Deciphering novel immuno-suppressive mechanisms adopted by *Leishmania donovani* parasites in the pathogenesis of *kala-azar*

A Thesis
Submitted in Partial
Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

By

Gundappa Saha

Department of Biosciences & Bioengineering
Indian Institute of Technology Guwahati
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AUGUST 2019

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Dedicated to

My wonderful and unconditional love pouring parents,

Inspiring and motivational teachers (“*gurus*”)

And the ever amazing lovely friends

Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world.

- Louis Pasteur

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOSCIENCES & BIOENGINEERING

CERTIFICATE

I hereby declare that the research informations embodied in this thesis titled **"Deciphering novel immunosuppressive mechanisms adopted by *Leishmania donovani* parasites in the pathogenesis of *kala-azar*''** is a result of investigations obtained from the research work carried out by me in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India under the supervision of **Prof. Vikash Kumar Dubey**.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work of other investigators are referred. Further the data in the thesis are collected by me. I certify that there is no fabrication or manipulation of data in the thesis.

Gundappa Saha

Date: *August 2019*

Gundappa Saha

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The Care Continues...

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Signing off
Gundappa Saha
August, 2019

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ABBREVIATIONS

APAF-1 :	Apoptotic Protease Activating Factor 1
ASC :	Apoptosis-Associated Speck-Like Protein Containing CARD
BIR :	Baculovirus Inhibitor Of Apoptosis Repeat
BLIMP-1 :	B-lymphocyte induced maturation protein 1
CARD :	Caspase Recruitment Domain
CD :	Cluster of differentiation
CL :	Cutaneous leishmaniasis
CLRs :	C – lectin type receptors
DAMPs :	Danger Associated Molecular Patterns
DCL :	Diffuse cutaneous leishmaniasis
DCs :	Dendritic Cells
DMEM :	Dulbecco's Modified Eagle's Medium
DNA :	Deoxy ribonucleic acid
FBS :	Fetal Bovine Serum
GLA-SE :	Gluco-pyranosyl lipid adjuvant formulated in a stable emulsion
gp29 :	Glycoprotein 29
HIV :	Human Immunodeficiency Virus
HRP :	Horse Radish Peroxidase
HSV-1 :	Herpes Simplex Virus Type 1
ICAM :	Intercellular Adhesion Molecule
IFN-γ	Interferon-γ
IgG :	Gamma Immunoglobulin
IKK :	Inhibitor of κappa β kinase
ILs :	Interleukins
IPAF :	Interstitial pneumonia with autoimmune features
IRAK :	IL-1 receptor-associated kinase
IRFs :	Interferon Regulatory Factors
KMP-11 :	Kinetoplastid membrane protein 11

LDH :	Lactate Dehydrogenase
LPS :	Lipopolysaccharides
LRRs :	Leucine Rich Repeats
MAPK:	Mitogen Activated Protein Kinases
MCL :	Mucocutaneous leishmaniasis
mCMV :	Mouse Cytomegalovirus
MPL-SE :	Monophosphoryl lipid A (MPL) plus squalene
MSU :	Monosodium Urate
NAC :	N-Acetyl Cysteine
NACHT :	NAIP (neuronal apoptosis inhibitory protein), CIITA (MHC class II transcription activator), HET-E (incompatibility locus protein from Podospora anserina) and TP1 (telomerase-associated protein)
NEK7 :	NIMA related kinase 7
NEMO :	NF-kappa β Essential Modulator
NFκβ :	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3 :	Nucleotide-binding domain and leucine-rich repeat containing (NLR) protein 3
NLRs :	Nucleotide oligomerization domain (NOD) – like receptors
NO :	Nitric oxide
PAMPs :	Pathogen Associated Molecular Patterns
PGN :	Peptidoglycan
PKDL :	Post *kala-azar* dermal leishmaniasis
PMA :	12 – Phorbol 13 – myristate acetate
PRRs :	Pathogen Recognition Receptors
PYD :	Pyrin Domain
RIG-I :	Retinoic Acid-Inducible Receptor
RLRs :	RIG-I like receptors
ROS :	Reactive Oxygen Species
RPMI :	Roswell Park Memorial Institute
RT :	Room Temperature
SbIII :	Trivalent antimonite

