exposure and perinatal outcomes in inner Mongolia, China. *J Epidemiol Community Health, 64*(4), 325-329. doi:10.1136/jech.2008.084392

Nadeau, K. C., Li, Z., Farzan, S., Koestler, D., Robbins, D., Fei, D. L., . . . Karagas, M. R. (2014). In utero arsenic exposure and fetal immune repertoire in a US pregnancy cohort. *Clin Immunol, 155*(2), 188-197. doi:10.1016/j.clim.2014.09.004

Naranmandura, H., Rehman, K., Le, X. C., & Thomas, D. J. (2013). Formation of methylated oxyarsenicals and thioarsenicals in wild-type and arsenic (+3 oxidation state) methyltransferase knockout mice exposed to arsenate. *Anal Bioanal Chem, 405*(6), 1885-1891. doi:10.1007/s00216-012-6207-0

Navas-Acien, A., Sharrett, A. R., Silbergeld, E. K., Schwartz, B. S., Nachman, K. E., Burke, T. A., & Guallar, E. (2005). Arsenic exposure and cardiovascular disease: a systematic review of the epidemiologic evidence. *Am J Epidemiol, 162*(11), 1037-1049. doi:10.1093/aje/kwi330

Nayak, A. S., Lage, C. R., & Kim, C. H. (2007). Effects of low concentrations of arsenic on the innate immune system of the zebrafish (Danio rerio). *Toxicol Sci, 98*(1), 118-124. doi:10.1093/toxsci/kfm072

Nebert, D. W., & Vasiliou, V. (2004). Analysis of the glutathione S-transferase (GST) gene family. *Hum Genomics, 1*(6), 460-464. doi:10.1186/1479-7364-1-6-460

Nemeti, B., Anderson, M. E., & Gregus, Z. (2012). Glutathione synthetase promotes the reduction of arsenate via arsenolysis of glutathione. *Biochimie, 94*(6), 1327-1333. doi:10.1016/j.biochi.2012.02.033

Ng, J. C., Wang, J., & Shraim, A. (2003). A global health problem caused by arsenic from natural sources. *Chemosphere, 52*(9), 1353-1359.

Nusrat, A., Chen, J. A., Foley, C. S., Liang, T. W., Tom, J., Cromwell, M., . . . Mrsny, R. J. (2000). The coiled-coil domain of occludin can act to organize structural and functional elements of the epithelial tight junction. *J Biol Chem, 275*(38),

29816-29822. doi:10.1074/jbc.M002450200

O'Bryant, S. E., Edwards, M., Menon, C. V., Gong, G., & Barber, R. (2011). Long-term low-level arsenic exposure is associated with poorer neuropsychological functioning: a Project FRONTIER study. *Int J Environ Res Public Health, 8*(3), 861-874. doi:10.3390/ijerph8030861

Okumura, T., Nozu, T., Ishioh, M., Igarashi, S., Kumei, S., & Ohhira, M. (2021). Centrally administered butyrate improves gut barrier function, visceral sensation and septic lethality in rats. *J Pharmacol Sci, 146*(4), 183-191. doi:10.1016/j.jphs.2021.04.005

Ostrosky-Wegman, P., Gonsebatt, M. E., Montero, R., Vega, L., Barba, H., Espinosa, J., . . . et al. (1991). Lymphocyte proliferation kinetics and genotoxic findings in a pilot study on individuals chronically exposed to arsenic in Mexico. *Mutat Res, 250*(1-2), 477-482. doi:10.1016/0027-5107(91)90204-2

Ouellette, A. J. (2011). Paneth cell alpha-defensins in enteric innate immunity. *Cell Mol Life Sci, 68*(13), 2215-2229. doi:10.1007/s00018-011-0714-6

Padovani, A. M., Molina, M. F., & Mann, K. K. (2010). Inhibition of liver x receptor/retinoid X receptor-mediated transcription contributes to the proatherogenic effects of arsenic in macrophages in vitro. *Arterioscler Thromb Vasc Biol, 30*(6), 1228-1236. doi:10.1161/ATVBAHA.110.205500

Parvez, F., Chen, Y., Brandt-Rauf, P. W., Slavkovich, V., Islam, T., Ahmed, A., . . . Ahsan, H. (2010). A prospective study of respiratory symptoms associated with chronic arsenic exposure in Bangladesh: findings from the Health Effects of Arsenic Longitudinal Study (HEALS). *Thorax, 65*(6), 528-533. doi:10.1136/thx.2009.119347

Peng, L., He, Z., Chen, W., Holzman, I. R., & Lin, J. (2007). Effects of butyrate on intestinal barrier function in a Caco-2 cell monolayer model of intestinal barrier. *Pediatr Res, 61*(1), 37-41. doi:10.1203/01.pdr.0000250014.92242.f3

Peng, L., Li, Z. R., Green, R. S., Holzman, I. R., & Lin, J. (2009). Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. *J Nutr, 139*(9), 1619-1625. doi:10.3945/jn.109.104638

Pickard, J. M., Zeng, M. Y., Caruso, R., & Nunez, G. (2017). Gut microbiota: Role in pathogen colonization, immune responses, and inflammatory disease. *Immunol Rev, 279*(1), 70-89. doi:10.1111/imr.12567

Pinyayev, T. S., Kohan, M. J., Herbin-Davis, K., Creed, J. T., & Thomas, D. J. (2011). Preabsorptive metabolism of sodium arsenate by anaerobic microbiota of mouse cecum forms a variety of methylated and thiolated arsenicals. *Chemical research in toxicology, 24*(4), 475-477.

Quatrehomme, G., Ricq, O., Lapalus, P., Jacomet, Y., & Ollier, A. (1992). Acute arsenic intoxication: forensic and toxicologic aspects (an observation). *J Forensic Sci, 37*(4), 1163-1171. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/1506832

Quigley, E. M. (2013). Gut bacteria in health and disease. *Gastroenterol Hepatol (N Y), 9*(9), 560-569. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/24729765

Radabaugh, T. R., & Aposhian, H. V. (2000). Enzymatic reduction of arsenic compounds in mammalian systems: reduction of arsenate to arsenite by human liver arsenate reductase. *Chemical research in toxicology, 13*(1), 26-30.

Rahman, A., Islam, M. S., Tony, S. R., Siddique, A. E., Mondal, V., Hosen, Z., . . . Hossain, K. (2021). T helper 2-driven immune dysfunction in chronic arsenic-exposed individuals and its link to the features of allergic asthma. *Toxicol Appl Pharmacol, 420*, 115532. doi:10.1016/j.taap.2021.115532

Rahman, A., Vahter, M., Ekstrom, E. C., & Persson, L. A. (2011). Arsenic exposure in pregnancy increases the risk of lower respiratory tract infection and diarrhea during infancy in Bangladesh. *Environ Health Perspect, 119*(5), 719-724. doi:10.1289/ehp.1002265

Rahman, M. M., Ng, J. C., & Naidu, R. (2009). Chronic exposure of arsenic via drinking water and its adverse health impacts on humans. *Environ Geochem Health, 31 Suppl 1*, 189-200. doi:10.1007/s10653-008-9235-0

Ramos Elizagaray, S. I., & Soria, E. A. (2014). Arsenic immunotoxicity and immunomodulation by phytochemicals: potential relations to develop chemopreventive approaches. *Recent Pat Inflamm Allergy Drug Discov, 8*(2), 92-103. doi:10.2174/1872213x08666140619104944

Ramsey, K. A., Foong, R. E., Sly, P. D., Larcombe, A. N., & Zosky, G. R. (2013). Early life arsenic exposure and acute and long-term responses to influenza A infection in mice. *Environ Health Perspect, 121*(10), 1187-1193. doi:10.1289/ehp.1306748

Raqib, R., Ahmed, S., Ahsan, K. B., Kippler, M., Akhtar, E., Roy, A. K., . . . Vahter,

M. (2017). Humoral Immunity in Arsenic-Exposed Children in Rural Bangladesh: Total Immunoglobulins and Vaccine-Specific Antibodies. *Environ Health Perspect, 125*(6), 067006. doi:10.1289/EHP318

Raqib, R., Ahmed, S., Sultana, R., Wagatsuma, Y., Mondal, D., Hoque, A. M., . . . Vahter, M. (2009). Effects of in utero arsenic exposure on child immunity and morbidity in rural Bangladesh. *Toxicol Lett, 185*(3), 197-202. doi:10.1016/j.toxlet.2009.01.001

Reichard, J. F., & Puga, A. (2010). Effects of arsenic exposure on DNA methylation and epigenetic gene regulation. *Epigenomics, 2*(1), 87-104. doi:10.2217/epi.09.45

Ren, X., Gaile, D. P., Gong, Z., Qiu, W., Ge, Y., Zhang, C., . . . Wu, H. (2015). Arsenic responsive microRNAs in vivo and their potential involvement in arsenic-induced oxidative stress. *Toxicol Appl Pharmacol, 283*(3), 198-209. doi:10.1016/j.taap.2015.01.014

Ren, X., McHale, C. M., Skibola, C. F., Smith, A. H., Smith, M. T., & Zhang, L. (2011). An emerging role for epigenetic dysregulation in arsenic toxicity and carcinogenesis. *Environ Health Perspect, 119*(1), 11-19. doi:10.1289/ehp.1002114

Rossman, T. G. (2003). Mechanism of arsenic carcinogenesis: an integrated approach. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 533*(1-2), 37-65.

Runtsch, M. C., Round, J. L., & O'Connell, R. M. (2014). MicroRNAs and the regulation of intestinal homeostasis. *Front Genet, 5*, 347. doi:10.3389/fgene.2014.00347

Saha, A., Chowdhury, M. I., Nazim, M., Alam, M. M., Ahmed, T., Hossain, M. B., . . . Qadri, F. (2013). Vaccine specific immune response to an inactivated oral cholera vaccine and EPI vaccines in a high and low arsenic area in Bangladeshi children. *Vaccine, 31*(4), 647-652. doi:10.1016/j.vaccine.2012.11.049

Saha, D., & Ray, R. K. (2019). Groundwater resources of India: Potential, challenges and management. In *Groundwater development and management* (pp. 19-42): Springer.

Saitou, M., Furuse, M., Sasaki, H., Schulzke, J. D., Fromm, M., Takano, H., . . . Tsukita, S. (2000). Complex phenotype of mice lacking occludin, a component

of tight junction strands. *Mol Biol Cell, 11*(12), 4131-4142. doi:10.1091/mbc.11.12.4131

Salgado-Bustamante, M., Ortiz-Perez, M. D., Calderon-Aranda, E., Estrada-Capetillo, L., Nino-Moreno, P., Gonzalez-Amaro, R., & Portales-Perez, D. (2010). Pattern of expression of apoptosis and inflammatory genes in humans exposed to arsenic and/or fluoride. *Sci Total Environ, 408*(4), 760-767. doi:10.1016/j.scitotenv.2009.11.016

Salnikow, K., & Zhitkovich, A. (2008). Genetic and epigenetic mechanisms in metal carcinogenesis and cocarcinogenesis: nickel, arsenic, and chromium. *Chem Res Toxicol, 21*(1), 28-44. doi:10.1021/tx700198a

Sanchez de Medina, F., Romero-Calvo, I., Mascaraque, C., & Martinez-Augustin, O. (2014). Intestinal inflammation and mucosal barrier function. *Inflamm Bowel Dis, 20*(12), 2394-2404. doi:10.1097/MIB.0000000000000204

Santra, A., Maiti, A., Das, S., Lahiri, S., Charkaborty, S. K., & Mazumder, D. N. (2000). Hepatic damage caused by chronic arsenic toxicity in experimental animals. *J Toxicol Clin Toxicol, 38*(4), 395-405. doi:10.1081/clt-100100949

Sasaki, A., Oshima, Y., & Fujimura, A. (2007). An approach to elucidate potential mechanism of renal toxicity of arsenic trioxide. *Exp Hematol, 35*(2), 252-262. doi:10.1016/j.exphem.2006.10.004

Schmuck, E. M., Board, P. G., Whitbread, A. K., Tetlow, N., Cavanaugh, J. A., Blackburn, A. C., & Masoumi, A. (2005). Characterization of the monomethylarsonate reductase and dehydroascorbate reductase activities of Omega class glutathione transferase variants: implications for arsenic metabolism and the age-at-onset of Alzheimer's and Parkinson's diseases. *Pharmacogenet Genomics, 15*(7), 493-501. doi:10.1097/01.fpc.0000165725.81559.e3

Schneeberger, E. E., & Lynch, R. D. (2004). The tight junction: a multifunctional complex. *Am J Physiol Cell Physiol, 286*(6), C1213-1228. doi:10.1152/ajpcell.00558.2003

Schroeder, H. A., & Mitchener, M. (1971). Toxic effects of trace elements on the reproduction of mice and rats. *Arch Environ Health, 23*(2), 102-106. doi:10.1080/00039896.1971.10665963

Schulthess, J., Pandey, S., Capitani, M., Rue-Albrecht, K. C., Arnold, I., Franchini, F., . . . Powrie, F. (2019). The Short Chain Fatty Acid Butyrate Imprints an

Antimicrobial Program in Macrophages. *Immunity, 50*(2), 432-445 e437. doi:10.1016/j.immuni.2018.12.018

Schwarzenbach, R. P., Egli, T., Hofstetter, T. B., Von Gunten, U., & Wehrli, B. (2010). Global water pollution and human health. *Annual review of environment and resources, 35*(1), 109-136.

Segain, J. P., Raingeard de la Bletiere, D., Bourreille, A., Leray, V., Gervois, N., Rosales, C., . . . Galmiche, J. P. (2000). Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. *Gut, 47*(3), 397-403. doi:10.1136/gut.47.3.397

Selgrade, M. K. (2007). Immunotoxicity: the risk is real. *Toxicol Sci, 100*(2), 328-332. doi:10.1093/toxsci/kfm244

Shaji, E., Santosh, M., Sarath, K., Prakash, P., Deepchand, V., & Divya, B. (2021). Arsenic contamination of groundwater: A global synopsis with focus on the Indian Peninsula. *Geoscience frontiers, 12*(3), 101079.

Shen, S., & Wong, C. H. (2016). Bugging inflammation: role of the gut microbiota. *Clin Transl Immunology, 5*(4), e72. doi:10.1038/cti.2016.12

Sikorski, E. E., Burns, L. A., Stern, M. L., Luster, M. I., & Munson, A. E. (1991). Splenic cell targets in gallium arsenide-induced suppression of the primary antibody response. *Toxicol Appl Pharmacol, 110*(1), 129-142. doi:10.1016/0041-008x(91)90296-q

Silva, J. P. B., Navegantes-Lima, K. C., Oliveira, A. L. B., Rodrigues, D. V. S., Gaspar, S. L. F., Monteiro, V. V. S., . . . Monteiro, M. C. (2018). Protective Mechanisms of Butyrate on Inflammatory Bowel Disease. *Curr Pharm Des, 24*(35), 4154-4166. doi:10.2174/1381612824666181001153605

Singh, B., Halestrap, A. P., & Paraskeva, C. (1997). Butyrate can act as a stimulator of growth or inducer of apoptosis in human colonic epithelial cell lines depending on the presence of alternative energy sources. *Carcinogenesis, 18*(6), 1265-1270. doi:10.1093/carcin/18.6.1265

Singh, V., Gera, R., Kushwaha, R., Sharma, A. K., Patnaik, S., & Ghosh, D. (2016). Hijacking microglial glutathione by inorganic arsenic impels bystander death of immature neurons through extracellular cystine/glutamate imbalance. *Sci Rep, 6*, 30601. doi:10.1038/srep30601

Singh, V., Mitra, S., Sharma, A. K., Gera, R., & Ghosh, D. (2014). Isolation and characterization of microglia from adult mouse brain: selected applications for

ex vivo evaluation of immunotoxicological alterations following in vivo xenobiotic exposure. *Chem Res Toxicol, 27*(5), 895-903. doi:10.1021/tx500046k

Smedley, P. L., & Kinniburgh, D. G. (2001). Source and behaviour of arsenic in natural waters. *United Nations synthesis report on arsenic in drinking water. World Health Organization, Geneva, Switzerland. http://www. who. int/water_sanitation_health/dwq/arsenicun1. pdf*, 1-61.

Smith, P. M., Howitt, M. R., Panikov, N., Michaud, M., Gallini, C. A., Bohlooly, Y. M., . . . Garrett, W. S. (2013). The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science, 341*(6145), 569-573. doi:10.1126/science.1241165

Smith, Z. D., Chan, M. M., Mikkelsen, T. S., Gu, H., Gnirke, A., Regev, A., & Meissner, A. (2012). A unique regulatory phase of DNA methylation in the early mammalian embryo. *Nature, 484*(7394), 339-344. doi:10.1038/nature10960

Sommer, F., & Backhed, F. (2013). The gut microbiota--masters of host development and physiology. *Nat Rev Microbiol, 11*(4), 227-238. doi:10.1038/nrmicro2974

Soto-Pena, G. A., Luna, A. L., Acosta-Saavedra, L., Conde, P., Lopez-Carrillo, L., Cebrian, M. E., . . . Vega, L. (2006). Assessment of lymphocyte subpopulations and cytokine secretion in children exposed to arsenic. *FASEB J, 20*(6), 779-781. doi:10.1096/fj.05-4860fje

Sousa, T., Paterson, R., Moore, V., Carlsson, A., Abrahamsson, B., & Basit, A. W. (2008). The gastrointestinal microbiota as a site for the biotransformation of drugs. *Int J Pharm, 363*(1-2), 1-25. doi:10.1016/j.ijpharm.2008.07.009

States, J. C., Srivastava, S., Chen, Y., & Barchowsky, A. (2009). Arsenic and cardiovascular disease. *Toxicol Sci, 107*(2), 312-323. doi:10.1093/toxsci/kfn236

Stensballe, L. G., Simonsen, J., Jensen, S. M., Bonnelykke, K., & Bisgaard, H. (2013). Use of antibiotics during pregnancy increases the risk of asthma in early childhood. *J Pediatr, 162*(4), 832-838 e833. doi:10.1016/j.jpeds.2012.09.049

Suhag, R. (2016). Overview of ground water in India. *PRS Legislative Research, 9504*(February), 12.

Suzuki, T. (2013). Regulation of intestinal epithelial permeability by tight junctions.

Cell Mol Life Sci, 70(4), 631-659. doi:10.1007/s00018-012-1070-x

Tanaka, T. (1988). Distribution of arsenic in the natural environment with emphasis on rocks and soils. *Applied Organometallic Chemistry, 2*(4), 283-295.

Tokar, E. J., Qu, W., Liu, J., Liu, W., Webber, M. M., Phang, J. M., & Waalkes, M. P. (2010). Arsenic-specific stem cell selection during malignant transformation. *J Natl Cancer Inst, 102*(9), 638-649. doi:10.1093/jnci/djq093

Tremaroli, V., & Backhed, F. (2012). Functional interactions between the gut microbiota and host metabolism. *Nature, 489*(7415), 242-249. doi:10.1038/nature11552

Tsai, S. Y., Chou, H. Y., The, H. W., Chen, C. M., & Chen, C. J. (2003). The effects of chronic arsenic exposure from drinking water on the neurobehavioral development in adolescence. *Neurotoxicology, 24*(4-5), 747-753. doi:10.1016/S0161-813X(03)00029-9

Tsuji, J. S., Garry, M. R., Perez, V., & Chang, E. T. (2015). Low-level arsenic exposure and developmental neurotoxicity in children: A systematic review and risk assessment. *Toxicology, 337*, 91-107. doi:10.1016/j.tox.2015.09.002

Turner, J. R. (2009). Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol, 9*(11), 799-809. doi:10.1038/nri2653

Vahter, M. (1994). Species differences in the metabolism of arsenic compounds. *Applied Organometallic Chemistry, 8*(3), 175-182.