SbV :	Pentavalent antimonite
siRNA :	Small interfering ribonucleic acid (RNA)
TAK1 :	Transforming growth factor beta-activated kinase 1
TIR :	Toll/IL-1 receptor
TLRs :	Toll – like receptors
TNF :	Tumor Necrosis Factor
TRAF :	TNF Receptor Associated Factor
TRIF :	TIR-domain-containing adapter-inducing interferon-β
TSA :	Thiol specific antioxidant antigen
VL :	Visceral leishmaniasis
VSV :	Vesicular Stomatitis Virus
WHO :	World Health Organization
zmp1 :	Zn^{2+} metalloprotease

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GENERAL ABSTRACT

Leishmaniasis *(*commonly termed as "*kala azar*" in Indian subcontinent*)* is one of the most neglected parasitic diseases in the world. Despite advances in chemotherapeutic inventions, high cost, toxicity, duration of treatment, route of administration and development of drug resistance are the major drawbacks of chemotherapy. Thus, there is a huge urge to develop novel strategy for long term treatment which can be addressed by the development of novel immuno-therapeutics. To do so, thorough knowledge of immunosuppressive mechanism during infection has to be evaluated. Numerous advances have been made in highlighting the regulation of inflammasomes and its involvement in innate immunity during any pathogenic infections. Inflammasome activation is a tightly regulated process in providing defense against pathogenic insults. Few studies on the involvement of nucleotide-binding domain and leucine-rich repeat containing (NLR) proteins, NLRP3 inflammasome have been reported in leishmanial infections with contradictory results and without much mechanistic insights. However, the role of NLRP3 inflammasome and its components has not been well deciphered in *Leishmania donovani* infection. Here we report for the first time a detailed mechanism and plausible impairment of caspase 1 activation during *L. donovani* infection leading to the survival of these parasites inside the host cells. We demonstrate the importance of caspase 1 in the host defense mechanism *in-vitro* via siRNA mediated knock-down of caspase 1 in macrophage cell lines resulting in significantly higher parasitic burden. In addition, we have also shown that parasite can exploit a host molecule BLIMP-1 to mediate impairment of NLRP3 inflammasome activation by its overexpression. This upregulation of BLIMP-1 has been found to inhibit various other molecules like NFκβ, NEK7, TAK1 and p53 leading to escape of parasite from host immune response. Cells deficient in BLIMP-1 expression were used to validate its role during infection and suggest these parasites are tweaking the tight regulation of NFκβ – NLRP3 signaling pathway by BLIMP-1 overexpression. Eventually, the parasites disrupt an inflammation-mediated pyroptosis cell death pathway in infected cells and evade the inflammatory response of the host cells. The detailed understanding of this novel mechanism can play a vital role in designing future immunotherapeutics to combat Leishmaniasis.

CHAPTER 1

Leishmaniasis in the Context of Immunological Responses

1.1 History & Overview of Leishmaniasis

Leishmaniasis is a group of vector borne neglected tropical disease caused by a protozoan parasites of the genus *Leishmania* (Herwaldt, 1999). Leishmaniasis has an extended history dating back to the extent that of first century AD. Visceral leishmaniasis (VL) is commonly referred to as *kala-azar* (due to dark pigmentation in the skin post fever) in the Indian subcontinent. A British surgeon William Twinning reported the occurrence of *kala-azar* dating back to 1824 in Calcutta with symptoms like splenic enlargement, acute anemia and intermittent fever in the local population. In the year 1858, an epidemic broke out in the regions of Assam and Bengal, claiming numerous lives and the disease was also resistant to quinine and had malaria like symptoms. In the year 1903, William Boog Leishman and Charles Donovan both independently demonstrated the parasite in the splenic tissue from patients in India who died from "dum-dum fever" and named them as trypanosomatids, which were popularly known as "Leishman-Donovan bodies" (Leishman, 1903). But it was Ronald Ross who anticipated that Leishman-Donovan bodies were the new parasites and named as *Leishmania donovani* (Ross, 1903). Later in 1904, Dr. Sheffield Neave identified the parasite in Sudan. Post kala azar dermal leishmaniasis (PKDL) was described by Upendranath Brahmachari in India in 1922. This disease is mostly transmitted by 1 to 30 species of sand flies of the genera *Phlebotomus* or *Lutzomyia* (Chappuis et al., 2007) and was demonstrated by Swaminathan, Shortt and Anderson in the year 1942 and later proved by Gibson in 1983 (Gibson, 1983). There are several factors that govern the spread of this disease such as human migrations, socio-economic factors, malnutrition, climatic change and

environmental changes and also lack of awareness. Poor living habitats (i.e. open sewage, spread of waste and poor sanitary conditions) intensifies the threat for leishmaniasis infection which provide perfect condition for sandfly breeding. Malnutrition is another major factor that increases the risk of infection by *Leishmania*. Lack of efficient chemotherapeutics and no vaccine candidates in the market has worsen the situation with more number of epidemic breakouts globally. Detailed understanding of the biology of leishmaniasis and its associated molecular mechanisms will help in efficient management and eradication of the disease.

1.2 Systemic position of *Leishmania*

The genus *Leishmania* are unicellular organisms which uses flagella for their locomotion, feeds by pinocytosis or phagocytosis, contain a large kinetoplast - a portion of the single mitochondrion containing a large amount of DNA. Based on these characteristics, members of the genus *Leishmania* are classified into following nomenclature:

Domain	: *Eukarya*
Kingdom	: *Protista*
Sub-kingdom	: *Protozoa*
Phylum	: *Sarcomastigophora*
Sub-phylum	: *Mastigophora*
Class	: *Zoomastigophora*
Order	: *Kinetoplastida*
Family	: *Trypanosomatidae*
Genus	: *Leishmania*

1.3 Morphology & life cycle of *Leishmania* parasite

The digenetic or heteroxenic lifecycle of *Leishmania* parasites alternate between elongated promastigotes and ovoid amastigotes that have adapted to live in the extracellular space of the intestinal cavities of the insect vector or in an intracellular habitat inside the mammalian macrophages respectively (Kamhawi, 2006). A cartoon sketch of promastigote and amastigote has been shown in Figure 1.1A along with their live scanning electron microscopy image in Figure 1.1B. The infective stages of the parasites also called the "metacyclic promastigotes" are inoculated into the host by sand flies of *Phlebotomus* genus

during their blood meal. The promastigotes adhere to the plasma membrane of the host and trigger a phagocytic process (Lodge and Descoteaux, 2008). Thereafter, promastigotes make a silent entry to the macrophage and reside inside a parasitophorous vacuole and develop during its life cycle. Then, the promastigotes differentiate into amastigotes that live in an acidic pH habitat where they divide by binary fission. After multiple divisions, a large number of amastigotes leads to the lyses of the macrophages, releasing parasites that can infect new macrophages or be ingested by sandflies during new blood meals. Morphologically, amastigotes are smaller in size than promastigote with no flagella present. Amastigotes are ovoid in shapes that measure ~2-4 μm in diameter. The amastigotes transform into procyclic promastigotes in the gut of the sand flies which are long slender cells of ~15-20 μm x 1.5-3.5 μm measurements and a 15-28 μm anterior flagellum helps in attachment and locomotion of the parasite in the gut wall of sand-fly. This process quickly enters into a differentiation process known as "metacyclogenesis". In this process, non-infective forms differentiate into infective metacyclic promastigotes that migrate to the proboscis, thereby starting a new round of infection again (Bates, 2007). The life cycle of *Leishmania* alternating between sandfly vector and mammalian host has been shown in Figure 1.2.