Vahter, M. (2008). Health effects of early life exposure to arsenic. *Basic Clin Pharmacol Toxicol, 102*(2), 204-211. doi:10.1111/j.1742-7843.2007.00168.x

Van de Wiele, T., Gallawa, C. M., Kubachka, K. M., Creed, J. T., Basta, N., Dayton, E. A., . . . Bradham, K. (2010). Arsenic metabolism by human gut microbiota upon in vitro digestion of contaminated soils. *Environ Health Perspect, 118*(7), 1004-1009. doi:10.1289/ehp.0901794

Vega, L., Ostrosky-Wegman, P., Fortoul, T. I., Diaz, C., Madrid, V., & Saavedra, R. (1999). Sodium arsenite reduces proliferation of human activated T-cells by inhibition of the secretion of interleukin-2. *Immunopharmacol Immunotoxicol, 21*(2), 203-220. doi:10.3109/08923979909052758

von Ehrenstein, O. S., Guha Mazumder, D. N., Hira-Smith, M., Ghosh, N., Yuan, Y., Windham, G., . . . Smith, A. H. (2006). Pregnancy outcomes, infant mortality, and arsenic in drinking water in West Bengal, India. *Am J Epidemiol, 163*(7), 662-669. doi:10.1093/aje/kwj089

Wall, S. (1980). Survival and mortality pattern among Swedish smelter workers. *Int J Epidemiol, 9*(1), 73-87. doi:10.1093/ije/9.1.73

Wang, B. J., Lee, Y. Y., Mak, C. P., Kao, H. F., Hsu, M. L., & Hsien, J. R. (1991). Quantitative and morphological changes of Langerhans cells in Bowen's disease from patients with chronic arsenicism. *J Formos Med Assoc, 90*(11), 1093-1098. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/1687057

Wang, J. P., Qi, L., Zheng, B., Liu, F., Moore, M. R., & Ng, J. C. (2002). Porphyrins as early biomarkers for arsenic exposure in animals and humans. *Cell Mol Biol (Noisy-le-grand), 48*(8), 835-843. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/12699241

Wang, L., Xu, J., Ji, C., Gu, S., Lv, Y., Li, S., . . . Mao, Y. (2005). Cloning, expression and characterization of human glutathione S-transferase Omega 2. *Int J Mol Med, 16*(1), 19-27. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/15942673

Wang, Z., Zhou, J., Lu, X., Gong, Z., & Le, X. C. (2004). Arsenic speciation in urine from acute promyelocytic leukemia patients undergoing arsenic trioxide treatment. *Chem Res Toxicol, 17*(1), 95-103. doi:10.1021/tx0341714

Waxman, S., & Anderson, K. C. (2001). History of the development of arsenic derivatives in cancer therapy. *The oncologist, 6*(S2), 3-10.

Weber, C. R. (2012). Dynamic properties of the tight junction barrier. *Ann N Y Acad Sci, 1257*, 77-84. doi:10.1111/j.1749-6632.2012.06528.x

Welch, A. H., Lico, M. S., & Hughes, J. L. (1988). Arsenic in ground water of the western United States. *Groundwater, 26*(3), 333-347.

Welch, K., Higgins, I., Oh, M., & Burchfiel, C. (1982). Arsenic exposure, smoking, and respiratory cancer in copper smelter workers. *Arch Environ Health, 37*(6), 325-335. doi:10.1080/00039896.1982.10667586

WHO. (2000). Air quality guidelines for Europe.

Worsley, S. F., Davies, C. S., Mannarelli, M. E., Hutchings, M. I., Komdeur, J., Burke, T., . . . Richardson, D. S. (2021). Gut microbiome composition, not alpha diversity, is associated with survival in a natural vertebrate population. *Anim Microbiome, 3*(1), 84. doi:10.1186/s42523-021-00149-6

Wu, M. M., Chiou, H. Y., Ho, I. C., Chen, C. J., & Lee, T. C. (2003). Gene expression of inflammatory molecules in circulating lymphocytes from arsenic-exposed human subjects. *Environ Health Perspect, 111*(11), 1429-1438.

doi:10.1289/ehp.6396

Yan, L., Yang, C., & Tang, J. (2013). Disruption of the intestinal mucosal barrier in Candida albicans infections. *Microbiol Res, 168*(7), 389-395. doi:10.1016/j.micres.2013.02.008

Yang, C. Y., Chang, C. C., Tsai, S. S., Chuang, H. Y., Ho, C. K., & Wu, T. N. (2003). Arsenic in drinking water and adverse pregnancy outcome in an arseniasis-endemic area in northeastern Taiwan. *Environ Res, 91*(1), 29-34. doi:10.1016/s0013-9351(02)00015-4

Yang, H. T., Chou, H. J., Han, B. C., & Huang, S. Y. (2007). Lifelong inorganic arsenic compounds consumption affected blood pressure in rats. *Food Chem Toxicol, 45*(12), 2479-2487. doi:10.1016/j.fct.2007.05.024

Yang, P., Ebbert, J. O., Sun, Z., & Weinshilboum, R. M. (2006). Role of the glutathione metabolic pathway in lung cancer treatment and prognosis: a review. *J Clin Oncol, 24*(11), 1761-1769. doi:10.1200/JCO.2005.02.7110

Yang, W., Xu, H. W., Lu, X. R., Xu, Q. F., Tao, M. H., & Dai, Y. M. (2022). Overexpression of miR-122 Impairs Intestinal Barrier Function and Aggravates Acute Pancreatitis by Downregulating Occludin Expression. *Biochem Genet, 60*(1), 382-394. doi:10.1007/s10528-021-10106-2

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., . . . Gordon, J. I. (2012). Human gut microbiome viewed across age and geography. *Nature, 486*(7402), 222-227. doi:10.1038/nature11053

Ye, D., Guo, S., Al-Sadi, R., & Ma, T. Y. (2011). MicroRNA regulation of intestinal epithelial tight junction permeability. *Gastroenterology, 141*(4), 1323-1333. doi:10.1053/j.gastro.2011.07.005

Yeh, S., How, S. W., & Lin, C. S. (1968). Arsenical cancer of skin. Histologic study with special reference to Bowen's disease. *Cancer, 21*(2), 312-339. doi:10.1002/1097-0142(196802)21:2<312::aid-cncr2820210222>3.0.co;2-k

Yip, W., Hughes, M. R., Li, Y., Cait, A., Hirst, M., Mohn, W. W., & McNagny, K. M. (2021). Butyrate Shapes Immune Cell Fate and Function in Allergic Asthma. *Front Immunol, 12*, 628453. doi:10.3389/fimmu.2021.628453

Yoon, M. Y., Lee, K., & Yoon, S. S. (2014). Protective role of gut commensal microbes against intestinal infections. *J Microbiol, 52*(12), 983-989. doi:10.1007/s12275-014-4655-2

Young, J. L., Cai, L., & States, J. C. (2018). Impact of prenatal arsenic exposure on

chronic adult diseases. *Syst Biol Reprod Med, 64*(6), 469-483. doi:10.1080/19396368.2018.1480076

Yudovich, Y. E., & Ketris, M. (2005). Arsenic in coal: a review. *International Journal of Coal Geology, 61*(3-4), 141-196.

Zakharyan, R. A., & Aposhian, H. V. (1999). Enzymatic reduction of arsenic compounds in mammalian systems: the rate-limiting enzyme of rabbit liver arsenic biotransformation is MMAV reductase. *Chemical research in toxicology, 12*(12), 1278-1283.

Zakharyan, R. A., Sampayo-Reyes, A., Healy, S. M., Tsaprailis, G., Board, P. G., Liebler, D. C., & Aposhian, H. V. (2001). Human monomethylarsonic acid (MMA(V)) reductase is a member of the glutathione-S-transferase superfamily. *Chem Res Toxicol, 14*(8), 1051-1057. doi:10.1021/tx010052h

Zhang, C., Zhang, M., Wang, S., Han, R., Cao, Y., Hua, W., . . . Zhao, L. (2010). Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. *ISME J, 4*(2), 232-241. doi:10.1038/ismej.2009.112

Zhao, Y., Zhang, J., Huang, W., Wang, Z., Li, Y., Song, D., . . . Zheng, C. (2008). Arsenic emission during combustion of high arsenic coals from Southwestern Guizhou, China. *Energy Conversion and Management, 49*(4), 615-624.

Zheng, L., Kuo, C. C., Fadrowski, J., Agnew, J., Weaver, V. M., & Navas-Acien, A. (2014). Arsenic and Chronic Kidney Disease: A Systematic Review. *Curr Environ Health Rep, 1*(3), 192-207. doi:10.1007/s40572-014-0024-x

Publications and Proceedings

Prenatal arsenic exposure interferes in postnatal immunocompetence despite an absence of ongoing arsenic exposure.

Mainak Chakraborty and Moumita Bhaumik. ***Journal of Immunotoxicology***, 17(1), 135-143.

Prenatal arsenic exposure stymies gut butyrate production and enhances gut permeability in post natal life even in absence of arsenic deftly through miR122-Occludin pathway.

Mainak Chakraborty, Anupam Gautam, Oishika Das, Aaheli Masid and Moumita Bhaumik. ***Toxicology Letters***, 374, 19-30.

AUF-1 knockdown in mice undermines gut microbial butyrate-driven hypocholesterolemia through AUF-1–Dicer-1–mir-122 hierarchy.

Oishika Das, Jayanta Kundu, Atanu Ghosh, Anupam Gautam, Souradeepa Ghosh, **Mainak Chakraborty**, Aaheli Masid, Samiran Sona Gauri, Debmalya Mitra, Moumita Dutta, Budhaditya Mukherjee, Surajit Sinha, Moumita Bhaumik. ***Frontiers in Cellular and Infection Microbiology***, 12, 1864.

Journal of Immunotoxicology

Taylor & Francis
Taylor & Francis Group

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/iimt20

Prenatal arsenic exposure interferes in postnatal immunocompetence despite an absence of ongoing arsenic exposure

Mainak Chakraborty & Moumita Bhaumik

To cite this article: Mainak Chakraborty & Moumita Bhaumik (2020) Prenatal arsenic exposure interferes in postnatal immunocompetence despite an absence of ongoing arsenic exposure, Journal of Immunotoxicology, 17:1, 135-143, DOI: 10.1080/1547691X.2020.1767238

To link to this article: https://doi.org/10.1080/1547691X.2020.1767238

Published online: 13 Jun 2020.

JOURNAL OF IMMUNOTOXICOLOGY
2020, VOL. 17, NO. 1, 135–143
https://doi.org/10.1080/1547691X.2020.1767238

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

OPEN ACCESS Check for updates

Prenatal arsenic exposure interferes in postnatal immunocompetence despite an absence of ongoing arsenic exposure

Mainak Chakraborty and Moumita Bhaumik

Division of Immunology, ICMR-National Institute of Cholera and Enteric Diseases, Kolkata, India

ABSTRACT

Arsenic (As) readily crosses the placenta and exposure of the fetus may cause adverse consequences later in life, including immunomodulation. In the current study, the question was asked how the immune repertoire might respond in postnatal life when there is no further As exposure. Here, pregnant mice (Balb/c [H-2^d]) were exposed to arsenic trioxide (As_2O_3) through their drinking water from time of conception until parturition. Their offspring, 4-week-old mice who had not been exposed again to As, were used for functional analyses of innate, humoral and cellular immunity. Compared to cells from non-As-exposed dam offspring, isolated peritoneal macro-phages (Mϕ) displayed no differences in T-cell stimulating ability. Levels of circulating IgG_{2a} but not IgG_1 were decreased in As-exposed dam offspring as compared to control offspring counterparts. Mixed-leukocyte reactions (MLR) indicated that $CD4^+$ T-cells from the prenatal As-exposed mice were significantly less responsive to allogenic stimulation as evidenced by decreases in interferon (IFN)-γ and IL-2 production and in expression of CD44 and CD69 (but not CD25) activation markers. Interestingly, the Mϕ from the prenatal As-exposed mice were capable of stimulating normal allogenic T-cells, indicating that T-cells from these mice were refractory to allogenic signals. There was also a significant decrease in absolute numbers of splenic $CD4^+$ and $CD8^+$ T-cells due to prenatal As exposure (as compared to control). Lastly, the impaired immune function of the prenatal As-exposed mice was correlated with a very strong susceptibility to *Escherichia coli* infection. Taken together, the data from this study clearly show that *in utero* As exposure may continue to perpetuate a dampening effect on the immune repertoire of offspring, even into the early stages of postnatal life.

ARTICLE HISTORY
Received 16 January 2020
Revised 20 April 2020
Accepted 6 May 2020

KEYWORDS
Arsenic; prenatal; CD4+ T cell; IL-2; immunosuppression

Introduction

Arsenic (As) can induce a wide range of health effects and so is a major concern world-wide. As arsenic is reported to cross the placenta, the fetus is particularly vulnerable to toxic insult *in utero* (Concha et al. 1998; Davis et al. 2015). Immunotoxic effects of arsenic in early life have been widely studied in human and animal models. For instance, immunosuppression due to prenatal As exposure was identified (i.e. poor cellular immune function reported among cord blood cells) in human cohorts (Nadeau et al. 2014). Impaired function of T-cell subsets has also shown to be associated with prenatal As exposure. The presence of maternal cells in the cord blood limits the observation to be conclusive on immunosuppression in early childhood.

Substantial childhood morbidity that has been documented in As-polluted areas is probably due to compromised immune status. *In utero* exposure to As has been reported to reduce CD4/CD8 ratios in offspring (Soto-Pena et al. 2006). In preschool students in rural Bangladesh, As exposure appeared to cause reductions in cell-mediated immunity and in formation of T-helper (T_H) Type-1 cytokines (Raqib et al. 2017). Other studies reported alterations in fetal thymic function induced by oxidative stress and apoptosis (Ahmed et al. 2012). Such changes in immune function/thymic development have a potential to increase host susceptibility to infection, as well as to autoimmune diseases/chronic diseases in childhood and also later in life.

The prevalence of infectious diseases has been reported to be increased dramatically in As-impacted regions of the world (Ferrario et al. 2016; Gera et al. 2017). Chronic low-level As exposure has been reported to increase host risk of lung infections, influenza, diarrhea, etc. (Raqib et al. 2009; Rahman et al. 2011; Smith et al. 2012, 2013; Farzan et al. 2016), especially among children. While in many cases these outcomes might be associated with ongoing As exposure of the children, it should be recalled that As exposure can affect fetal development (Vahter 2008; Kippler et al. 2016; Attreed et al. 2017) and even cause long-term epigenetic re-programming of the fetus (Bailey and Fry 2014). One epidemiological study showed an association between *in utero* As exposure and development of adult diseases (Young et al. 2018). If one of the results of these changes was alterations in child immunocompetence, this might mean these hosts could be impacted even if they were no longer exposed to As in their environments.

A few studies have indicated that As exposure is associated with changes in the immune system (Ferrario et al. 2016). In various models, direct As exposure caused suppressed immunoglobulin production (Selgrade 2007), decreased cytokine expression (Conde et al. 2007), defective antigen-driven T-cell

CONTACT Moumita Bhaumik drmoumitabhaumik@gmail.com Division of Immunology, ICMR-National Institute of Cholera and Enteric Diseases, P-33 C.I.T Rd Beleghata, Kolkata 700010, India

Supplemental data for this article can be accessed here.

proliferation, and reduced macrophage reactive oxygen species generation (Vega et al. 1999). It was also shown that As exposure *in vitro* can reduce IL-2 secretion by lymphocytes (Vega et al. 1999; Galicia et al. 2003). One mechanism proposed to explain this observation was that there is a delayed activation of T-cells due to As-induced accumulation of IL-2 in these cells (Galicia et al. 2003). There is also mounting evidences of an increased risk of infections due to prenatal As exposures (Ramsey et al. 2013). Thus, it is evident from the literature – from data derived from different experimental conditions and in different mammalian species – that all three arms of the host immune repertoire, i.e. innate, humoral, and cellular, can be affected by As exposure. While most of this data is based on *de facto* As exposures, less is known about what may happen as a result of *in utero* exposures to As.

In the investigation reported here, all three arms of immune system i.e. innate, humoral, and cellular, were evaluated to better understand how/if *in utero* As exposure influences the postnatal host immune repertoire. To address this question, As was given via drinking water to pregnant Balb/c mice and their resulting pups (who underwent no further As exposure) were examined.

Materials and methods

Reagents

Arsenic trioxide (As_2O_3) was purchased from MP Biomedicals (Irvine, CA). Concanavalin A (ConA), and most other general reagents were procured from Sigma (St. Louis, MO). Biotin-conjugated anti-mouse IgG_{2a} and anti-IgG_1 were also procured from Sigma. Cell culture reagents and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Waltham, MA) and Sigma. ELISA kits used for analyses of IL-2 and IFNγ were purchased from BD Biosciences (San Jose, CA). PerCP (peridinin-chlorophyll-protein)-conjugated-anti-CD4, APC (allophycocyanin)-anti-CD8, FITC (fluorescein isothiocyanate)-anti-CD25, APC-anti-CD44, FITC-anti-CD69, FITC-anti-CD11b, and PE (phycoerythrin)-anti-I-A^d were purchased from eBiosciences (San Diego, CA).

Animals

Male and female Balb/c (H-2^d) and C57BL/6 (H-2^b) mice (all 6-week-of-age) were procured from the ICMR-National Institute of Cholera and Enteric Diseases (Kolkata, India). All mice were housed in cages containing straw bedding held in pathogen-free facilities maintained at 24 °C with a 50% relative humidity and 12-h light:dark cycle. All mice had *ad libitum* access to standard rodent chow. After 2 weeks of acclimatization, the Balb/c (H-2^d) mice were bred by housing two females with a male. Once pregnancy was confirmed, the dams were given *ad libitum* access to drinking water containing 4 ppm As as described in He et al. (2007). The As-containing water was changed twice weekly. After birth, the mothers were then given *ad libitum* access to clean As-free water. For the experiments, when pups reached 4 week-of-age, groups were randomly collected, and processed for biomaterials. Age-matched juvenile mice whose mothers were never exposed to As were processed in parallel as controls. For each experiment, 5–6 juvenile mice were randomly chosen (without any sex bias) for evaluation (Aung et al. 2016).

All protocols were approved by the Institutional Animal Ethics Committee of ICMR-NICED (PRO/151/July 2018–June 2021). All experiments were done in accordance with the guidelines set by the committee for the purpose of control and supervision of experiments on animals (CPCSEA; Ministry of Environment and Forests, New Delhi, India).

Histopathologic examination of offspring spleen and thymus

At 28 days-of-age, after their body weights were recorded, five mice in each group were selected, blood was drawn from their tail vein, and then they were euthanized by cervical dislocation. At necropsy, both macroscopic observations and weights of the spleen and thymus from each mouse were taken. Each organ was then fixed in 4% paraformaldehyde for 48 h at 4 °C. The fixed tissues were then dehydrated through graded alcohols, embedded in paraffin, and routine microtomy then carried out to generate 5-μM sections. The sections, in turn, were stained with hematoxylin and eosin for later microscopic examination.

Analysis of serum levels of IgG_1 and IgG_{2a}

Each collected tail vein blood sample was allowed to stand for 3 h at room temperature and then serum was isolated by centrifugation at 1800 rpm. Serum samples were then analyzed to determine total IgG_1 and IgG_{2a} titers. For this, an aliquot of each sample was diluted 1:10, 1:100, and 1:1000 with PBS (phosphate-buffered saline, pH 7.4) + 10% FBS solution. For each paired sets of diluted sample, an aliquot of the diluted serum was added to a 96-well plate whose wells had been pre-coated with biotin-conjugated mouse anti-mouse IgG_1 or mouse anti-mouse IgG_{2a}. The plates were then incubated for 1 h at room temperature before unbound serum components were removed by repeated washings with PBS (and centrifugation). Detection reagent (avidin-conjugated horseradish peroxidase) was then added to each well and the plates were incubated a further 1 h. The absorbance in each tube was then evaluated at 450 nm in an iMark™ Microplate Absorbance Reader (BioRad, Hercules, CA). Results were reported in terms of mean OD values. Each mouse sample was analyzed in triplicate.

Isolation of peritoneal exudate macrophages (PEMϕ)

For use in the MLR assays outlined below, sets of five 28-day-old Balb/c (As-exposed and unexposed) as well as naive C57BL/6 mice were each injected intraperitoneally (IP) once with 3 ml of a 4% (w/v) starch solution in water. After 48 hr, PEMϕ were collected by peritoneal lavage, pelleted, re-suspended in RPMI medium supplemented with 10% FBS, 100 U penicillin/ml, and 100 μg streptomycin/ml (the latter two from Gibco [Waltham, MA]), and then seeded into 24-well plates at 5×10^4 cells/ml (0.5 ml/well) (Zhang et al. 2008). The cells were then cultured for 48 h at 37 °C in a humidified 5% CO_2 incubator to dampen/mitigate any residual effects of the starch. Non-adherent cells were removed thereafter by gentle washing of the wells with serum-free medium. The remaining adherent cells were then collected by gentle scraping with a plastic scraper for use in the MLR reactions (see below).

One-way mixed-leukocyte reactions (MLR)

Allo-antigens on antigen-presenting cells (APC) can be recognized by T-cells and studied in mixed-leukocyte reactions (MLR) using methods first defined by Lause et al. (1976). For the current study, Balb/C and C57BL/6 mice (MHC disparate

[allogenic]) T-cells were utilized. In one sub-study, PEM from the 28-day old Balb/c hosts were cultured with allogenic normal $CD4^+$ T-cells of C57BL/6 mice. Specifically, PEMϕ derived from the Balb/c mice (As-exposed and unexposed; see above) were treated in their wells with mitomycin c (10 μg/ml) for 6 h, and then washed thoroughly with complete RPMI medium (Sui et al. 2017). Thereafter, each well received aliquots of allogenic normal splenic $CD4^+$ T-cells from C57BL/6 mice (at a ratio of 1:5; isolation of cells outlined below) and then were incubated for 72 h at 37 °C in a humidified CO_2 incubator. At indicated timepoints over this period, samples from each well were collected for later measures of IL-2 in order to assess $CD4^+$ T-cell activation. In a parallel sub-study, naive C57BL/6 PEMϕ were cultured with allogenic $CD4^+$ T-cells from the As-exposed or unexposed Balb/c mice. As above, the C57BL/6 PEMϕ were first treated with mitomycin c and then incubated with splenic $CD4^+$ T-cells from the As-exposed or unexposed 28-day-old Balb/c mice. As a control, C57BL/6 PEMϕ were cultured with syngeneic T-cells in parallel. Culture samples were collected during the 72-hr period to permit measures of IL-2.