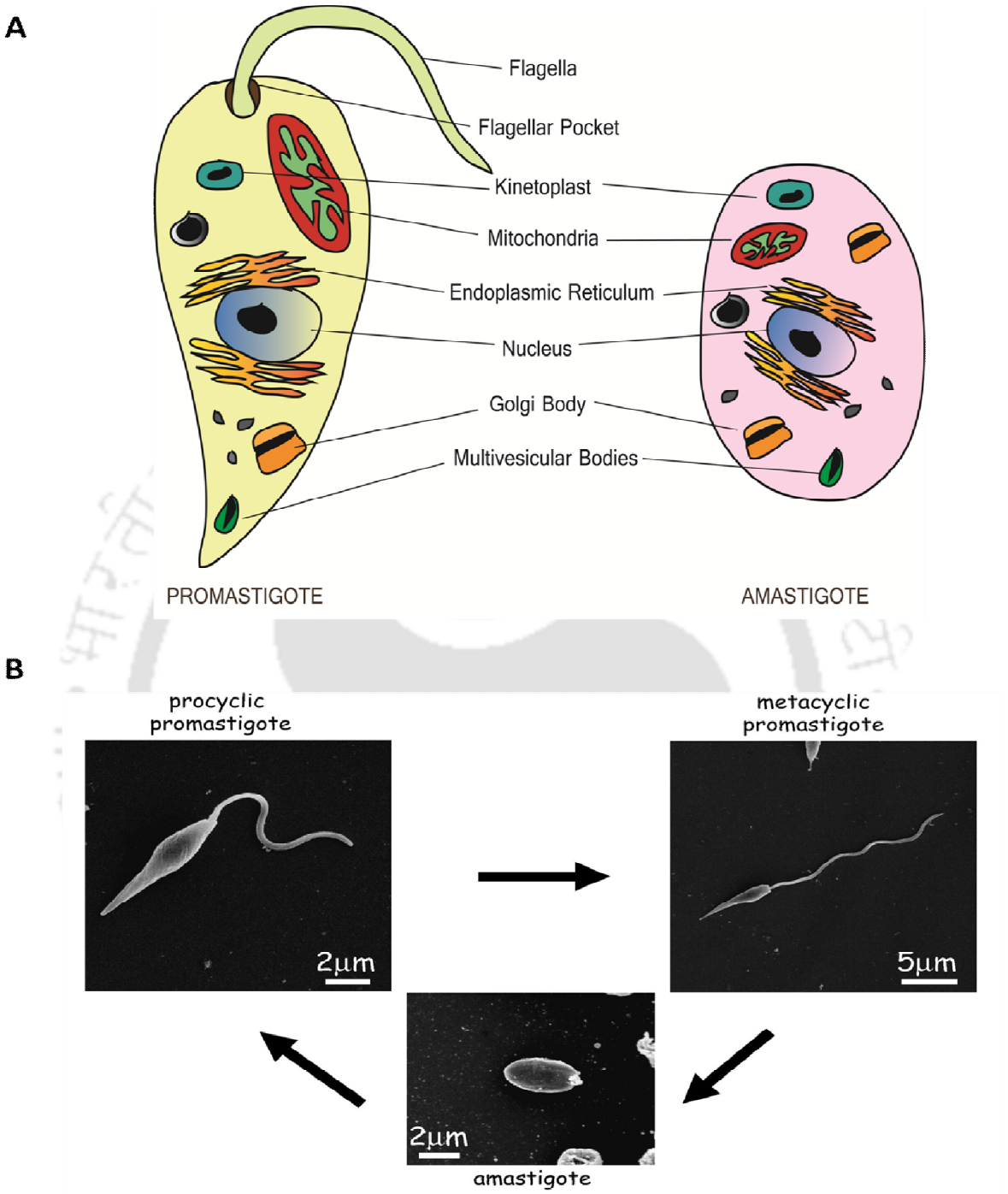

Figure 1.1: Morphology of different forms of *Leishmania* parasite. (A) A cartoon sketch representing the intracellular organelles of *Leishmania* promastigote (left) and amastigote (right), (B) Scanning electron microscope images of *Leishmania major* life-cycle stages; the procyclic and metacyclic promastigotes were grown in culture, the amastigote was isolated from an infected macrophage of a mouse [Adapted from (Besteiro et al., 2007)].