Analysis of splenic T-cell populations

For analysis of potential effects from maternal exposure to As on their T-cell populations, at 28-days-of-age, sets of As-exposed and unexposed mice were euthanized. At necropsy, each had their spleen aseptically removed and processed to generate single cell suspensions for use as indicated below (Ferrario et al. 2016). The numbers of nucleated cells were then counted using a hemocytometer. To estimate splenic levels of $CD4^+$ and $CD8^+$ T-cells, aliquots containing 10^6 splenocytes/mouse were stained for 0.5 h on ice with a solution of PerCP-anti-CD4 and APC-anti-CD8 monoclonal antibody (1:500 dilutions) in PBS. The cells were then immediately analyzed in a FACS Aria II system using FACSDiva software (both Becton Dickinson, San Jose, CA). A minimum of 10,000 events per sample was acquired. Absolute numbers of splenic $CD4^+$ and $CD8^+$ T-cells were then determined as described in Helmby et al. (2000).

For their use in the MLR above and for analysis of activation marker expression, splenic $CD4^+$ T-cells were isolated from among the starting sets of splenocytes (from Balb/c as well as from the naive C57BL/6 mice) using an Easy Sep $CD4^+$ T-cell enrichment kit (Stem Cell Technologies, Vancouver, Canada), following manufacturer protocols. For the analyses of T-cell activation markers, parallel sets of purified $CD4^+$ T-cells from the Balb/c mice were incubated with allogenic PEMϕ (5:1 ratio). After 24 hr of incubation, the T-cells were collected, and then stained and analyzed for CD44 and CD69 expression by flow cytometry (as above). In another experiment, the co-incubations were allowed to proceed for 72 h before the T-cells were collected, stained, and analyzed for CD25 expression by flow cytometry.

Host resistance to infection

To assess the impact of the prenatal As exposures on the intact host immune system, additional sets of control and prenatal As-exposed Balb/c mice ($n = 3$/group) were injected IP with a potent strain of *Escherichia coli* and then levels of the bacteria were assessed in several immunologically-active sites in the body (i.e. spleen, liver, peritoneal cavity). For the assay, *E. coli* strain E14 were grown overnight at 37 °C in Luria Bertani broth. After determining their concentration by optical density (derived from turbidity at 600 nm), the bacteria were washed by centrifugation and re-suspended in PBS for subsequent IP injection of 10^8 *E. coli* (in 100 μl PBS). At 24 h post-infection, each mouse was euthanized by cervical dislocation, and their peritoneal cavity was rinsed with PBS (2 ml) that was in turn placed on ice. The liver and spleen were then aseptically removed and dissociated in PBS using a tissue homogenizer. Aliquots of each homogenate and of the peritoneal wash (20 μl wash/mouse) were then plated onto LB agar plates for estimation of bacterial load after overnight incubation at 37 °C. All data are reported in terms of absolute counting colony forming units (CFU) per g organ or per ml peritoneal wash.

Statistical analysis

All data (apart from contour plots) are reported as mean ± SE. All statistical analyses were performed using Prism-5 Software (GraphPad, San Diego, CA). All data were analyzed using a non-parametric *t*-test with 95% confidence intervals. A $p < 0.05$ was considered significant.

Results

Prenatal As exposure effects on body weight and thymic/splenic weight and histology

In this study, arsenic (As_2O_3) was given to the dams in drinking water during the length of their pregnancy (Figure 1(A)). The data showed that the As-exposed pups did not differ in body weight from control pups (Figure 1(B)), nor did weights of their spleen or thymus differ (Figures 1(C,D)). Histologic examination of each organ revealed no gross changes (Figure 1(E)).

Prenatal As exposure effects on circulating levels of IgG_{2a} and IgG_1

To assess potential shifts in future humoral immunity-related events, circulating levels of IgG_{2a} and IgG_1 in the As-exposed and unexposed Balb/c offspring were evaluated. No differences in IgG_1 status was noted between the groups (Figure 2(A)). However, IgG_{2a} levels were decreased significantly in the prenatal As-exposed Balb/c pups as compared to levels seen with control pups (Figure 2(B)). IgG_{2a} levels were ≈30% lower due to maternal exposure to As.

Prenatal As exposure effects on splenic $CD4^+$ and $CD8^+$ T-cells levels

The frequencies of splenic $CD4^+$ and $CD8^+$ T-cells were evaluated in the mice. The data show that the absolute number of nucleated splenic cells remained unaltered as a result of the prenatal As exposures (Supplemental Table 1). In contrast, the absolute numbers of splenic $CD4^+$ and $CD8^+$ T-cells were decreased significantly in the pups whose dams underwent As exposure (Figure 3(A)). Specifically, there was a ≈ 20% reduction in $CD4^+$ T-cells (Figure 3(B)) and a 5% reduction in $CD8^+$ T-cells (Figure 3(C)) vs. levels in control pup spleens.

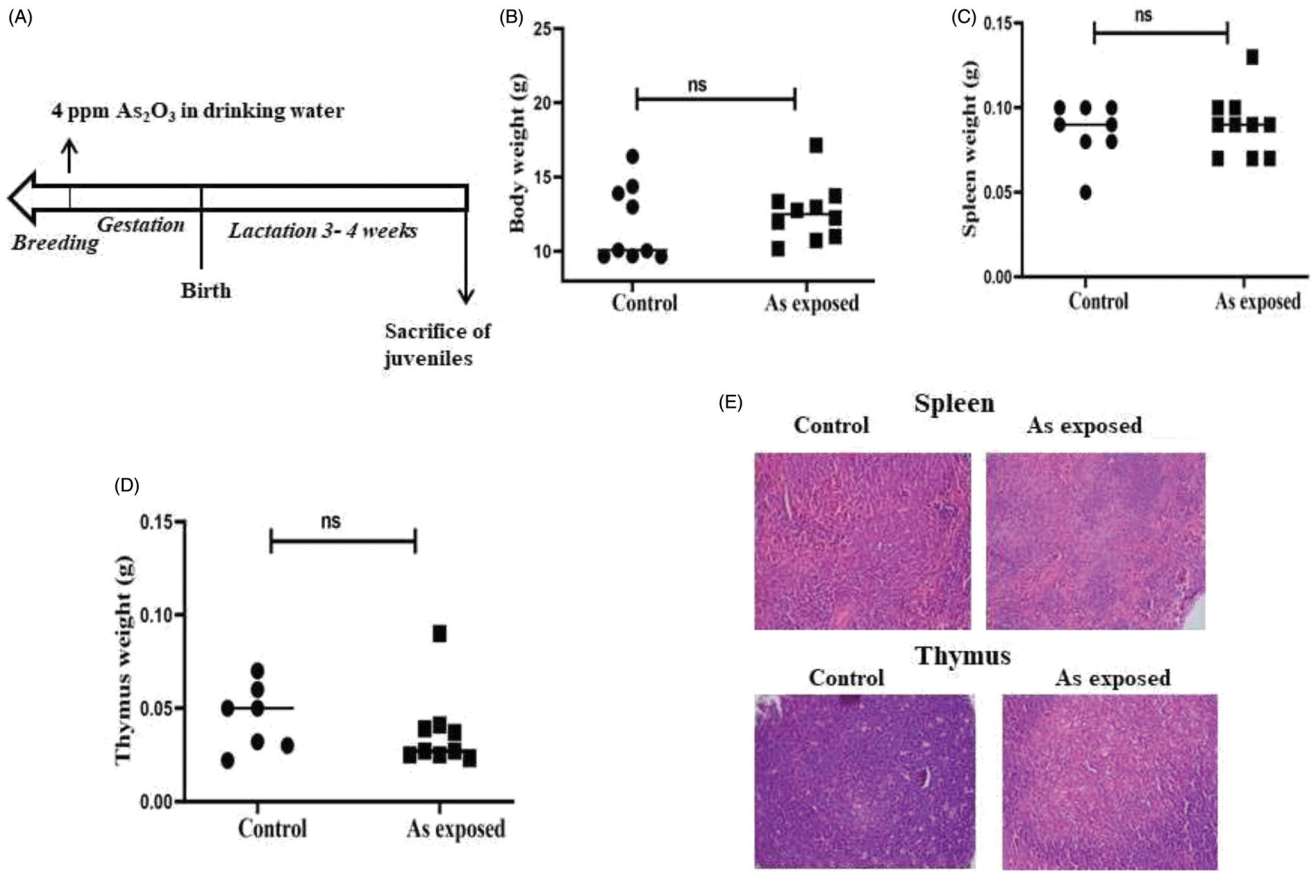


Figure 1. Effect of prenatal As exposure on pup body weight, and splenic/thymic weights and histology. (A) Schematic diagram of experiment. (B) Body weights, (C, D) spleen and thymus weights, and (E) representative micrographs of the spleen and thymus of control and As-exposed offspring (H&E; 20× magnification). All samples were collected from mice at 28 days-of-age ($n \geq 7$/group).

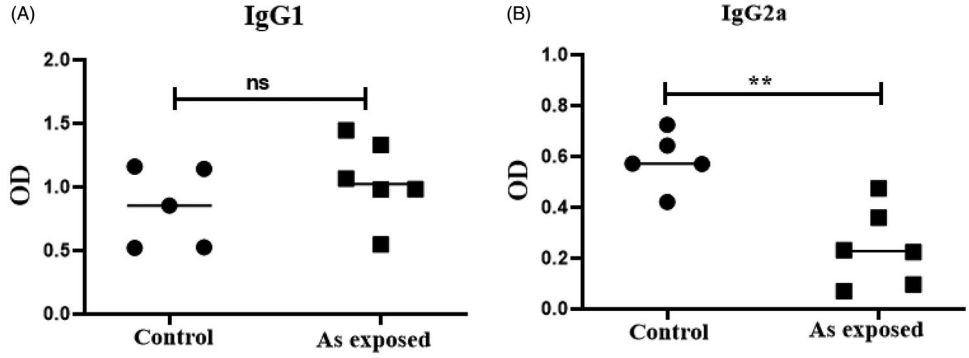


Figure 2. Effect of prenatal As exposure on circulating IgG levels. (A) IgG_1 and (B) IgG_{2a} in sera (dilution 10^{-3} presented) collected at 28 days-of-age. Data shown is representative of five mice/group ($n = 5$/group). Values shown are mean ± SE (OD). Value significantly different from control at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Effects of maternal As exposures on offspring ex vivo mixed-lymphocyte reactions (MLR)

Mitomycin c-treated PEMϕ from As-exposed or unexposed Balb/c offspring were used to stimulate allogenic T-cells (from naive C57BL/6 mice) in a one-way MLR. After 48 h of co-culture, wells were sampled and IL-2 levels subsequently assessed. It was seen that there was no significant effect on IL-2 production from the T-cells regardless of PEM ϕ source (Figure 4).

In the second MLR, splenic $CD4^{+}$ T-cells from As-exposed or unexposed Balb/c offspring were incubated with PEMϕ from allogenic naive C57BL/6 mice for 48 hr and then levels of T-cell-derived IL-2 and IFNγ were evaluated. The results show allogenic stimulation caused significant IL-2 and IFNγ production compared to levels from syngeneic co-cultures. Allogenic stimulation of T-cells from prenatal As-exposed mice resulted in ≈50% less IL-2 production compared to that of T-cells from control pup counterparts (Figure 5(A)). Similarly, there was a 30% reduction in IFNγ production from As-exposed offspring T-cells compared to that of control pup T-cells (Figure 5(B)).

Prenatal As exposure effects on splenic T-cell activation marker expression

In parallel with the MLR, expression of T-cell activation markers CD25, CD44, and CD69 were evaluated in the harvested splenic T-cells. Expression of each marker was reported in the context

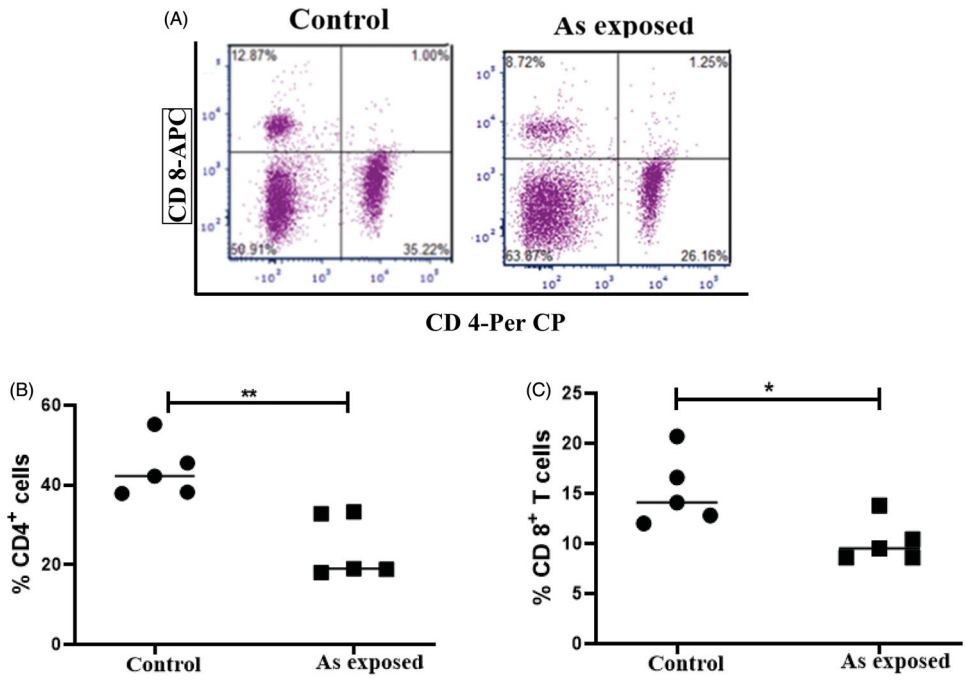


Figure 3. Effect of prenatal As exposure on splenic $CD4^+$ and $CD8^+$ T-cell frequencies. Splenocytes from individual mice were collected at 28 days-of-age, stained, and $CD4^+$ and $CD8^+$ populations evaluated using flow cytometry. (A) Representative dot-plots of $CD4^+$ and $CD8^+$ T-cells in control and prenatally As-exposed offspring. Percentage (B) $CD4^+$ T-cells and (C) $CD8^+$ T-cells ($n = 5$/group). Value significantly different from control at $^*p < 0.05$, $^{**}p < 0.01$.

of both syngeneic and allogenic co-cultures. The data showed that when control T-cells were mixed with allogenic PEMϕ, while there were significant increases in CD44 and CD69 expression on the T-cells, this change was reduced on prenatal As-exposed offspring cells (Figure 6(A,B)). Accordingly, the frequency of double positive $CD44^+CD69^+$ cells was also decreased significantly (~20%) as compared to in normal counterpart offspring T-cells (Figure 6(C)). In contrast, CD25 expression remained comparable between the two groups (Figure 6(D,E)).

Prenatal As exposure effects on host response to infection

To obtain a more "global" evaluation of host immunocompetence, host resistance to a pathogenic challenge was evaluated among pups in both groups. Mice were infected IP with 10^8 viable *E. coli* (strain E14) and then organ parasite load was enumerated 24 h later. Bacterial load was expressed as cfu/g liver (Figure 7(A)), cfu/g spleen (Figure 7(B)), or as cfu/ml peritoneal wash (Figure 7(C)). In general, bacterial loads were significantly higher (~20%) in the prenatal As-exposed pups (all sites) as compared to in the respective site in their normal control counterparts.

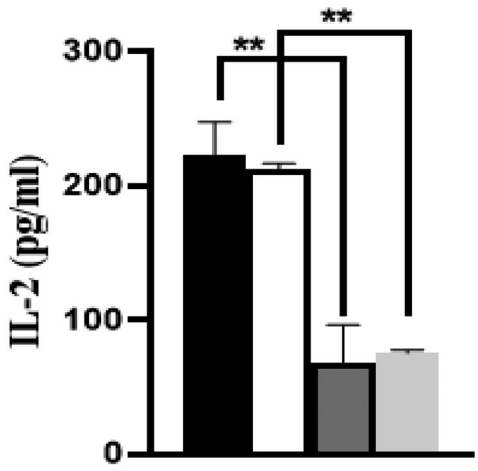
Discussion

There is mounting evidence describing the detrimental effects of arsenic (As) exposure on human health (Abdul et al. 2015). Studies in experimental animals have also yielded evidence of these effects (Santra et al. 2000). In humans, the durations of exposure and levels of As in the drinking water often dictate the breadth of ill effects induced. Many of these effects also seem to impact on children who may have been exposed *in utero* or as infants/neonates/young children. One study in children with varying levels of As exposure in Bangladesh found that total white blood cell (WBC) levels were most significantly reduced among those children who had the highest As exposures (Saha et al. 2013). Similarly, animal studies have shown that early-life As exposure gave rise to subsequent decreases in host resistance to influenza (Ramsey et al. 2013).

The World Health Organization (WHO) has indicated that a "safe" level of As in drinking water is 10 µg/L or 0.01 ppm for adults. In the experiments here, much higher levels of arsenic trioxide (As_2O_3) in drinking water were employed so as to mimic a scenario of highly exposed local populations in Bangladesh. Studies using animal models of As exposure have utilized different approaches to assess immunotoxicities induced by the metalloid. A prevailing approach to assess alterations in the immune repertoire caused by As exposure was to examine changes in disease susceptibility; Farzan et al. (2013) found that As exposures of young rats resulted in increased susceptibility to pathogen-based respiratory diseases. A study of preschool children in rural Bangladesh showed that repeated As exposures (in water) led to altered BCG vaccination efficacy, i.e. BCG-specific $CD4^+$ T-cells that failed to respond to PPD, the antigen that had been seen earlier by the hosts (Ahmed et al. 2014). Based on these above findings, it is reasonable to conclude that As causes immunosuppression in exposed children.

A developing fetus is particularly vulnerable to toxic insult because of the rapid rate of *in utero* development. Similarly, gestation is a critical period for immune development (Dietert and Piepenbrink 2006). Accordingly, the current study attempted to address a specific point not evaluated systematically before, i.e. does prenatal exposure to As (in trivalent form) influence the immune repertoire of offspring at the innate, humoral, and/or cell-mediated immunity levels? Further, the study sought to assess whether immunomodulation developed and/or persisted during post-natal periods wherein there was no further As exposure.

One measure here of potential immunotoxic effects from As exposure during prenatal development were changes in immune system organ (thymus, spleen) weights and gross changes in their



BALB/c	MΦ	Normal	+	-	+	-
		As exposed	-	+	-	+
	CD4 T cells	Normal	-	-	+	+
C57BL/6	CD4 T cells	Normal	+	+	-	-

Figure 4. One-way MLR. PEMϕ from exposed or normal Balb/c mice and allogenic C57BL/6 mice naive T-cells were used (MLR-I). PEMϕ either from As-exposed or normal Balb/c mice collected at 28 days-of-age were pooled, treated with mitomycin C (10 μg/ml) for 6 h, washed, and then combined with allogenic naive C57BL/6 splenic $CD4^+$ T-cells at a ratio of 1:5 PEMϕ:T-cells. Cells were cultured for 48 h at 37 °C and supernatant was then harvested for IL-2 analysis. Values shown are mean (pg/ml)±SD. Value significantly different from control at $^{**}p < 0.01$. All experiments were performed three times using pooled cells from each group ($n = 5$/group).

histological architecture. In the mice that no longer were in contact with As after birth, no significant changes were noted in their immune system organ weights or their histology. Studies have also noted that total numbers of splenic nucleated cells in male C57BL/6J mice were not impacted by As exposures (Xu et al. 2016). Those outcomes do not concur with findings in a study of preschool children from As-exposed areas of Bangladesh whose thymuses were altered by oxidative stress and apoptosis (Ahmed et al. 2012). Of course, that study was examining effects of ongoing As exposures and not one in which the exposure occurred well before the exposed hosts were analyzed (apart from also looking at spleen, not thymus and being in animal models).