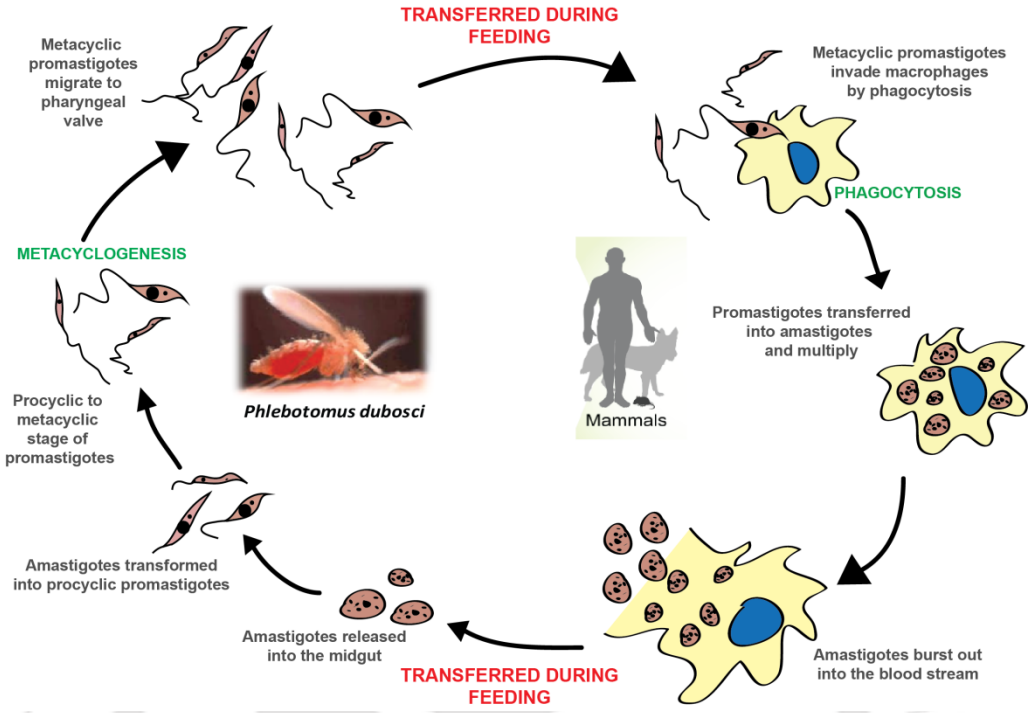

Figure 1.2: Life cycle of *Leishmania* parasite. The procyclic promastigote forms of *Leishmania* differentiate in sand-fly into metacyclic promastigotes which are non-dividing and infective stage. At the time of blood feeding, the sand-fly spit up metacyclic promastigotes, along with various salivary components. The macrophage cells that are present at the bite site phagocytose metacyclic promastigotes. After forming an intracellular habitation, metacyclic promastigotes transform into a flagellate amastigotes. Amastigotes undergo replication within host cells, which break when too many amastigotes are present, allowing reinfection of local macrophage cells. The transmission cycle is complete when infected macrophage cells are ingested up in a blood by a new sand-fly, and amastigotes then transform into promastigotes in the sand-fly midgut.

1.4 Epidemiology & Clinical Manifestations of Leishmaniasis

At least 21 species of *Leishmania* can cause disease transmitted to humans by sand flies of 1 of 30 species from the genera *Phlebotomus* or *Lutzomyia*. *Leishmania* species are divided in two subgenera: *Leishmania* and *Viannia*. Together they are responsible for five main clinical manifestations: Visceral leishmaniasis (VL) or *kala-azar*, cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL), and post *kala-azar* dermal leishmaniasis (PKDL). Leishmaniasis has multiple manifestations that range from ulcerative self-healing skin lesions called cutaneous leishmaniasis to life threatening disseminated visceral leishmaniasis. The other form of the disease is mucocutaneous leishmaniasis in which the oral and nasal mucosal inflammation occurs. Different clinical

manifestations depend on the type of species of *Leishmania* which has been tabulated in Table 1.1. The detailed informations has been listed below and shown in Figure 1.3.

a) **Visceral Leishmaniasis (VL):** This manifestation is considered the most severe form of the disease characterized by undulating fever, weight loss, splenomegaly (enlarged spleen), hepatomegaly (enlarged liver), lymphadenopathy, anemia and night sweating are common symptoms and progress with time (Osman et al., 2000, Salam et al., 2014). The parasites display a marked tropism for visceral organs, such as the liver, spleen, bone marrow and lymphatic system. The blackening of the skin is one of the symptom from which the disease got its name "*kala-azar*" in India (*kala-azar* means black fever in Hindi). A new emerging problem of concern with visceral infection is its development as an opportunistic infection in immunocompromised patients, such as AIDS. In VL/HIV co-infection patients, the chances of treatment are less as both infections mutually destroy the immunity of the patient and eventually die (Pintado and López-Vélez, 2001, Alvar et al., 2008).

b) **Cutaneous Leishmaniasis (CL):** This is the most common and widespread infections of *Leishmania* and is characterized by development of red lesions at the site of the bite by infected sand-fly (Reithinger et al., 2007). These lesions develop as papules, to nodules and ulcerate with central depression and raised borders ultimately to atrophic scars, leading to significant disfigurement and social stigmatization. Generally this are self curing without any specific treatment but has a devastating social stigma. In endemic countries, diagnosis is often made clinically and, if possible, by microscopic examination of lesion biopsy smears to visually confirm leishmania parasites as the cause (Dowlati, 1996). Fewer options are available for effective vector control because of the complexity of cutaneous leishmaniasis epizoology.