Similarly, there were no structural/organizational changes in the splenic white pulp of the *in utero* As-exposed hosts (nor in their levels of nucleated cells), but there was a significant decrease in splenic $CD4^+$T-cells and $CD8^+$ T-cells. Interestingly, the reduction in $CD4^+$T-cell levels was more pronounced than that of $CD8^+$ cells. This change would be more in keeping with the findings of Kile et al. (2014) who noted that for each $\log_{10}$ increase in drinking water As in Bangladesh, $CD4^+$ T-cells in infant subjects were decreased by 9.2% (Kile et al. 2014). There is another report of As-exposure induced downward trends in IL-2 production among splenocytes stimulated with a mitogen *in vitro* (Conde et al. 2007); this finding implies either fewer numbers or functionality of $CD4^+$ T-cells from As-exposed hosts. Still, the question remains as to why decreases in splenic $CD4^+$ and $CD8^+$ T-cells were not reflected in the white pulp architecture or in the absolute numbers of nucleated cells of the *in utero* As-exposed pups. Regarding the latter point, the present study did not quantify levels of other common cell types present in the spleen (i.e. B-cells, NK cells, macrophages, etc.). Whether or not these various immune cell subtypes were altered so as to yield no net negative change in overall splenic nucleated cell levels will be the subject of a follow-on investigation here. Regarding the white pulp, it is possible that gross architecture may not reflect any degree of change until a later time, i.e. if

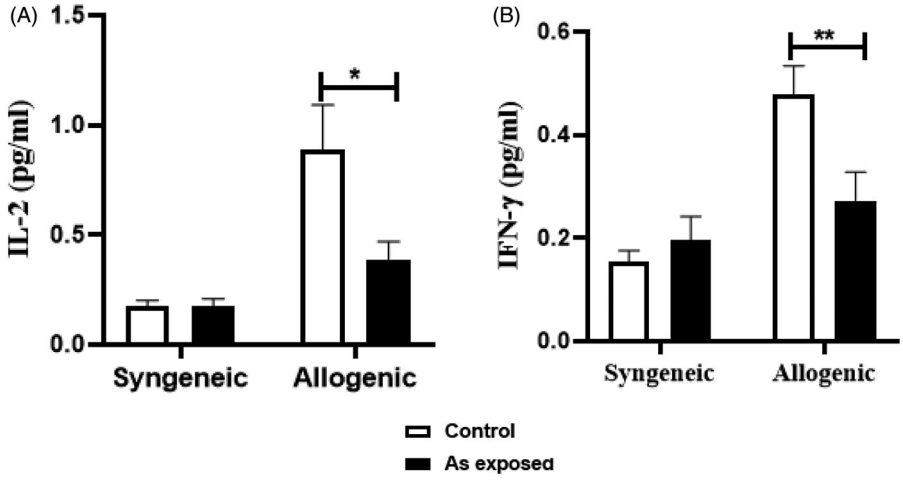


Figure 5. Cytokine production in one-way MLR. $CD4^+$ T-cells from normal and As-exposed Balb/c offspring were used as responder cells and naive C57BL/6 PEMϕ as stimulators (MLR-II). $CD4^+$ T-cells were purified from spleens of the mice at 28 days-of-age and then pooled. PEMϕ were isolated from C57BL/6 mice, treated with mitomycin C (10 μg/ml) for 6 h, washed, and then combined with the Balb/c $CD4^+$ T cells derived at a ratio of 1:5 PEMϕ:T-cells. A control assay was performed in parallel using $CD4^+$ T-cells from each group in combination with syngeneic PEMϕ (Balb/c). After 48 h, culture supernatants were collected to permit analyses of released cytokines. Results are represented for both syngeneic and allogenic cultures. (A) IL-2. (B) IFNγ. Values shown are mean (pg/ml)±SD. Value significantly different from control at $^{*}p < 0.05$, $^{**}p < 0.01$. All experiments were performed three times using pooled cells from each group ($n = 5$/group).

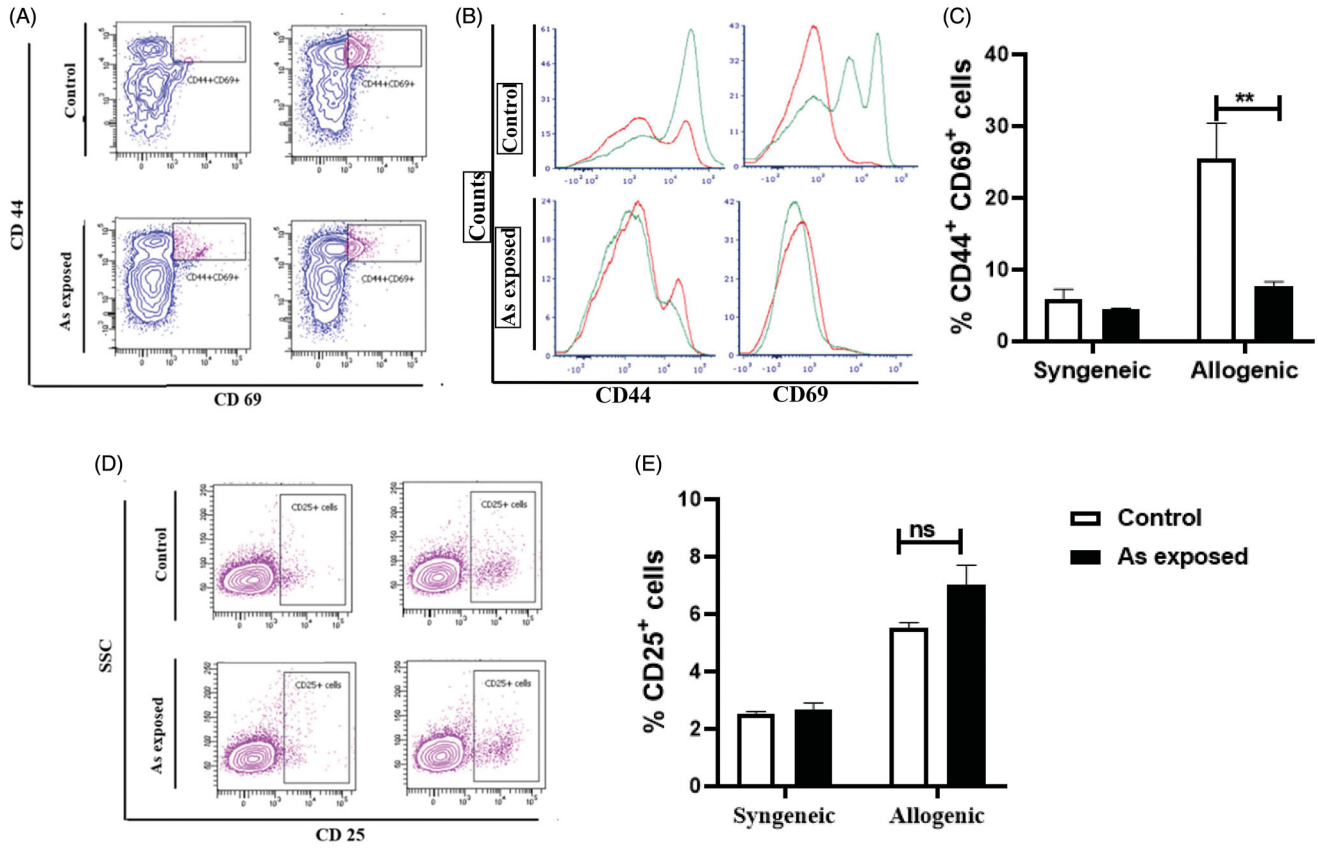


Figure 6. T-cell activation in one-way MLR. $CD4^+$ T-cells from normal or As-exposed offspring were used as responder cells and naive C57BL/6 PEMϕ served as stimulator cells (MLR-II). Protocols followed those outlined in Figures 3 and 4, using cells collected from mice at 28 days-of-age. Here, after 24 h of co-incubation, cells were analyzed for CD44 and CD69 expression; after 72 h, CD25 expression was assessed. (A) $CD44^+$ and $CD69^+$ expression in syngeneic and allogenic reactions (contour plot). (B) Representative histograms of stimulated $CD44^+$ and $CD69^+$ expression. Red = syngeneic reaction, green = allogenic stimulation. (C) Percentage $CD44^+CD69^+$ T-cells in syngeneic and allogenic co-culture. (D) $CD25^+$ expression in syngeneic and allogenic reactions (contour plot). (E) Percentage $CD25^+$ T-cells in syngeneic and allogenic reactions. Values shown are mean ± SE ($n = 3$). Value significantly different from control at $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. All experiments were performed three times using pooled cells from each group ($n = 5$/group).

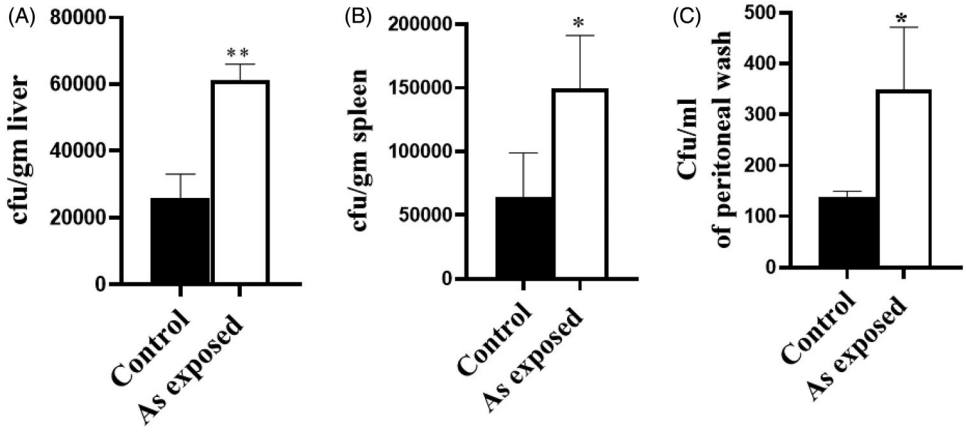


Figure 7. Effect of prenatal As exposure on susceptibility to septicemic *E coli* infection. At 28 days-of-age, mice in each group were infected intraperitoneally with 10^8 septicemic *E. coli* strain E14 and then evaluated at 24 h post-infection. (A) Liver and (B) spleen were each isolated, homogenized, and plated to permit colony-forming unit (cfu) estimations. (C) Peritoneal cavity lavages were also plated. *E. coli* cfu in each organ or cavity lavage are reported as mean ± SE ($n = 3$ per group; cfu/g or cfu/ml). Value significantly different from control at $^*p < 0.05$, $^{**}p < 0.01$.

trends continue and T-cell levels fall off dramatically. Further studies (i.e. longer-term postpartum) are warranted.

Having seen that splenic T-cell frequency was impacted by maternal As exposures, and that decreases in levels of $CD4^+$ cells were more pronounced than those of $CD8^+$ cells in the As-exposed offspring, studies on $CD4^+$ T-cell function were undertaken. Specifically, one-way Mixed Leukocyte Reactions (MLR) were done using PEMϕ from prenatal As-exposed pups as stimulator cells to drive responses among responder allogenic normal T-cells (C57BL/6). It was observed in the MLR reaction that PEMϕ (regardless of the source being As-exposed or normal hosts) were capable of driving allogenic T-cells to produce IL-2. This indicated that prenatal As exposure did not induce any generalized defect in PEMϕ function in the context of stimulating

T-cells. This observation clearly indicated that there was, at least in this one functional regard, no long-term defect induced in the innate function of PEMϕ due to the *in utero* As exposure.

In contrast, when PEMϕ from naive allogenic mice (C57BL/6) were used to stimulate T-cells from As-exposed Balb/c pups (responders), it was seen that these cells failed to produce IL-2 and IFNγ and there were reductions in inducible activation markers CD44 and CD69. Oddly, CD25 expression was not affected by the host *in utero* As exposures. An explanation for this could be that all surface molecules/markers were not equally affected by dam As exposure, i.e. each can differ in how they are assembled in lymphocyte membrane bi-layers. Although we have not yet studied the potential impact of *in utero* As exposure on membrane fluidity, it is known that As-induced "stress" can cause changes in cell membrane fluidity (Ghosh et al. 2018).

Because it was clear that the *in utero* As exposure led to some longer-term effects on T-cells/compartment composition, the present study also looked for any potential impacts on B-cell-related outcomes in the offspring. For this, non-stimulated host circulating levels of IgG_{2a} and IgG_1 were evaluated in the offspring as these isotypes have been accepted as markers for potential T_H1 and T_H2 type responses, respectively (Mountford et al. 1994). Despite no impact on IgG_1 levels, there were decreases in IgG_{2a} levels, suggesting to us that *in utero* As exposure potentially impacted on T_H1 responses well after daily encounters with As had discontinued. This suggestion gained support from the findings of the studies here of allo-stimulation of T-cells from the As-exposed mice, i.e. they failed to produce IFNγ important for T_H1 cell expansion.

Conclusions

Development of T_H1 responses is essential for cell-mediated immunity and, ultimately, host resistance against pathogenic infections. Various animal studies have shown that early-life As exposure decreases host resistance to influenza (Ramsey et al. 2013). In the present study, an defect in T-cell repertoire development due to As exposure manifest as increased bacterial burdens in several organs/sites in As-exposed mice, even when the exposure had only occurred *in utero*. This clearly indicated to us that As induced *in utero* a generalized defect in immune function that then persisted – even in the absence of any further As exposures.

Acknowledgments

The authors are grateful to Prof. Syamal Roy (NIPER, Kolkata) and Dr. Shanta Dutta (ICMR-NICED, Kolkata) for support, helpful discussion, and suggestions for the manuscript. A grateful acknowledgement is also made to Dr. Sulagna Basu for providing the *E. coli* sE14 strain. MC is a recipient of a Council of Scientific and Industrial Research (CSIR) fellowship.

Disclosure statement

The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

Funding

This work was funded by the ICMR-National Institute of Cholera and Enteric Diseases.

ORCID

Mainak Chakraborty http://orcid.org/0000-0002-9814-308X

References

Abdul K, Jayasinghe S, Chandana E, Jayasumana C, de Silva P. 2015. Arsenic and human health effects: A review. Environ Toxicol Pharmacol. 40(3): 828–846.

Ahmed S, Ahsan K, Kippler M, Mily A, Wagatsuma Y, Hoque A, Ngom P, El Arifeen S, Raqib R, Vahter M. 2012. *In utero* arsenic exposure is associated with impaired thymic function in newborns possibly via oxidative stress and apoptosis. Toxicol Sci. 129(2):305–314.

Ahmed S, Moore S, Kippler M, Gardner R, Hawlader M, Wagatsuma Y, Raqib R, Vahter M. 2014. Arsenic exposure and cell-mediated immunity in preschool children in rural Bangladesh. Toxicol. Sci. 141(1):166–175.

Attreed S, Navas-Acien A, Heaney C. 2017. Arsenic and immune response to infection during pregnancy and early life. Curr Environ Health Rep. 4(2): 229–243.

Aung K, Kyi-Tha-Thu C, Sano K, Nakamura K, Tanoue A, Nohara K, Kakeyama M, Tohyama C, Tsukahara S, Maekawa F. 2016. Prenatal exposure to arsenic impairs behavioral flexibility and cortical structure in mice. Front Neurosci. 10:137.

Bailey K, Fry R. 2014. Long-term health consequences of prenatal arsenic exposure: Links to the genome and the epigenome. Rev Environ Health. 29(1–2):9–12.

Concha G, Vogler G, Lezcano D, Nermell B, Vahter M. 1998. Exposure to inorganic arsenic metabolites during early human development. Toxicol Sci. 44(2):185–190.

Conde P, Acosta-Saavedra L, Goytia-Acevedo R, Calderon-Aranda E. 2007. Sodium arsenite-induced inhibition of cell proliferation is related to inhibition of *IL-2* mRNA expression in mouse activated T cells. Arch Toxicol. 81(4):251–259.

Davis M, Higgins J, Li Z, Gilbert-Diamond D, Baker E, Das A, Karagas M. 2015. Preliminary analysis of *in utero* low-level arsenic exposure and fetal growth using biometric measurements extracted from fetal ultrasound reports. Environ. Health. 14:12.

Dietert R, Piepenbrink M. 2006. Perinatal immunotoxicity: Why adult exposure assessment fails to predict risk. Environ Health Perspect. 114(4): 477–483.

Farzan S, Karagas M, Chen Y. 2013. *In utero* and early-life arsenic exposure in relation to long-term health and disease. Toxicol Appl Pharmacol. 272(2):384–390.

Farzan SF, Li Z, Korrick SA, Spiegelman D, Enelow R, Nadeau K, Baker E, Karagas MR. 2016. Infant infections and respiratory symptoms in relation to *in utero* arsenic exposure in a U.S. cohort. Environ Health Perspect. 124(6):840–847.

Ferrario D, Gribaldo L, Hartung T. 2016. Arsenic exposure and immunotoxicity: A review including the possible influence of age and sex. Curr Environ Health Rep. 3(1):1–12.

Galicia G, Leyva R, Tenorio E, Ostrosky-Wegman P, Saavedra R. 2003. Sodium arsenite retards proliferation of PHA-activated T-cells by delaying the production and secretion of IL-2. Intl. Immunopharmacol. 3(5): 671–682.

Gera R, Singh V, Mitra S, Sharma AK, Singh A, Dasgupta A, Singh D, Kumar M, Jagdale P, Patnaik S, et al. 2017. Arsenic exposure impels CD4 commitment in thymus and suppress T-cell cytokine secretion by increasing regulatory T cells . Sci Rep. 7(1):7140

Ghosh D, Bhadury P, Routh J. 2018. Coping with arsenic stress: Adaptations of arsenite-oxidizing bacterial membrane lipids to increasing arsenic levels. Microbiologyopen. 7(5):e00594

He W, Greenwell R, Brooks D, Calderón-Garciduenas L, Beall H, Coffin J. 2007. Arsenic exposure in pregnant mice disrupts placental vasculogenesis and causes spontaneous abortion. Toxicol Sci. 99(1):244–253.

Helmby H, Jonsson G, Troye-Blomberg M. 2000. Cellular changes and apoptosis in the spleens and peripheral blood of mice infected with blood-stage *Plasmodium chabaudi chabaudi* AS. Infect Immun. 68(3):1485–1490.

Kile M, Houseman E, Baccarelli A, Quamruzzaman Q, Rahman M, Mostofa G, Cardenas A, Wright R, Christiani D. 2014. Effect of prenatal arsenic exposure on DNA methylation and leukocyte sub-populations in cord blood. Epigenetics. 9(5):774–782.

Kippler M, Skröder H, Rahman S, Tofail F, Vahter M. 2016. Elevated childhood exposure to arsenic despite reduced drinking water concentrations: A longitudinal cohort study in rural Bangladesh. Environ. Intl. 86: 119–125.

Lause D, Waksal S, Waksal H, Pierre R. 1976. Thymocyte sub-populations as stimulators in the mixed-lymphocyte reaction. Adv. Exp. Med. Biol. 66: 123–127.

Mountford A, Fisher A, Wilson R. 1994. The profile of IgG_1 and IgG_{2a} antibody responses in mice exposed to *Schistosoma mansoni*. Parasite Immunol. 16(10):521–527.

Nadeau KC, Li Z, Farzan S, Koestler D, Robbins D, Fei DL, Malipatlolla M, Maecker H, Enelow R, Korrick S, et al. 2014. *In utero* arsenic exposure and fetal immune repertoire in a U.S. pregnancy cohort. Clin Immunol. 155(2):188–197.

Rahman A, Vahter M, Ekström E, Persson L. 2011. Arsenic exposure in pregnancy increases the risk of lower respiratory tract infection and diarrhea during infancy in Bangladesh. Environ. Health Perspect. 119(5):719–724.

Ramsey K, Foong R, Sly P, Larcombe A, Zosky G. 2013. Early life arsenic exposure and acute and long-term responses to influenza A infection in mice. Environ Health Perspect. 121(10):1187–1193.

Raqib R, Ahmed S, Ahsan K, Kippler M, Akhtar E, Roy A, Lu Y, Arifeen S, Wagatsuma Y, Vahter M. 2017. Humoral immunity in arsenic-exposed children in rural Bangladesh: Total immunoglobulins and vaccine-specific antibodies. Environ Health Perspect. 125(6):067006.

Raqib R, Ahmed S, Sultana R, Wagatsuma Y, Mondal D, Hoque A, Nermell B, Yunus M, Roy S, Persson L, et al. 2009. Effects of *in utero* arsenic exposure on child immunity and morbidity in rural Bangladesh. Toxicol Lett. 185(3):197–202.

Saha A, Chowdhury M, Nazim M, Alam M, Ahmed T, Hossain M, Hore S, Sultana G, Svenner-Holm A, Qadri F. 2013. Vaccine specific immune response to an inactivated oral cholera vaccine and EPI vaccines in a high and low arsenic area in Bangladeshi children. Vaccine. 31(4):647–652.

Santra A, Maiti A, Das S, Lahiri S, Charkaborty SK, Guha Mazumder DN, Guha Mazumder D. 2000. Hepatic damage caused by chronic arsenic toxicity in experimental animals. J. Toxicol. Clin. Toxicol. 38(4):395–405.

Selgrade M. 2007. Immunotoxicity: The risk is real. Toxicol Sci. 100(2): 328–332.

Smith A, Marshall G, Liaw J, Yuan Y, Ferreccio C, Steinmaus C. 2012. Mortality in young adults following *in utero* and childhood exposure to arsenic in drinking water. Environ. Health Perspect. 120(11):1527–1531.

Smith AH, Yunus M, Khan AF, Ercumen A, Yuan Y, Smith MH, Liaw J, Balmes J, von Ehrenstein O, Raqib R, et al. 2013. Chronic respiratory symptoms in children following *in utero* and early-life exposure to arsenic in drinking water in Bangladesh. Intl. J. Epidemiol. 42(4):1077–1086.

Soto-Pena G, Luna A, Acosta-Saavedra L, Conde P, López-Carrillo L, Cebrián M, Bastida M, Calderón-Aranda E, Vega L. 2006. Assessment of lymphocyte subpopulations and cytokine secretion in children exposed to arsenic. Faseb J. 20(6):779–781.

Sui T, Ge D, Yang L, Tang J, Cao X, Ge Y. 2017. Mitomycin c induces apoptosis in human epidural scar fibroblasts after surgical decompression for spinal cord injury. Neural Regen Res. 12(4):644–653.

Vahter M. 2008. Health effects of early life exposure to arsenic . Basic Clin Pharmacol Toxicol. 102(2):204–211.

Vega L, Ostrosky-Wegman P, Fortoul TI, Díaz C, Madrid V, Saavedra R. 1999. Sodium arsenite reduces proliferation of human activated T-cells by inhibition of the secretion of interleukin-2. Immunopharmacol Immunotoxicol. 21(2):203–220.

Xu H, McClain S, Medina S, Lauer F, Douillet C, Liu K, Hudson L, Styblo M, Burchiel S. 2016. Differential sensitivities of bone marrow, spleen and thymus to genotoxicity induced by environmentally relevant concentrations of arsenite. Toxicol Lett. 262:55–61.

Young J, Cai L, States J. 2018. Impact of prenatal arsenic exposure on chronic adult diseases. Syst Biol Reprod Med. 64(6):469–483.

Zhang X, Goncalves R, Mosser D. 2008. The isolation and characterization of murine macrophages. Curr. Protocol. Immunol. Chapter 14:Unit 14:1.

Toxicology Letters 374 (2023) 19–30

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.journals.elsevier.com/toxicology-letters

Prenatal arsenic exposure stymies gut butyrate production and enhances gut permeability in post natal life even in absence of arsenic deftly through miR122-Occludin pathway

Mainak Chakraborty [a], Anupam Gautam [b,c], Oishika Das [a], Aaheli Masid [a], Moumita Bhaumik [a,*]

[a] Division of Immunology, ICMR-National Institute of Cholera and Enteric Diseases, Beleghata, Kolkata 700010, India
[b] Department of Algorithms in Bioinformatics, Institute for Bioinformatics and Medical Informatics, University of Tübingen, Sand 14, 72076 Tübingen, Germany
[c] International Max Planck Research School "From Molecules to Organisms", Max Planck Institute for Biology Tübingen, Max-Planck-Ring~5, 72076 Tübingen, Germany

ARTICLE INFO

Keywords:
Prenatal
Arsenic
Butyrate
Occludin
Tight junction
MiR122

ABSTRACT

This discourse attempts to capture a few important dimensions of gut physiology like microbial homeostasis, short chain fatty acid (SCFA) production, occludin expression, and gut permeability in post-natal life of mice those received arsenic only during pre-natal life. Adult Balb/c mice were fed with 4 ppm arsenic trioxide in drinking water during breeding and gestation. After the birth of the pups, the arsenic water was withdrawn and replaced with clean drinking water. The pups were allowed to grow for 28 days (pAs-mice) and age matched Balb/c mice which were never exposed to arsenic served as control The pAs-mice showed a striking reduction in Firmicutes to Bacteroidetes (F/B) ratio coupled with a decrease in tight junction protein, occludin resulting in an increase in gut permeability, increased infiltration of inflammatory cells in the colon and decrease in common SCFAs in which butyrate reduction was quite prominent in fecal samples as compared to normal control. The above phenotypes of pAs-mice were mostly reversed by supplementing 5% sodium butyrate (w/w) with food from 21st to 28th day. The ability of butyrate in enhancing occludin expression, in particular, was dissected further. As miR122 causes degradation of Occludin mRNA, we transiently overexpressed miR122 by injecting appropriate plasmids and showed reversal of butyrate effects in pAs-mice. Thus, pre-natal arsenic exposure orchestrates variety of effects by decreasing butyrate in pAs-mice leading to increased permeability due to reduced occludin expression. Our research adds a new dimension to our understanding that pre-natal arsenic exposure imprints in post-natal life while there was no further arsenic exposure.

1. Introduction

Arsenic is a highly potent environmental toxin affecting more than 200 million people globally where south east Asian countries including India, Bangladesh, Pakistan, Nepal, and China are largely affected (Mukherjee et al., 2006). As arsenic is readily absorbed *in utero* the intake of arsenic by children (per unit body mass) is higher than that of adults increasing the risk of infant mortality, health risks and impaired intellectual development with associated impacts later in life (Donohoe et al., 2012; Cherry et al., 2010; Myers et al., 2010). Earlier we have shown that prenatal arsenic exposure causes compromised immune response in juvenile mice which leads to susceptibility to pathogen infection (Chakraborty and Bhaumik, 2020). Recent reports showed that environmentally relevant level of arsenic exposure in mice causes gut microbial dysbiosis (Chi et al., 2017). Although the children in arsenic affected areas showed altered gut microbial composition (Dong et al., 2017), however there is no report on *in utero* arsenic exposure and effect on gut microbiome.

Maternal–offspring microbiota exchanges play a significant role in the development and maturation of the neonatal microbiome (Dong et al., 2015). Epidemiological studies also indicate antibiotic usage during pregnancy has also been linked to an increased incidence of juvenile obesity (Mor et al., 2015) and asthma (Stensballe et al., 2013). It has also been reported that the children born to mothers suffering from ulcerative colitis whose disease was active during pregnancy have a higher risk of developing childhood illnesses (Hashash and Kane, 2015).

* Correspondence to: Division of Immunology, ICMR-National Institute of Cholera and Enteric Diseases, P-33 C.I. T Road, Beleghata, Kolkata 700010, West Bengal, India.
E-mail address: drmoumitabhaumik@gmail.com (M. Bhaumik).

https://doi.org/10.1016/j.toxlet.2022.11.011
Received 25 May 2022; Received in revised form 15 November 2022; Accepted 21 November 2022
Available online 5 December 2022

Recent reports showed that arsenic exposure in early life alters the gut microbiome during the critical window of infant development (Hoen et al., 2018; Dong et al., 2017). These observations festoon an impending issue which nudge us to investigate the impact of prenatal arsenic exposure on the gut physiology in post-natal life. Germane with the idea that mother's microbiome plays a role in an infant's gut microbial establishment; it is plausible that subtle alterations in microbiota in early life may act as a vulnerability factor, impacting on disturbances in gut physiological functions which may lead to disorders in adult hood.

Short chain fatty acids (SCFA), an important metabolite produced by the gut microbes after fermentation of dietary fibres are significantly decreased in arsenic exposure (Chi et al., 2017). Butyrate, one of the important SCFA controls pleotropic functions in the body including the development of the immune system (Yip et al., 2021; Schulthess et al., 2019). Butyrate can down-regulate inflammation by inhibiting the growth of pathobionts (Chen and Vitetta, 2020), increasing mucosal barrier integrity (Okumura et al., 2021), encouraging obligate anaerobic bacteria dominance (Chen and Vitetta, 2020) and decreasing oxygen availability in the gut (Kelly et al.,2015). In an in vitro model of the intestinal epithelial barrier employing Caco-2 cells, SCFA, particularly butyrate, has been demonstrated to enhance intestinal barrier function as measured by an increase in transepithelial electrical resistance (TEER) and a decrease in inulin permeability (Peng et al., 2007; Peng et al., 2009). Inflammatory bowel disease (IBD), obesity, non-alcoholic steatohepatitis (NASH), and non- alcoholic fatty liver disease (NAFLD) are all linked to a defective intestinal tight junction (TJ) barrier, which can be corrected by butyrate (Silva et al., 2018; Coppola et al., 2021; Endo et al., 2013). Reports showed that butyrate enhances the intestinal barrier by regulating the assembly of TJ proteins like occludin (Peng et al., 2009). Occludin, having four transmembrane domains is highly expressed at cell-cell contact sites and is important in the assembly and maintenance of TJ (Al-Sadi et al., 2011). Importantly, occludin knockout mice showed exhibited elevated inflammation, hyperplasia, and growth retardation (Saitou et al., 2000). Dysregulation of TJ has been implicated in various gut associated diseases including, IBD and colon cancer (Casalino et al., 2016). TJ protein expression at the cellular level is being governed by microRNAs, small regulatory RNAs (Ye et al., 2011). MiR122 is one of the microRNA that promotes occludin mRNA decay by binding to its 3'UTR (Jingushi et al., 2017; Yang et al., 2022). MiR122 is abundantly present in liver but also reported to be found in gut where it targets NOD2 and plays important role in colon cancer (Li et al., 2019).

The study is designed to investigate the effect of prenatal arsenic exposure on gut functional phenotype in the post natal life. We studied the microbial composition, metabolite production, and TJ protein expression and gut permeability in prenatally arsenic exposed mice and compared with normal control. Our results showed a decrease in Firmicutes to Bacteriodes ratio in gut microbiota coupled with a decrease in SCFA production of prenatally arsenic exposed mice. The lack of butyrate increased miR122 expression in gut, causing decrease in occludin and resulted in increased gut permeability. By replenishing butyrate, the prominently decreased SCFA due to pre-natal arsenic exposed (pAs-mice) we showed that butyrate renders a crucial base in the maintenance of gut physiology. Further leverage on the role of miRNA122 was stemmed from over-expressing miR122 to butyrate treated prenatally arsenic exposed mice which led to decrease in occludin. By harmonising narratives from our experimental studies, we showed that in prenatal arsenic exposure, imbalance of the gut microbiota resulted in decrease in gut butyrate, a "critical" denominator for maintaining general gut homeostasis. We showed a lasting imprint of prenatal arsenic exposure on post-natal gut physiology in adult life even in absence of butyrate.

2. Materials and methods

2.1. Reagents, chemicals, and buffers

Chow diet (Harlan Teklad LM-485), was purchased from ICMR-NIN, Hyderabad, India. Dulbecco's modified Eagle's medium (DMEM) and foetal calf serum (FCS) were purchased from GIBCO (Waltham, MA, USA). BCA protein assay kit was purchased from Thermofisher (Waltham, MA, USA). Penicillin, streptomycin, Triton X100, PMSF, leupeptin, glycine, acrylamide, bis-acrylamide, para-formaldehyde, sodium butyrate, sodium propionate, sodium acetate, Hoechst 33342, and FITC-Dextran 4000 were purchased from Sigma (St. Louis, MO, USA). PVDF membrane, Trizol, were purchased from Invitrogen (Carlsbad, CA, USA). Super Reverse Transcriptase MuLV Kit, RT^2 SYBR® Green qPCR Mastermix were purchased from Qiagen (Hilden, Germany). Ripa lysis buffer, Occludin (rabbit, polyclonal) were purchased from Abcam (Cambridge, UK). Anti-GAPDH antibody (rabbit polyclonal) was purchased from Bio-Bharati (Kolkata, India). All primers were purchased from ID (Lowa, USA). Human colon carcinoma cell line HT-29 cells were a kind gift from Dr. Amit Pal (ICMR- NICED, India). miR122 expression plasmid was a kind gift from Dr. Suvendranath Bhattacharya (CSIR-IICB).

2.2. Mice and animal ethics

Balb/c mice (6 weeks old) were procured from ICMR-NICED animal facility of the institute. All the protocol for the study was approved by the Institutional Animal Ethics Committee of ICMR-NICED, Kolkata, India, (PRO/151/July 2018–June2021). Experiments have been carried out in accordance with the guidelines laid down by the committee for the purpose of control and supervision of experiments on animals (CPCSEA), Ministry of Environment and Forests, Government of India, New Delhi, India.

2.3. Arsenic treatment to dams

All Balb/c mice were housed in cages containing straw bedding held in pathogen-free facilities maintained at 24 °C with a 50% relative humidity and 12-h light:dark cycle. All mice had ad libitum access to standard rodent chow. After 2 weeks of acclimatization, the Balb/c mice were bred by housing two females with a male and given ad libitum access to drinking water containing with/ without 4 ppm Arsenic trioxide (As_2O_3) as described (Chakraborty and Bhaumik, 2020). The water was changed twice weekly. Each dam gave birth to average 4 litters. After birth, the mothers were then given ad libitum access to As-free water. For the experiments, when pups reached 4week-of-age, groups were randomly collected, and processed for biomaterials. Age-matched juvenile mice whose mothers were never exposed to Arsenic were processed in parallel as controls. For each experiment, 5–6 juvenile mice of same age were randomly chosen (without any sex bias) from 2 or more dams for evaluation. The experiments were set in duplicate at a time to increase the chances of pregnancy in mice and to get the pAs- mice of same age.

2.4. Dietary supplementation of sodium butyrate

The dietary supplementation studies were performed as reported earlier with minor modifications (Lin et al., 2012). Briefly, a group of 5 prenatally arsenic exposed mice pups (3 weeks old) were fed with 5% sodium butyrate (w/w) (Sodium butyrate in solid form were thoroughly blended into chow diet) for next 7 days (pAs-butyrate-mice) (Xu et al., 2018).

2.5. MiR122 overexpression in mice

MiR-122 was overexpressed in mouse gut in an identical procedure as described earlier (Ghosh et al., 2013). The miR-122 expression plasmid or empty plasmid (mock) was injected through the tail vein of pAs-butyrate-mice at a dose of 25 μg plasmid DNA dissolved in 100 μl saline. Following sacrifice of the mice after 4 days, the expression of occludin and miR122 in the colon was determined by qPCR.

2.6. Determination of in vivo gut permeability

The gut permeability assay was performed following previously reported protocol (Rangan et al., 2019). Briefly, mice were starved 16 h prior to FITC Dextran 4000 (FD-4) administration. FD-4 (44 mg/100 g body weight) was administered by oral gavage with a needle attached to a 1 ml syringe. A gap of 30 min between each mouse was kept for the FD-4 oral gavage. After 4 h, the blood was collected from tail vein. The blood was immediately transferred to a Microtainer SST tube and was mixed by inverting the tube 3–4 times and was stored at 4 °C in the dark. Once blood was collected from all the mice, SST tubes are processed to separate the serum following the manufacturer's instruction. The serum was then diluted with an equal amount of PBS. The concentration of FD-4 in sera was determined by spectrophotofluorometry with an excitation of 485 nm (20 nm band width) and an emission wavelength of 528 nm (20 nm band width) in FluoroskanTM Microplate Fluorometer (Thermo Fisher Scientific).

2.7. Mice fecal samples collection

Fresh fecal samples of all mice pups were collected at 15:00–17:00 p. m. to minimize possible circadian effects. Samples were collected into empty sterile microtubes on ice and stored at − 80 °C within 1 h for future use.

2.8. 16 S rRNA gene sequencing and analysis

For extraction of fecal DNA, fecal pellets were incubated for 24 hr at 56 °C with proteinase K. DNA was then isolated using QIAamp DNA Mini Kits (Qiagen) using ~25 mg of feces.

2.9. Evaluation of changes in the gut microbiome

Faecal pellets were collected from juvenile mice (irrespective of sex) from both the control and prenatally Arsenic exposed group. Bacterial genomic DNA was extracted using QIAamp Fast DNA Stool Mini Kit (Qiagen, Valencia, CA, United States) by following the manufacturer's protocol. DNA concentration was evaluated by NanoDrop spectrophotometer from Thermo Fisher Scientific (Waltham, MA). Illumina standardized V3-V4 regions of 16 s rRNA library protocol were employed for the preparation of the library. The library which was generated contained V3-V4 amplicons were then sequenced on an Illumina MiSeq platform following the manufacturer's protocol. The generated reads (data) were processed by using QIIME2 (Bolyen et al., 2019) (version 2021.8.0). Filtering, merging and denoising was carried out by using DADA2 (Callahan et al., 2016) plugin within QIIME2. RESCRIPt (Robeson et al., 2021) plugin was used for processing and filtering of SILVA 138 (Quast et al., 2013) database to make it compatible with QIIME2 for carrying out taxonomy assignment. V3-V4 region primers were used for trimming SILVA sequences. and classify-sklearn (Pedregosa et al., 2011) was used for taxonomical classification. Further, the biome and taxonomy table was exported and diversity analyses were carried out by using phyloseq (McMurdie and Holmes, 2013) in R Data. Alpha diversity and rarefaction curve were studied on samples (biom table) rarefy to a depth of 10,952 reads per sample. Samples generated during this experiment were submitted to Sequence Read Archives (SRA) of National Centre for Biotechnology Information (NCBI) under accession numbers (Control1 =SRR19309430, Control 2 = SRR19309429, pAs1 = SRR19309428 and pAs2 =SRR19309427). The raw data is available in bioproject accession PRJNA839617.

2.10. Estimation of faecal SCFA by GC/MS

The faecal concentrations of butyrate were measured by GC/MS as previously described (Li et al., 2020). Briefly, 50 mg of faecal samples from both groups were homogenized in 200 µl of distilled water. The samples were then centrifuges at 4000 rpm for 5 min and the resulting supernatant was collected. 200 µl of a benzyl alcohol–pyridine mixture (3:2) and 100 µl DMSO was added to the supernatant and the mixture was vortexed for 5 s 100 µl of derivatizing agent benzyl chloroformate was added very carefully and the tube lids were kept open for 1 min to release the gas formed during the reaction. The tube lids were then closed and the resulting mixture was vortexed for 3 min. After the derivatization, 200 µl of cyclohexane was added and the resulting mixture was vortexed for 1 min which was then followed by centrifugation at 4000 rpm for 5 min. 100 µl of the resulting derivative extract (upper cyclohexane layer) was isolated and 1 µl was injected into GC-MS instrument for further analysis. The samples were analysed using Shimadzu GCMS-QP2020 (Shimadzu Corporation, Kyoto, Japan) with an AOC-20i auto injector. An InertCap WAX capillary column (30 m × 0.32 mm × 0.25 µm; GL Sciences Inc., Tokyo, Japan) was used for separation. Helium was used as a carrier gas with a flow rate of 1 ml/min. The temperature of the front inlet was set at 250 ºC while the temperatures of the transfer line and the ion source were set to 280 ºC and 230 ºC respectively. The initial column temperature was held at 70 °C for 3 min and then was increased to 200 °C at a rate of 10 °C /min and was finally increased to 290 °C at a rate of 35 °C /min and then was held at this temperature for 7 min. A single run took 25.5 min. The solvent delay time was set to 6.7 min. The electron energy was − 70 eV and the gain factor was set to 2.0.

2.11. Immunofluorescence Staining and imaging

Colon samples were collected from all the groups during necroscopy examination and were fixed in 4% paraformaldehyde. The sections were then embedded in paraffin and 5 µm sections were generated. The paraffin embedded sections were then de-paraffinized in xylene and were rehydrated by passing through a graded series of ethanol followed by rinsing with distilled water. Antigen retrieval was performed by immersing the slides in 1 mM EDTA buffer pH 8.0 for 5 min.at sub boiling temperatures. The slides were then washed with distilled water. The sections were then permeabilized by 0.1% sodium citrate and 0.5% Triton-X in Tris Buffered Saline with 0.1% Tween 20 detergent (TBST) for 15 min. The sections were then blocked with 5% fetal bovine sera in TBST for 1 h at room temperature. The primary antibody to Occludin was then diluted (1:200) in the blocking solution and added to the sections which were then incubated overnight in a humidified chamber at 4ºC. Following incubation, the sections were washed Tris Buffered Saline (TBS) and TBST alternatively for 5 min. The sections were then incubated with goat anti-mouse or goat-anti rabbit secondary antibody conjugated with Alexa Fluor 488 for 2 h at room temperature while being protected from light. Following incubation, the slides were again washed with TBS and TBST alternatively for 5 min. The sections were then mounted with 1 µg/ml Hoechst 33342 stain which acted as a nuclear counterstain. Fluorescence images were then captured using Carl Zeiss microscope equipped with a CCD camera and controlled by Zen software (Carl Zeiss, Gottingen, Germany) (Gumber et al., 2014).

2.12. Histopathologic examination of proximal colon

The proximal colon samples were fixed in 4% paraformaldehyde for 48 h at 4°C. The fixed tissues were then dehydrated through graded alcohols, embedded in paraffin, and routine microtomy then carried out to generate 5 µm sections. The sections, in turn, were stained with hematoxylin and eosin for later microscopic examination. (Chakraborty and Bhaumik, 2020).

2.13. Propagation of HT-29 cells and SCFA treatment

HT-29 cells were cultured in DMEM supplemented with 10% FCS and 50 µg/ml penicillin and streptomycin at 37°C with 5% CO_2. Cellular viability was assayed by MTT. Cells (10^6 cells/ml) were treated with

either sodium butyrate (butyrate) or sodium propionate (propionate) or sodium acetate (acetate) at concentrations indicated in the figures for 24 h. Thereafter the cells were washed and processed for further analysis.

2.14. Tissue homogenisation and RNA and protein isolation

Colon samples (dissected into small pieces) or the cells were resuspended either in RIPA Lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% Sodium deoxycholate, 2.5 mM Sodium Pyrophosphate, 1 mM β-glycophosphate, 1 mM Na_3VO_4 1 μg/m^l leupeptin with 1 mM of phenylmethylsulfonyl fluoride (PMSF) immediately before use) for protein isolation or in Trizol (Invitrogen, US) for RNA isolation. The tissue was homogenized using a micropestle and centrifuged at 13,000 g for 15 min at 4 °C. The clear supernatant was collected and either stored as protein lysate in − 80 °C or further processed to isolate RNA using the standard protocol (Wu et al. 2018). The concentration of the extracted RNA was analyzed by Nanodrop and RNA was stored at −80 °C.

2.15. RNA extraction and reverse transcription

cDNA was prepared from total RNA by reverse specific primers using Super Reverse Transcriptase MuLV Kit. The primers for the reverse transcription are listed in Table 1. U6 and GAPDH were normalized for the expressions of miR122 and other genes of interest. The total reaction volume for reverse transcription was 20 μl in which 1 μM of reverse primer, 5 ng of RNA template, 1 μl dNTP mix, 12 μl of Diethyl pyrocarbonate (DEPC) treated water, 4 μl of 5X first strand buffer, 1 μl of 0.1 M Dithiothreitol (DTT), 1 μl of RNase inhibitor and 1 μl Super RT MuLV. Reverse transcription was carried out at 65 °C for 5 min, followed by incubation at 55 °C for 1 h and then heat inactivating the reaction at 70 °C for 15 min (Abdelmohsen et al., 2012).

2.16. Quantitative real-time PCR

Total RNA was extracted with Trizol reagent from snap frozen colon and RNA concentration was determined using a nanodrop. The miR-122, occludin, claudin1, claudin 2, claudin 4, ZO-1, GAPDH, and U6 levels were quantified with Applied Biosystems™ StepOne™ Real Time PCR System with RT^2 SYBR® Green qPCR Mastermix following the manufacturer's instructions. Each 20 μl qPCR reaction contained an amount of cDNA equivalent to 5 ng of total RNA, 10 μl of RT^2 SYBR® Green qPCR Mastermix, 1 μM of the forward and reverse primer (each) and nuclease free water. Real-time PCR was performed with the following conditions: 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min PCR product was calculated according to the $2^{-\Delta\Delta Ct}$ method described previously (Abdelmohsen et al., 2012).

2.17. Microbial analysis via PCR

To compare the relative abundances of various bacterial taxa in fecal DNA from female mice, we used qPCR analysis quantified by SYBR green and normalized the data to total bacterial abundance using pan-bacterial primers. The data is represented as $2^{-\Delta\Delta ct}$ (Guo et al., 2008). The primer sequences use for PCR amplification are as follows:

2.18. Western blot

Colon tissue protein and cell lysate were extracted in RIPA Lysis buffer. Protein concentration was measured using Pierce ™ BCA Protein Assay Kit. Proteins (50 μg/lane) were separated by using SDS-PAGE on 10% gel under reducing condition and electro transferred to polyvinylidene difluoride (PVDF) membrane in atransferred buffer (25 mM Tris-HCl, 150 mM Glycine, 20% Methanol). Membranes were blocked at room temperature with 5% non fat skim milk in TBS for 2 h, and then incubated with primary antibody against specific protein. The membranes were incubated with the horseradish peroxidase (HRP)-conjugated secondary antibodies at 37 °C for 1 h. SuperSignal West Pico chemiluminescent substrate kit (Thermo) was used to visualize the blotting results. The blots were imaged with Fluor Chem R system (ProteinSimple, San Jose, CA, USA) (Abdelmohsen et al., 2012). Antibody used for Western Blots:

Name of Antigen	Raised in	Source	Dilutions used
Anti-mouse-HRP	Horse	Cell Signalling Technology	1:5000

(continued on next page)

Table 1
Primers used for Real time PCR.

Gene	Primer Sequence	
	Forward Primer	Reverse Primer
GAPDH (Human)	5`-GAGAAGGCTGGGGCTCATTT3`	5`AGTGATGGCATGGACTGTGG3`
U6SnRNA (Human)	5`-CTCGCTTCGGCAGCACATATACT3`	5`ACGCTTCACGAATTTGCGTGTC 3`
miRNA122 (Human)	5`TAGCAGAGCTGTGGAGTGTG3`	5`GCCTAGCAGTAGCTATTTAGTG TG3`
U6snRNA(Mouse)	5-CTCGCTTCGGCAGCACATATACT-3`	5`ACGCTTCACGAATTTGCGTGTC-3`
GAPDH (Mouse)	5`-AGAGAGGCCCAGCTACTCG-3`	5`GGCACTGCACAAGAAGATGC-3`
miRNA122(Mouse)	5`-GCTCGACCTCTCATGGGC-3`	5`TTAAGCCCTGCGTGTCCTCC-3`
Occludin (human)	5'- AAGAGTTGACAGTCCCATGGCATA C -3'	5'ATCCACAGGCGAAGTTAATGG AAG − 3'
Occludin (mice)	5'-TCACTTTTCCTGCGGTGACT-3'	5'- GGGAACGTGGCCGATATAATG-3'
Claudin1 (mouse)	5'- CTGGAAGATGATGAGGTGCAGAAG A-3'	5'- CCACTAATGTCGCCAGACCTGA A-3'
Claudin 2 (mouse)	5'-GGCTGTTAGGCACATCCAT − 3'	5'-TGGCACCAACATAGGAACTC − 3'
JAM-A(mouse)	5'-ACCCTCCCTCCTTTCCTTAC − 3'	5'-CTAGGACTCTTGCCCAATCC − 3'
ZO-1 (mouse)	5'- AGCTCATAGTTCAACACAGCCTCCA G-3'	5'- TTCTTCCACAGCTGAAGGACTCA CAG-3'
16 SrRNA panprimer	5'- AGAGTTTGATCCTGGCTCAG-3'	5'- AAGGAGGTGWTCCARCC-3'
Firmicutes	5'-GGAGAATGTGGTTTAATTCGAAGC A-3'	5'- AGCTGACGACAACCATGCAC-3'
Bacteroidetes	5'- GTTTAATTCGATGATACGCGAG-3'	5'- TTAACCCGACACCTCACGG-3'

(*continued*)

Name of Antigen	Raised in	Source	Dilutions used
conjugatedsecondary antibody			
Anti-rabbit-HRP conjugatedsecondary antibody	Goat	Cell Signalling Technology	1:5000
Occludin	Rabbit	Abcam	1:1000
GAPDH	Rabbit	Biobharati	1:1000

2.19. Statistical analysis

All values in the figures and text are expressed as arithmetic mean ± SE. Data were analyzed with GraphPad Prism Version 8.01 software and statistical significance between groups was determined using unpaired student's t-test. Significance of more than two groups was determined by one way analysis of variance (ANOVA) followed by Tukey's post hoc test. The p values of < 0.05 were considered statistically significant. In the experiment involving Western blot, the figures shown are representative of at least 3 experiments performed on different days.

3. Results

3.1. Arsenic trioxide (arsenic) in drinking water decreases Firmicutes and increases Bacteroidetes in female mice

We investigated whether arsenic would perturb the abundance of *Firmicutes* (F) and *Bacteroidetes* (B) present in the gut microbiome of the dams. We observed 2.5 fold decrease ($p = 0.04$) in *Firmicutes* and 7 fold increase ($p = 0.0008$) in *Bacteroidetes* in As-exposed dams (Fig. S1 A and B) resulting in 18 fold decrease ($p = 0.005$) in F/B ratio compared to control (Fig. S1C). Next, we asked whether arsenic exposure during breeding and gestation would perturb the compositional profile of the gut microbiome of the offspring.

3.2. Prenatal arsenic exposure perturbs normal community composition of gut microbes

The pups from the As-exposed-dams were not exposed to arsenic any further after their birth and referred to as pAs-mice henceforth for convenience. The pups from the control-dams were referred as control-mice. The feaces were collected from the pups on day 21 post birth as represented pictorially (Fig. 1 A). The total number of taxonomically classified ASVs (Amplicon Sequence Variants) identified upon analysis of sequenced 16S-rRNA dataset from the feaces in both the groups is represented in a Venn diagram (Fig. 1 B). The identified genera are enlisted in Table S1. Around 13.2% ASV was found to be common in both the groups. But control-mice and pAs-mice showed the presence of 49.7% and 37% unique ASV respectively. Overall this observation indicates pAs-mice have lost about 12.7% of ASV compared to control-mice (Fig. 1 B). The predominant bacterial phyla in all the samples were *Firmicutes* and *Bacteroidetes* as assessed by 16S-rRNA sequencing (Fig. 1 C). Alpha diversity of the samples was measured by chao1 index. Following prenatal arsenic exposure, the microbial component of the gut microbiome was lowered significantly compared to control, along with a Species Rarefaction Curve (Fig. 1 D) showing reduction in the number of bacterial species in the pAs mice (line in red) with respect to control-mice (line in blue). There was also a decrease in diversity (chao1-values) in pAs exposed mice when compared with control (Fig. 1 E). At the phylum level, the prevalenceof *Firmicutes* and*Bacteroidetes* as determinedbymetagenomicanalysiswere significantly decreased and increased respectively in the pAs-mice compared to control-mice (Fig. 1 F). Considering the decrease in *Firmicutes* (F) and increase in *Bacteroidetes* (B) in pAs-mice, the F/B ratio is also decreased ($p = 0.0254$) in pAs-mice compared to control (Fig. 1 G). The abundance of these phyla were further validated by qPCR using phyla specific primers which also showed decrease and increase in *Firmicutes* and *Bacteroidetes* respectively in pAs-mice compared to control-mice (Fig. 1 H). We report 6.67 fold decreases ($p = 0.0013$) in F/B ratio in pAs-mice compared to control (Fig. 1 I).

3.3. pAs-mice showed reduced SCFA production compared to control-mice

The decrease in the abundance in *Firmicutes* in pAs mice faithfully reflected decrease in fecal SCFA compared to control as estimated by GC-MS., We observed 4 fold ($p = 0.0079$), 2 fold ($p = 0.0049$) and1.7 fold ($p = 0.025$) decrease in butyrate, acetate and propionate respectively in the feaces of pAs-mice compared to control-mice (Fig. 2). As SCFAs perform multiple functions including intestinal barrier integrity, mucous production and protect against inflammation in the gut it was important to study the physiological function of the gut in pAs-mice.

3.4. pAs-exposed mice showed increase in gut permeability and decrease in tight junction (TJ)

3.4.1. Protein, Occludin

To assess the potential shifts in the gut physiological function, if any, the gut barrier function, junctional protein expression and histology of gut in pAs-mice and control-mice were studied. The gut barrier function as studied by FITC dextran permeability showed 3.9 fold increase ($p = 0.0079$) in gut permeability in pAs-mice compared to the control-mice (Fig. 3 A). Further evaluation of tight junctional protein expression in the colon by qPCR showed 4 fold decrease ($p = 0.007$) in Occludin in pAs-mice compared to control-mice whereas, claudin 1, claudin-2, claudin-4, ZO-1, and JAM-A TJ protein expression remained unaltered (Fig. 3 B). Histological examination of the gut showed inflammatory cell infiltration in pAs-mice compared to the control-mice (Fig. 3C). The mucous secreting goblet cells also showed hyperplasia in pAs-exposed animals' gut histological sections. Recalling the gut microbial analysis and production of SCFA which depicted reduced SCFA production in pAs-mice, we sought to study if the lack of SCFA production in gut has any role on occludin expression in pAs-mice. Before that, it was essential to investigate the mechanism by which SCFA may influence the expression of occludin.

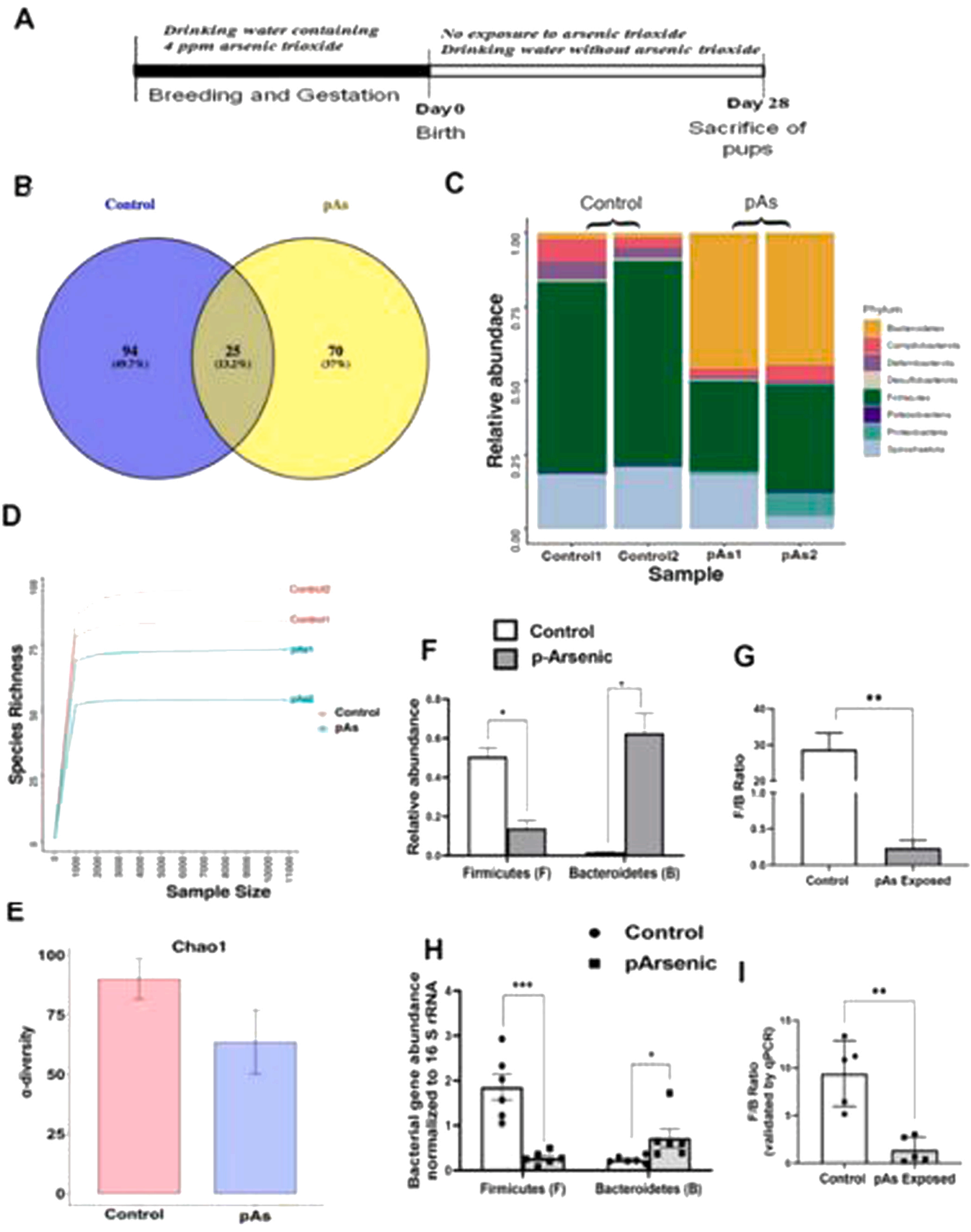
3.5. Butyrate treatment increases Occludin expression and decreases permeability in HT-29

3.5.1. Cells

HT-29 cells were treated with either butyrate or propionate or acetate with the function of concentration (0–20 mM) for 24 h. It was observed that unlike propionate and acetate, 10 mM and 20 mM butyrate treatment caused 45% and 58% increase in occludin expression respectively (Fig. S2A). These results provided initial evidence that butyrate but not propionate or acetate could increase occludin expression. For further deciphering the mechanism of regulation of occludin expression we studied miR122 expression upon butyrate treatment as it was shown previously that miR122 binds to the 3'UTR of the mRNA of Occludin, causing its degradation (Jingushi et al., 2017).

3.6. Butyrate treatment reduces miR122 expression in HT-29 cells

We monitored the status of miR122 in butyrate treated and untreated HT-29 cells by qPCR. It was observed that there was a progressive decrease in miR122 expression as a function of butyrate concentration (Fig. S2B). Notably, the expression of miR122 does not change on propionate and acetate treatment. Our result points towards the cascade of sine-qua-non events accomplished by butyrate action which is as follows: butyrate decreases miR122 which in turn increases occludin,



(caption on next page)

Fig. 1. Effect of prenatal Arsenic exposure on gut microbiome composition and diversity. Adult Balb/c mice were bred by housing two females with a male and given ad libitum access to drinking water containing 4 ppm arsenic trioxide (As). After birth of the pups, the mothers were then given ad libitum access to As-free water. When the pups reached 4weeks-of-age, their feaces were collected at random. Feaces from the age-matched pups of the dams that were never exposed to Arsenic were processed in parallel as control. The experimental plan is schematically represented (A).Venn- Diagram showing the number of microbial families' common and exclusive to control (control- mice) and prenatally As exposed mice (pAs-mice) (B) Stacked histogram showing major phylum abundant in control and pAs mice. (C). Species rarefaction curves outlining number of bacterial species in control and pAs samples (D). The Chao1 α diversity of microbial communities in control and pAs mice (E). Relative Abundance of Firmicutes and Bacteroidetes in control and pAs exposed mice (F).Firmicutes (F) to Bacteroidetes (B) ratio in control and pAs exposed mice (G) Firmicutes and Bacteroidetes abundance as measured by qPCR (H) and F/B ratio (I) Data represented as mean ± SE. n = 6/group for qpcr and n = 2/group for 16 s rRNA sequencing. Values significantly different from control at *p < 0.05, * *p < 0.01 and * **p < 0.001.

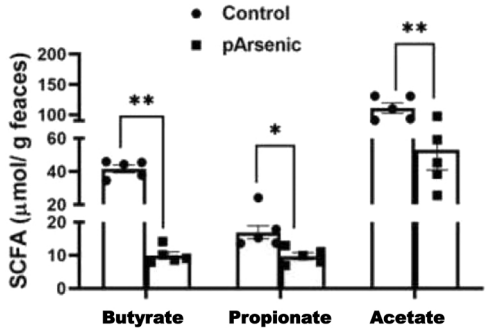


Fig. 2. Effect of prenatal arsenic exposure on concentration of faecal short chain fatty acids. Amount of faecal butyrate, acetate and propionate in control-mice and pAs-mice was determined by GC-MS. Data represented as mean ± SE (n = 5/group) *p < 0.05 and **p < 0.01.

which was further evaluated in pAs-exposed mice.

3.7. Butyrate treatment recovers gut permeability by reducing miR122 and increasing

3.7.1. Occludin expression in pAs-exposed mice

To show that decrease in gut-derived butyrate is intimately linked with decrease in barrier function in pAs mice, we supplemented butyrate orally to pAs mice and studied the reversal of barrier function. With this objective, we fed the pAs-mice from day 21–28 with 5% butyrate mixed with diet. The expression of Occludin in the gut was studied by western blot in control-mice, pAs-mice, and pAs-exposed mice fed with butyrate (pAs-butyrate-mice) (Fig. 4 A). The results showed that there was significant down regulation of occludin expression in the gut of pAs-mice which returned to normal with butyrate treatment (Fig. 4 B). Similarly, the immunohistochemical analysis showed that occludin protein expression in the gut samples of pAs-mice was 2.8 fold reduced compared to control-mice which were restored to normal in pAs-butyrate-mice (Fig. 4 C, D). The miR122 expression showed 3 fold increases in pAs-mice compared to control and after butyrate treatment the miR122 expression essentially decreased compared to pAs-mice and was equivalent to control (Fig. 4 E). As expected, there was significant increase in permeability in pAs-mice compared to control. Although in pAs-butyrate-mice the gut permeability decreased significantly compared to pAs-mice, it remains higher in respect to control-mice (Fig. 4 F). The colon histology sections revealed that butyrate restores gut heath by inhibiting recruitment of inflammatory cells like neutrophils that was induced by pAs-exposure (Fig. 4 G). Furthermore infiltration of inflammatory cells and goblet cell hyperplasia were reduced in pAs-butyrate-mice.

3.8. MiR122 over-expression rescues the effect of butyrate in pAs-butyrate-mice

From the previous experiments, it was indicated that butyrate downregulates miR122 and upregulates occludin expression in colon. The level of miR122 and occludin expression in the colon of control-mice, pAs-mice and pAs-butyrate-mice were similar to the previous experiment. MiR122 over-expression in pAs-butyrate-mice injected with miR122 expressing plasmid showed high levels of miR122 in colon indicating successful overexpression of miR122 (Fig. 5A). Corresponding analysis of occludin expression in the colon of pAs-butyrate-mice showed significant decrease with miR122-plasmid injection compared to mock-plasmid injection as studied by qPCR and western blot (Fig. 5B, C, D).

4. Discussion

The ambit of this investigation lies in answering an impending question whether *in utero* arsenic exposure can affect the gut microbiome which may alter gut physiology. Earlier reports showed that the environmentally relevant level of arsenic perturbs the normal gut microbiome in mice, decreasing abundance of *Firmicutes*, increase in *Bacteroidetes* and also changing its metabolites particularly SCFA (Chi et al., 2017). We validated this observation and showed that oral arsenic treatment causes decrease in *Firmicutes* and increase in *Bacteroidetes* in the dams. Considering the possibility of mother-offspring transmission of gut microbiome (Van Daele et al., 2019) we compared the broad microbiome composition of pAs-mice and control-mice. Overall, around 12.7% ASVs (Amplicon Sequence Variant) of normal gut bacteria was found to be lost in.

pAs-mice compared to control-mice. Arsenic is shown to prolong glycan residues of cell membrane glycoprotein of skin cancer cells (Lee et al., 2016). Glycosylation of the intestinal mucus and epithelium is quite complex and can change in response to microbial colonization (Pickard et al., 2017). This is interesting because host glycans can serve as nutrient sources or adhesion receptors for microbes (Pickard et al., 2017). Therefore it is tempting to speculate arsenic may reshape gut microbial composition in the mice by changing the extent of glycosylation pattern of the receptor proteins in the epithelial cell membrane. Diversity of a community depends on the intensity of sampling and Chao1is measured based on species richness (number of species in the community) in the groups. Although not significant, Chao1 alpha diversity of the microbiome showed a trend of decrease in pAs-mice compared to control-mice. In some cases, microbial compositions rather than diversity seems to be the key player to determine the phenotype as it was shown earlier with the survivability of wild vertebrate population (Worsley et al., 2021).

Our further analysis of the phylum composition of gut microbiota in pAs-mice and control-mice showed a significant shift in the relative abundance of *Firmicutes* (F) and *Bacteriodetes* (B) by metagenomics analysis and also by qPCR. Surprisingly, the extent of decrease and increase of *Firmicutes* (F) and *Bacteriodetes* (B) respectively in pAs-mice compared to control-mice as determined by the two methods were not hand in hand. This discrepancy may be due to low sensitivity of qPCR compared to metagenomics (Plaire et al., 2017; Andersen et al., 2017). We report decrease in *Firmicutes* (F) and increase in *Bacteriodetes* (B) leading to consolidated decrease in F/B ratio in the gut communities of pAs-mice compared to control-mice which essentially mirrors the F/B ratio of the dams. The difference in the magnitude of the drop in F/B ratio between dams and offsprings could be attributable to the dams' vaginal microbiome and environmental factors that also.

influence the establishment of gut microbiome in neonates (Tudor et al., 2017). The decrease in F/B ratio is a major biomarker of

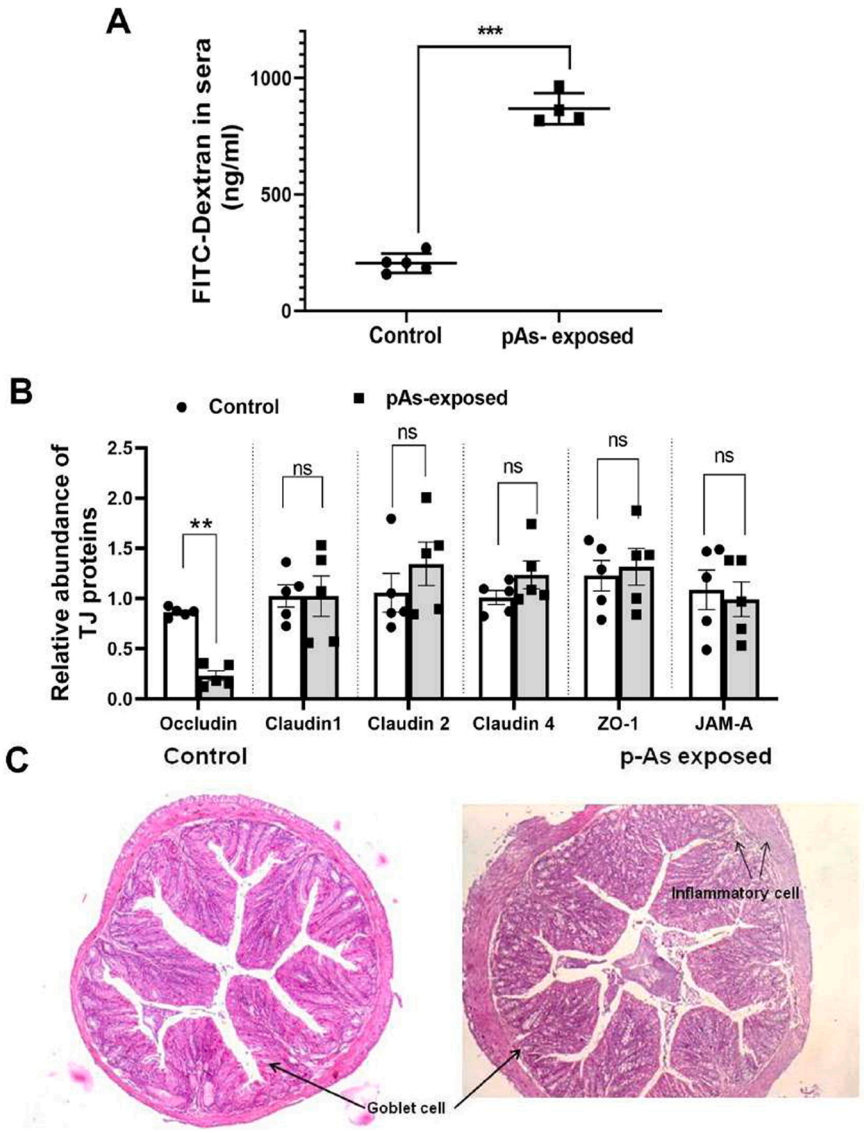


Fig. 3. Effect of prenatal Arsenic exposure on intestinal histoarchitecture and intestinal permeability. Control-mice and pAs-mice were starved for 16 h and then fed with FITC-Dextran 4000 (FD4) by oral gavage at a dose of 44 mg/100 g body weight. After 4 h the blood was collected and serum was extracted to determine the fluorescence of FD-4 in sera (A). The expression of tight junction proteins (Occludin, Claudin-1, Claudin-2, Claudin-4, ZO-1 and JAM-A) as determined by qPCR from colon tissue of Control-mice (fx1) and pAs-mice (fx2) (B). Representative micrograph of colon histology of control and pAs-mice (H&E; 10x magnification) (C). Arrows showing neutrophil infiltration. Data represented as mean ± SE. N = 5/ group. Values significantly different from control at * *$p < 0.01$ and * **$p < 0.001$. Values not significantly different from control are designated as 'ns'.

Inflammatory Bowel Disease (IBD) (Guo et al., 2021) suggesting a possibility that pAs-mice may have physiological dysfunction in the gut. The decrease in the abundance of *Firmicutes* in pAs-mice foretell the decrease in SCFA (den Besten et al., 2013). As expected, we observed significant decrease in all SCFAs including acetate, propionate and butyrate in the feaces of pAs-mice compared to control-mice. Notably, the decrease in butyrate was more pronounced than the decrease in acetate and propionate. The perturbations in the bacterial community and metabolites has overarching effect on the gut physiological functions as SCFAs particularly butyrate plays important role in multiple physiological processes in the host (Canani et al., 2011). Butyrate is an important energy source for intestinal epithelial cells (Singh et al., 1997) that maintains colonic homeostasis (Gasaly et al., 2021) and inhibits inflammation (Segain et al., 2000). The gastrointestinal epithelium forms the body's largest interface with the external environment (Groschwitz and Hogan, 2009). It effectively provides a barrier that selectively limits permeation of luminal toxins and antigens through the mucosa (Suzuki, 2013). The physical location of the intestinal epithelium, which is wedged between the luminal contents and the mucosal surface, supports the notion that a breach in the mucosal barrier causes mucosal inflammation (Ahmad et al., 2017). Studies with knockout mice with TJ proteins, develop inflammation in the gut epithelium (Ahmad et al., 2017; Lu et al., 2013) further studies support the key role of permeability, especially in its capacity to contribute to overall mucosal barrier function in regulating mucosal immune homeostasis (Castoldi et al., 2015).

Pertinent with this idea we studied the corpus of events of gut physiological functions of mice treated *in utero* with arsenic which revealed increased permeability in gut and down regulation of tight junction protein, Occludin whereas other TJ proteins like Claudin 1, claudin-2, claudin 4, ZO-1 and JaM-A remain unaltered. Tight junctions are complex signalling centres in a continually changing milieu (Weber, 2012) serving as a permeability barrier, preventing free passage of solutes via the intercellular space. Claudins, in combination with the cytoplasmic scaffold ZO, create TJ strands and perform critical roles in epithelial barrier assembly (Furuse, 2010). In addition to claudins, TJs are home to other integral membrane proteins such as occludin, a tetraspanning membrane protein, and immunoglobulin superfamily proteins, including junctional adhesion molecules (JAMs) (Furuse, 2010). They also play an important role in the regulation of paracellular permeability. The coiled coil domain of occludin acts to organize the structural and functional elements of TJ (Nusrat et al., 2000). Paracellular permeability is, to a great extent, controlled by tight junctions, and disrupting their integrity, assembly and expression results in increased permeability (Liang and Weber, 2014). Occludin being an important component of tight junction, the decrease in its expression or

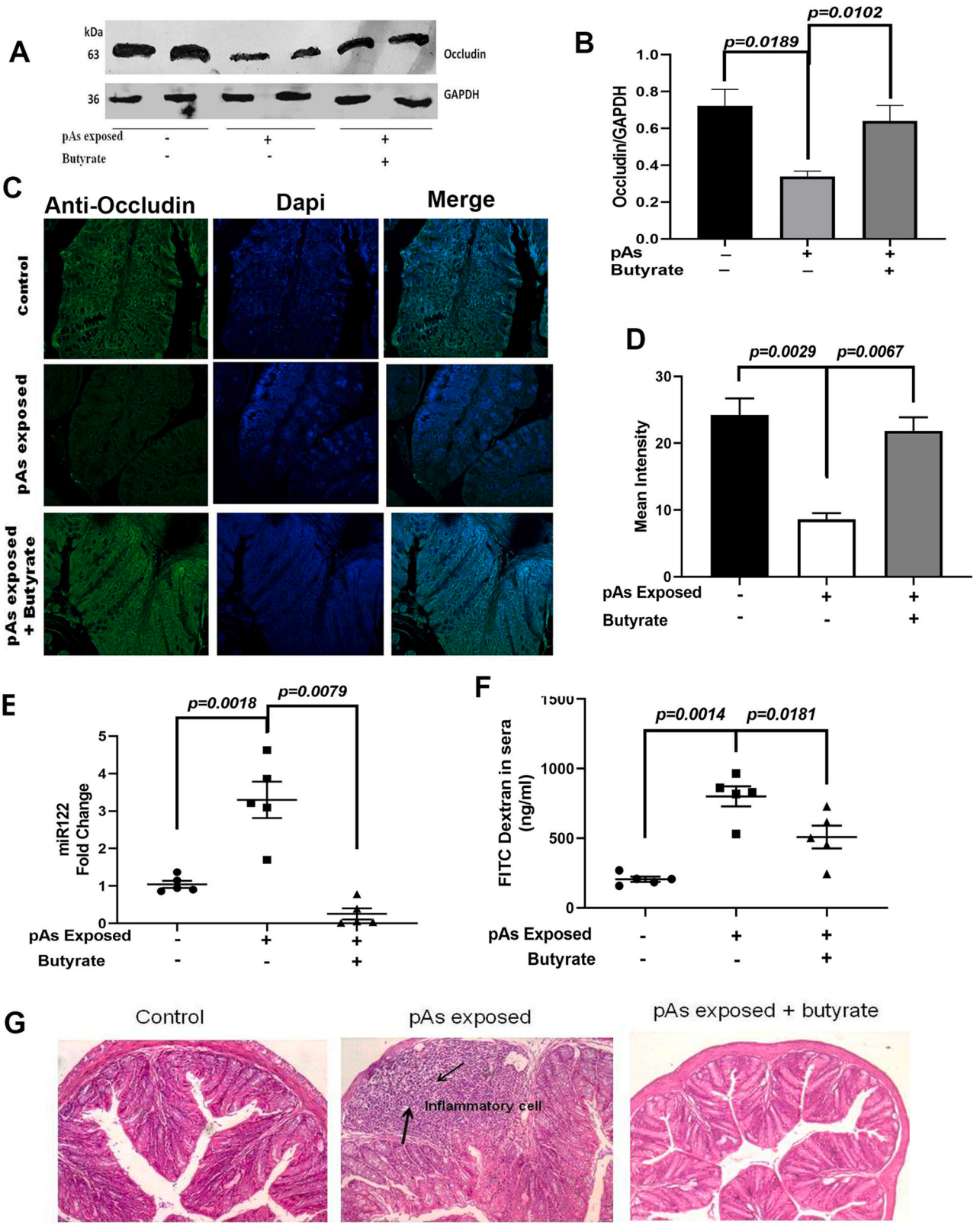


Fig. 4. Effect of oral supplementation of butyrate on colon histoarchitecture and intestinal permeability of prenatally arsenic exposed mice. Expression of Occludin as studied by Western blot (A) showing corresponding densitometry (B) and Immunofluorescence (C) showing mean intensity (D), in control-mice, pAs-mice and pAs-butyrate- mice. Expression of miR-122 quantified by qPCR in control-mice, pAs-mice and pAs-butyrate-mice (E). Intestinal permeability as measured by the presence of FITC- Dextran 4000 (FD4) in serum in control-mice, pAs-mice and pAs-butyrate-mice (F). Representative micrographs of colon sections from control-mice, pAs –mice and pAs- butyrate –mice (H&E; 20x magnification) (G). Arrows indicating neutrophil infiltration. Data represented as mean ± SE. N = 5/ group. Values (analyzed by one way ANOVA followed by Tukey's post hoc test) significantly different from control at $p < 0.05$ and $p < 0.01$.

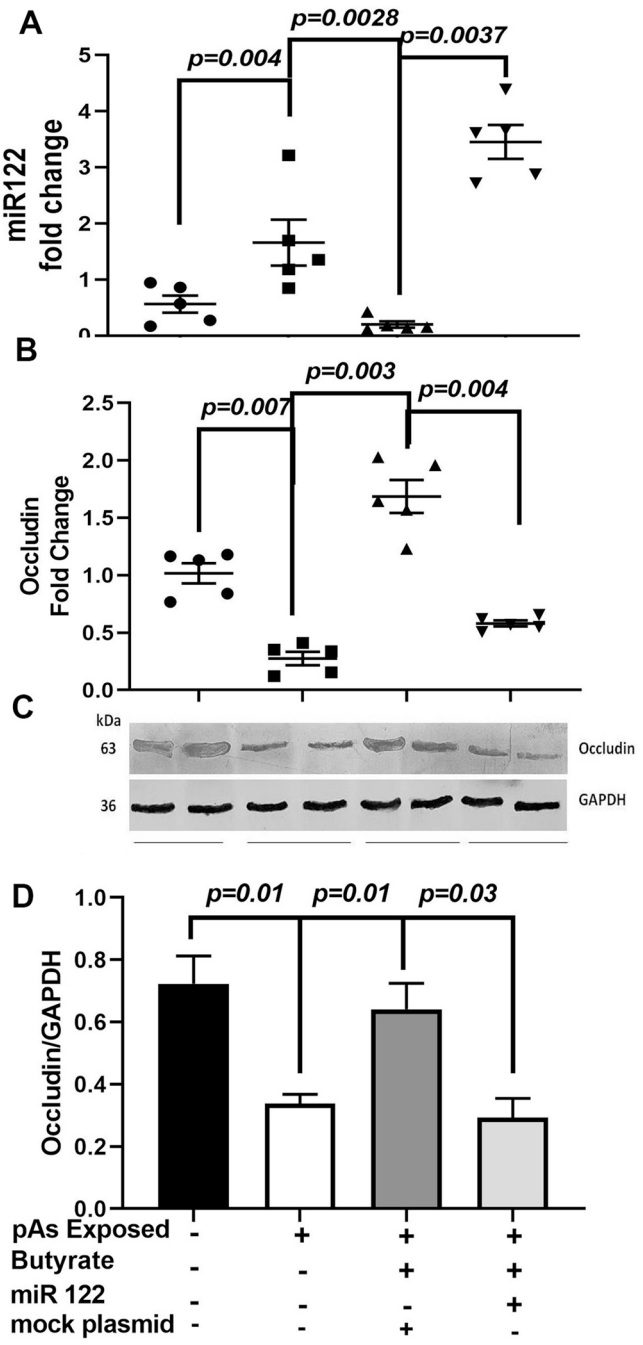


Fig. 5. Effect of overexpression of miR-122 on Occludin expression in mice colon. (A) Each butyrate treated pAs-mice were injected with either 25 μg in 100 μl of miR122 expressing plasmid or 25 μg in 100 μl of mock plasmid in tail vein. The mice were sacrificed 4 days post injection. The miR122 (A) and occludin (B) expression in colon was measured. N = 5, the data is represented as mean ± SE. The experiment was repeated twice. Values significantly different at $p < 0.05$ and $p < 0.01$.

function leads to increase in permeability. The translocation of luminal components into the host could cause both local and systemic inflammatory pathways in the case of enhanced intestinal permeability (Mu et al., 2017). Arsenic treated Caco2 cells were shown to increase paracellular permeability by redistribution of zona occludens and reduced claudin 1 expression (Chiocchetti et al., 2019). In contrast, our study showed that prenatal arsenic exposure does not change the expression of claudin 1 and ZO-1. Possibly, as the pAs-mice were not exposed to arsenic after birth, the toxic concentration of arsenic that is required to reduce claudin1 was not attained in the juvenile body. Recent report showed arsenic treatment impairs distinct population of intestinal stromal cells and intraepithelial and innate immune cells (Kellett et al., 2022; Medina et al., 2020). In conjunction, the histology of colon sections of pAs-mice showed infiltration of inflammatory cells and goblet cell hyperplasia. Gut barrier disruption and neutrophil infiltration are closely associated phenomena (Lin et al., 2020). The precise mechanism of goblet cell hyperplasia is unclear. A previous study showed that IL-13, the key regulator in type-2 mediated inflammation induces goblet cell hyperplasia to accelerate inflammation (Huang et al., 2020). Arsenic has been linked to IL-13 induction (Rahman et al., 2021), which could possibly lead to goblet cell hyperplasia. SCFA, particularly butyrate has been shown to strengthen barrier function and decrease intestinal permeability in several studies using cell culture models and animal models (Peng et al., 2009). Recalling the decrease of SCFA producing bacteria like members of the phylum Firmicutes and Spirochaetota and SCFA production in pAs mice we studied if SCFA has any effect on occludin expression. For deeper understanding of the effect of SCFA on occludin expression we undertook studies in colon carcinoma cell line, HT-29. The concentration of SCFA used in the treatment of HT-29 cells is effectively luminal concentration (Liu et al., 2018). Butyrate but not propionate or acetate treatment to HT-29 cells showed increase in Occludin expression. To further understand the mechanism by which butyrate regulates occludin expression we sought to study miRNA that plays a crucial role in regulating gene expression (Kaikkonen et al., 2011). It is reported that miR122, binds to the 3'UTR of Occludin mRNA causing its decay (Jingushi et al., 2017). Although miR122 abundantly found in liver, it is also reported to express in the intestinal tissue (Runtsch et al., 2014). Our study showed that butyrate decreases miR122 expression as function of its concentration. To confirm the inhibition of miR122 is specific to butyrate and not other SCFAs we showed that neither propionate nor acetate changes miR122 and Occludin expression. In another study from our group we have shown that a RNA binding protein, AUF1 plays a fulcrum point in miR122 regulation by butyrate (data not shown and the manuscript is under review).

Our findings capture transitivity of miR122 and Occludin in butyrate mediated decrease in paracellular permeability. The elegance of the interaction of these molecular players was further verified by providing butyrate orally in pAs-mice. As expected, following butyrate treatment to pAs-mice, Occludin expression in gut was increased with concomitant decrease in gut permeability. Butyrate fed pAs-mice not only restored miR122 level and occludin expression which resulted in decreased gut permeability and reduced infiltration of neutrophil in the gut. Further to understand the molecular events associated with butyrate and Occludin expression we over-expressed miR122 in butyrate treated pAs-mice resulted in appreciable recovery of Occludin expression. The limitation of our study is that it is focused only at a particular age of the post natal life. Further longitudinal studies are required to understand how microbial composition and gut functions changes with age in the post natal life if not exposed to arsenic anymore. Using technology involving CRISPR-based recording method by E coli sentinel cells to reveal transcriptional changes in intestinal and microbial physiology (Schmidt et al., 2022) will provide additional perspectives of arsenic induced changes in future.

5. Conclusion

Overall, the present study deals with an interesting connection of prenatal arsenic exposure and altered gut physiology in post natal life. We report gut microbial dysbiosis in pAs-mice leading to decrease in Firmicutes to Bacteroidetes ratio and decrease in production of SCFAs. We also document increase in gut permeability with decrease in Occludin expression which was reversed after butyrate treatment. Breach in the gut barrier function increases inflammatory gene expression in the gut of pAs-mice which was further reversed by butyrate

treatment. Employing in vitro and in vivo experiments we have shown that butyrate down regulates miR122 expression which is responsible for increase in Occludin expression leading to the decrease in permeability. By rescuing miR122 expression after butyrate treatment we further establish the sequential molecular partners- miR122 and Occludin that plays a role in butyrate mediated increase in barrier function in prenatal arsenic exposed mice.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

We acknowledge the support of Director, ICMR-NICED, Kolkata for carrying out the study. We thank Dr. P. Jaisankar, Sudip Dey and Sandip Chaudhury (CSIR-IICB) for GC-MS analysis. We also thank Dr. Anjan Das for analyzing the histology. We acknowledge Prof. Syamal Roy (CSIR-IICB) for critically reviewing the manuscript. MC and OD are recipient of fellowship from the CSIR and DST respectively.

Grant support

This work was supported by ICMR-NICED.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxlet.2022.11.011.

References

Abdelmohsen, K., Tominaga-Yamanaka, K., Srikantan, S., Yoon, J.H., Kang, M.J., Gorospe, M., 2012. RNA-binding protein AUF1 represses Dicer expression. Nucleic Acids Res 40, 11531–11544.

Ahmad, R., Sorrell, M.F., Batra, S.K., Dhawan, P., Singh, A.B., 2017. Gut permeability and mucosal inflammation: bad, good or context dependent. Mucosal Immunol. 10, 307–317.

Al-Sadi, R., Khatib, K., Guo, S., Ye, D., Youssef, M., Ma, T., 2011. Occludin regulates macromolecule flux across the intestinal epithelial tight junction barrier. Am. J. Physiol. Gastrointest. Liver Physiol. 300, G1054–G1064.

Andersen, S.C., Fachmann, M.S.R., Kiil, K., Moller Nielsen, E., Hoorfar, J., 2017. Gene-Based Pathogen Detection: Can We Use qPCR to Predict the Outcome of Diagnostic Metagenomics? Genes (Basel) 8.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodriguez, A.M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K.B., Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.X., Loftfield, E., Lozupone, C., Maher, M., Marotz, 615C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A.V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A., Robeson 2nd, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vazquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using 624 QIIME 2. In: Nat Biotechnol, 37, pp. 852–857.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583.

Canani, R.B., Costanzo, M.D., Leone, L., Pedata, M., Meli, R., Calignano, A., 2011. Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. World J. Gastroenterol. 17, 1519–1528.

Casalino, G., Del Turco, C., Corvi, F., Rafaeli, O., Bandello, F., Querques, G., 2016. Short-term retinal sensitivity and metamorphopsia changes following half-fluence photodynamic therapy in central serous chorioretinopathy. Ophthalmic Res 56, 23–29.

Castoldi, A., Favero de Aguiar, C., Moraes-Vieira, P.M., Olsen Saraiva Camara, N., 2015. They must hold tight: junction proteins, microbiota and immunity in intestinal mucosa. Curr. Protein Pept. Sci. 16, 655–671.

Chakraborty, M., Bhaumik, M., 2020. Prenatal arsenic exposure interferes in postnatal immunocompetence despite an absence of ongoing arsenic exposure. J. Immunotoxicol. 17, 135–143.

Chen, J., Vitetta, L., 2020. The role of butyrate in attenuating pathobiont-induced hyperinflammation. Immune Netw. 20, e15.

Cherry, N., Shaik, K., McDonald, C., Chowdhury, Z., 2010. Manganese, arsenic, and infant mortality in Bangladesh: an ecological analysis. Arch. Environ. Occup. Health 65, 148–153.

Chi, L., Bian, X., Gao, B., Tu, P., Ru, H., Lu, K., 2017. The effects of an environmentally relevant level of arsenic on the gut microbiome and its functional metagenome. Toxicol. Sci. 160, 193–204.

Chiocchetti, G.M., Velez, D., Devesa, V., 2019. Inorganic arsenic causes intestinal barrier disruption. Metallomics 11, 1411–1418.

Coppola, S., Avagliano, C., Calignano, A., Berni Canani, R., 2021. The protective role of butyrate against obesity and obesity-related diseases. Molecules 26.

den Besten, G., van Eunen, K., Groen, A.K., Venema, K., Reijngoud, D.J., Bakker, B.M., 2013. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J. Lipid Res 54, 2325–2340.

Dong, X., Gao, Y., Chen, W., Wang, W., Gong, L., Liu, X., Luo, J., 2015. Spatiotemporal distribution of phenolamides and the genetics of natural variation of hydroxycinnamoyl spermidine in rice. Mol. Plant 8, 111–121.

Dong, X., Shulzhenko, N., Lemaitre, J., Greer, R.L., Peremyslova, K., Quamruzzaman, Q., Rahman, M., Hasan, O.S., Joya, S.A., Golam, M., Christiani, D.C., Morgun, A., Kile, M.L., 2017. Arsenic exposure and intestinal microbiota in children from Sirajdikhan, Bangladesh. PLoS One 12, e0188487.

Donohoe, D.R., Collins, L.B., Wali, A., Bigler, R., Sun, W., Bultman, S.J., 2012. The Warburg effect dictates the mechanism of butyrate-mediated histone acetylation and cell proliferation. Mol. Cell 48, 612–626.

Endo, H., Niioka, M., Kobayashi, N., Tanaka, M., Watanabe, T., 2013. Butyrate-producing probiotics reduce nonalcoholic fatty liver disease progression in rats: new insight 667 into the probiotics for the gut-liver axis. PLoS One 8, e63388.

Furuse, M., 2010. Molecular basis of the core structure of tight junctions. Cold Spring Harb. Perspect. Biol. 2, a002907.

Gasaly, N., Hermoso, M.A., Gotteland, M., 2021. Butyrate and the Fine-Tuning of Colonic Homeostasis: Implication for Inflammatory Bowel Diseases. Int J. Mol. Sci. 22.

Ghosh, J., Bose, M., Roy, S., Bhattacharyya, S.N., 2013. Leishmania donovani targets 673 Dicer1 to downregulate miR-122, lower serum cholesterol, and facilitate murine liver 674 infection. Cell Host Microbe 13, 277–288.

Groschwitz, K.R., Hogan, S.P., 2009. Intestinal barrier function: molecular regulation and disease pathogenesis. J. Allergy Clin. Immunol. 124 (3–20; quiz), 21–22.

Gumber, S., Nusrat, A., Villinger, F., 2014. Immunohistological characterization of intercellular junction proteins in rhesus macaque intestine. Exp. Toxicol. Pathol. 66, 437–444.

Guo, X., Huang, C., Xu, J., Xu, H., Liu, L., Zhao, H., Wang, J., Huang, W., Peng, W., Chen, Y., Nie, Y., Zhou, Y., Zhou, Y., 2021. Gut Microbiota Is a Potential Biomarker in Inflammatory Bowel Disease. Front Nutr. 8, 818902.

Guo, X., Xia, X., Tang, R., Zhou, J., Zhao, H., Wang, K., 2008. Development of a real- time PCR method for Firmicutes and Bacteroidetes in faeces and its application to quantify intestinal population of obese and lean pigs. Lett. Appl. Microbiol 47, 367–373.

Hashash, J.G., Kane, S., 2015. Pregnancy and Inflammatory Bowel Disease. Gastroenterol. Hepatol. (N. Y) 11, 96–102.

Hoen, A.G., Madan, J.C., Li, Z., Coker, M., Lundgren, S.N., Morrison, H.G., Palys, T., Jackson, B.P., Sogin, M.L., Cottingham, K.L., Karagas, M.R., 2018. Sex-specific associations of infants' gut microbiome with arsenic exposure in a US population. Sci. Rep. 8, 12627.

Huang, Z.Q., Liu, J., Ong, H.H., Yuan, T., Zhou, X.M., Wang, J., Tan, K.S., Chow, V.T., Yang, Q.T., Shi, L., Ye, J., Wang, D.Y., 2020. Interleukin-13 Alters Tight Junction Proteins Expression Thereby Compromising Barrier Function and Dampens Rhinovirus Induced Immune Responses in Nasal Epithelium. Front Cell Dev. Biol. 8, 572749.

Jingushi, K., Kashiwagi, Y., Ueda, Y., Kitae, K., Hase, H., Nakata, W., Fujita, K., Uemura, M., Nonomura, N., Tsujikawa, K., 2017. High miR-122 expression promotes malignant phenotypes in ccRCC by targeting occludin. Int J. Oncol. 51, 289–297.

Kaikkonen, M.U., Lam, M.T., Glass, C.K., 2011. Non-coding RNAs as regulators of gene expression and epigenetics. Cardiovasc Res 90, 430–440.

Kellett, M.P., Jatko, J.T., Darling, C.L., Ventrello, S.W., Bain, L.J., 2022. Arsenic exposure impairs intestinal stromal cells. Toxicol. Lett. 361, 54–63.

Kelly, C.J., Zheng, L., Campbell, E.L., Saeedi, B., Scholz, C.C., Bayless, A.J., Wilson, K.E., Glover, L.E., Kominsky, D.J., Magnuson, A., Weir, T.L., Ehrentraut, S.F., Pickel, C., Kuhn, K.A., Lanis, J.M., Nguyen, V., Taylor, C.T., Colgan, S.P., 2015. Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function. Cell Host Microbe 17, 662–671.

Lee, C.H., Hsu, C.Y., Huang, P.Y., Chen, C.I., Lee, Y.C., Yu, H.S., 2016. Arsenite regulates prolongation of glycan residues of membrane glycoprotein: a pivotal study via wax physisorption kinetics and FTIR imaging. Int J. Mol. Sci. 17, 427.

Li, H., Zhang, X., Jin, Z., Yin, T., Duan, C., Sun, J., Xiong, R., Li, Z., 2019. MiR-122 Promotes the Development of Colon Cancer by Targeting ALDOA In Vitro. Technol. Cancer Res Treat. 18, 1533033819871300.
Li, M., Zhu, R., Song, X., Wang, Z., Weng, H., Liang, J., 2020. A sensitive method for the quantification of short-chain fatty acids by benzyl chloroformate derivatization combined with GC-MS. Analyst 145, 2692–2700.
Liang, G.H., Weber, C.R., 2014. Molecular aspects of tight junction barrier function. Curr. Opin. Pharm. 19, 84–89.
Lin, E.Y., Lai, H.J., Cheng, Y.K., Leong, K.Q., Cheng, L.C., Chou, Y.C., Peng, Y.C., Hsu, Y. H., Chiang, H.S., 2020. Neutrophil Extracellular Traps Impair Intestinal Barrier Function during Experimental Colitis. Biomedicines 8.
Lin, H.V., Frassetto, A., Kowalik Jr., E.J., Nawrocki, A.R., Lu, M.M., Kosinski, J.R., Hubert, J.A., Szeto, D., Yao, X., Forrest, G., Marsh, D.J., 2012. Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3- independent mechanisms. PLoS One 7, e35240.
Liu, H., Wang, J., He, T., Becker, S., Zhang, G., Li, D., Ma, X., 2018. Butyrate: a double-edged sword for health? Adv. Nutr. 9, 21–29.
Lu, Z., Ding, L., Lu, Q., Chen, Y.H., 2013. Claudins in intestines: Distribution and functional significance in health and diseases. Tissue Barriers 1, e24978.
McMurdie, P.J., Holmes, S., 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, e61217.
Medina, S., Lauer, F.T., Castillo, E.F., Bolt, A.M., Ali, A.S., Liu, K.J., Burchiel, S.W., 2020. Exposures to uranium and arsenic alter intraepithelial and innate immune cells in the 733 small intestine of male and female mice. Toxicol. Appl. Pharm. 403, 115155.
Mor, A., Antonsen, S., Kahlert, J., Holsteen, V., Jorgensen, S., Holm-Pedersen, J., Sorensen, H.T., Pedersen, O., Ehrenstein, V., 2015. Prenatal exposure to systemic antibacterials and overweight and obesity in Danish schoolchildren: a prevalence study. Int J. Obes. (Lond.) 39, 1450–1455.
Mu, Q., Kirby, J., Reilly, C.M., Luo, X.M., 2017. Leaky gut as a danger signal for autoimmune diseases. Front Immunol. 8, 598.
Mukherjee, A., Sengupta, M.K., Hossain, M.A., Ahamed, S., Das, B., Nayak, B., Lodh, D., Rahman, M.M., Chakraborti, D., 2006. Arsenic contamination in groundwater: a global perspective with emphasis on the Asian scenario. J. Health Popul Nutr. 24, 142–163.
Myers, S.L., Lobdell, D.T., Liu, Z., Xia, Y., Ren, H., Li, Y., Kwok, R.K., Mumford, J.L., Mendola, P., 2010. Maternal drinking water arsenic exposure and perinatal outcomes in inner Mongolia, China. J. Epidemiol. Community Health 64, 325–329.
Nusrat, A., Chen, J.A., Foley, C.S., Liang, T.W., Tom, J., Cromwell, M., Quan, C., Mrsny, R.J., 2000. The coiled-coil domain of occludin can act to organize structural and functional elements of the epithelial tight junction. J. Biol. Chem. 275, 29816–29822.
Okumura, T., Nozu, T., Ishioh, M., Igarashi, S., Kumei, S., Ohhira, M., 2021. Centrally administered butyrate improves gut barrier function, visceral sensation and septic lethality in rats. J. Pharm. Sci. 146, 183–191.
Peng, L., He, Z., Chen, W., Holzman, I.R., Lin, J., 2007. Effects of butyrate on intestinal barrier function in a Caco-2 cell monolayer model of intestinal barrier. Pedia Res 61, 37–41.
Peng, L., Li, Z.R., Green, R.S., Holzman, I.R., Lin, J., 2009. Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. J. Nutr. 139, 1619–1625.
Pickard, J.M., Zeng, M.Y., Caruso, R., Nunez, G., 2017. Gut microbiota: Role in pathogen colonization, immune responses, and inflammatory disease. Immunol. Rev. 279, 70–89.
Plaire, D., Puaud, S., Marsolier-Kergoat, M.C., Elalouf, J.M., 2017. Comparative analysis of the sensitivity of metagenomic sequencing and PCR to detect a biowarfare simulant (Bacillus atrophaeus) in soil samples. PLoS One 12, e0177112.
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glockner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41, D590–D596.
Rahman, A., Islam, M.S., Tony, S.R., Siddique, A.E., Mondal, V., Hosen, Z., Islam, Z., Hossain, M.I., Rahman, M., Anjum, A., Paul, S.K., Hossen, F., Sarker, M.K., Hossain, S., Salam, K.A., Haque, A., Hoque, M.A., Saud, Z.A., Xin, L., Sumi, D., Himeno, S., Hossain, K., 2021. T helper 2-driven immune dysfunction in chronic arsenic-exposed individuals and its link to the features of allergic asthma. Toxicol. Appl. Pharm. 420, 115532.
Rangan, P., Choi, I., Wei, M., Navarrete, G., Guen, E., Brandhorst, S., Enyati, N., Pasia, G., Maesincee, D., Ocon, V., Abdulridha, M., Longo, V.D., 2019. Fasting-Mimicking Diet Modulates Microbiota and Promotes Intestinal Regeneration to Reduce Inflammatory Bowel Disease Pathology. Cell Rep. 26 (2704–2719), e2706.
Robeson 2nd, M.S., O'Rourke, D.R., Kaehler, B.D., Ziemski, M., Dillon, M.R., Foster, J.T., Bokulich, N.A., 2021. RESCRIPt: Reproducible sequence taxonomy reference database management. PLoS Comput. Biol. 17, e1009581.
Runtsch, M.C., Round, J.L., O'Connell, R.M., 2014. MicroRNAs and the regulation of intestinal homeostasis. Front Genet 5, 347.
Saitou, M., Furuse, M., Sasaki, H., Schulzke, J.D., Fromm, M., Takano, H., Noda, T., Tsukita, S., 2000. Complex phenotype of mice lacking occludin, a component of tight junction strands. Mol. Biol. Cell 11, 4131–4142.
Schmidt, F., Zimmermann, J., Tanna, T., Farouni, R., Conway, T., Macpherson, A.J., Platt, R.J., 2022. Noninvasive assessment of gut function using transcriptional recording sentinel cells. Sci. 376, eabm6 038.
Schulthess, J., Pandey, S., Capitani, M., Rue-Albrecht, K.C., Arnold, I., Franchini, F., Chomka, A., Ilott, N.E., Johnston, D.G.W., Pires, E., McCullagh, J., Sansom, S.N., Arancibia-Carcamo, C.V., Uhlig, H.H., Powrie, F., 2019. The Short Chain Fatty Acid Butyrate Imprints an Antimicrobial Program in Macrophages. Immunity 50 (432–445), e437.
Segain, J.P., Raingeard de la Bletiere, D., Bourreille, A., Leray, V., Gervois, N., Rosales, C., Ferrier, L., Bonnet, C., Blottiere, H.M., Galmiche, J.P., 2000. Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. Gut 47, 397–403.
Silva, J.P.B., Navegantes-Lima, K.C., Oliveira, A.L.B., Rodrigues, D.V.S., Gaspar, S.L.F., Monteiro, V.V.S., Moura, D.P., Monteiro, M.C., 2018. Protective Mechanisms of Butyrate on Inflammatory Bowel Disease. Curr. Pharm. Des. 24, 4154–4166.
Singh, B., Halestrap, A.P., Paraskeva, C., 1997. Butyrate can act as a stimulator of growth or inducer of apoptosis in human colonic epithelial cell lines depending on the presence of alternative energy sources. Carcinogenesis 18, 1265–1270.
Stensballe, L.G., Simonsen, J., Jensen, S.M., Bonnelykke, K., Bisgaard, H., 2013. Use of antibiotics during pregnancy increases the risk of asthma in early childhood. J. Pedia 162 (832–838), e833.
Suzuki, T., 2013. Regulation of intestinal epithelial permeability by tight junctions. Cell Mol. Life Sci. 70, 631–659.
Tudor, K.I., Petravic, D., Jukic, A., Juratovac, Z., 2017. Skew deviation: case report and review of the literature. Semin Ophthalmol. 32, 734–737.
Van Daele, E., Knol, J., Belzer, C., 2019. Microbial transmission from mother to child: improving infant intestinal microbiota development by identifying the obstacles. Crit. Rev. Microbiol 45, 613–648.
Weber, C.R., 2012. Dynamic properties of the tight junction barrier. Ann. N. Y Acad. Sci. 1257, 77–84.
Worsley, S.F., Davies, C.S., Mannarelli, M.E., Hutchings, M.I., Komdeur, J., Burke, T., Dugdale, H.L., Richardson, D.S., 2021. Gut microbiome composition, not alpha diversity, is associated with survival in a natural vertebrate population. Anim. Micro 3, 84.
Wu, X., Yang, Y., Huang, Y., Chen, Y., Wang, T., Wu, S., Tong, L., Wang, Y., Lin, L., Hao, M., Zhong, Z.H., Zhang, F., Zhao, W., 2018. RNA-binding protein AUF1 suppresses miR- 122 biogenesis by down-regulating Dicer1 in hepatocellular carcinoma. Oncotarget 9, 14815–14827.
Xu, Y.H., Gao, C.L., Guo, H.L., Zhang, W.Q., Huang, W., Tang, S.S., Gan, W.J., Xu, Y., Zhou, H., Zhu, Q., 2018. Sodium butyrate supplementation ameliorates diabetic inflammation in db/db mice. J. Endocrinol. 238, 231–244.
Yang, W., Xu, H.W., Lu, X.R., Xu, Q.F., Tao, M.H., Dai, Y.M., 2022. Overexpression of miR-122 Impairs Intestinal Barrier Function and Aggravates Acute Pancreatitis by Downregulating Occludin Expression. Biochem Genet 60, 382–394.
Ye, D., Guo, S., Al-Sadi, R., Ma, T.Y., 2011. MicroRNA regulation of intestinal epithelial tight junction permeability. Gastroenterology 141, 1323–1333.
Yip, W., Hughes, M.R., Li, Y., Cait, A., Hirst, M., Mohn, W.W., McNagny, K.M., 2021. Butyrate shapes immune cell fate and function in allergic asthma. Front Immunol. 12, 628453.