c) **Diffuse Cutaneous Leishmaniasis (DCL):** This type of manifestation is rare even in leishmaniasis-endemic regions and occurs when the immune system fails to react against *Leishmania* antigens in individuals with a defective cell-mediated immune response (Bomfim et al., 1996). Multiple non-ulcerative lesions form around the body, sometimes resembling those found in lepromatous leprosy. These lesions never spontaneously heal, and unfortunately no treatment is currently available for it.

d) **Mucocutaneous Leishmaniasis (MCL):** In this type, the parasites have a marked tropism for the oral-nasal and pharyngeal cavities, often causing extensive destruction that involves mutilation of the face and great and painful suffering for the infected patients. In most of the cases, MCL is largely caused by parasites of the *Viannia* subgenus as a consequence of lymphatic or haematogenous distribution of parasite from the skin to the naso-oropharyngeal mucosa (NOLDER et al., 2007).

e) **Post *kala-azar* Dermal Leishmaniasis (PKDL):** PKDL is the reoccurrence of the VL and can appear up to 20 years after treatment has ended. The patients develop a chronic form of cutaneous leishmaniasis which requires long term treatment. Post miltefosine treatment develop PKDL as per report from Indian subcontinent (Das et al., 2009). Sometimes, PKDL appears as a co-infection with human immunodeficiency virus (HIV), which is another important feature of leishmaniasis (Zijlstra et al., 2003).

Table 1.1: Summary of different clinical manifestations specific to different *Leishmania* species

S. No.	Clinical Manifestations	*Leishmania* sp.
1.	Visceral leishmaniasis (*kala-azar*)	*L. donovani; L. infantum*
2.	Cutaneous leishmaniasis	*L. major; L. braziliensis; L. amazonensis*
3.	Diffuse cutaneous leishmaniasis	*L. amazonensis; L. mexicana; L. aethiopica*
4.	Mucocutaneous leishmaniasis	*L. braziliensis; L. guyanensis; L. aethiopica*
5.	Post *kala-azar* dermal leishmaniasis	*L. infantum; L. donovani; L. chagasi*

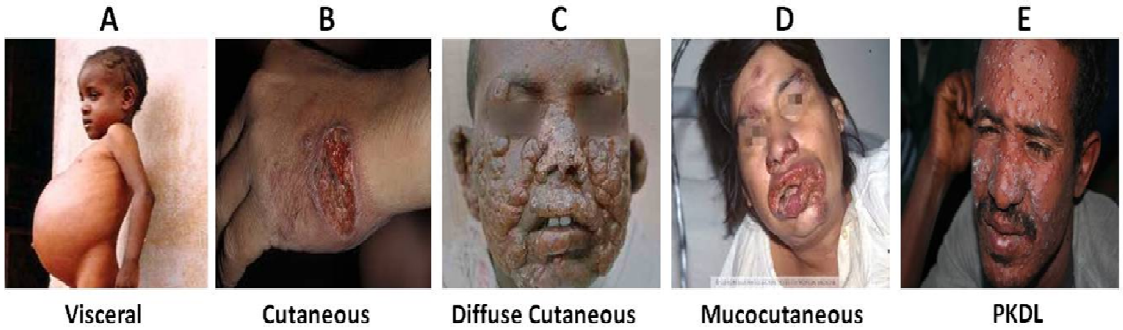

Figure 1.3: Different forms of clinical manifestations of Leishmaniasis (A) Visceral Leishmaniasis; (B) Cutaneous leishmaniasis; (C) Diffuse cutaneous leishmaniasis; (D) Mucocutaneous leishmaniasis; (E) Post kala-azar dermal leishmaniasis.

1.5 Global Burden of Leishmaniasis

Leishmaniasis is currently endemic in 88 nations ranging from deserts in western Asia to rain forests in Americas, covering intra-tropical temperate region of the globe with an estimated 350 million people at risk. Each year more than 90% of cases reported are contributed mainly by 11 nations, India, Nepal, Bangladesh, Brazil, Sudan, Peru, Bolivia, Iran, Afghanistan, Syria, and Saudi Arabia (Pigott et al., 2014). Globally the disease is associated with poor socio economic condition of the nations which can impairs economic growth and development of the nation as per reports from World Health Organization (WHO) – 2006. According to the latest estimation, nearly 2 million fresh cases are reported annually, among which cutaneous leishmaniasis accounts for 1.5 million and visceral form claims the remaining 500,000 cases, with overall prevalence around 12 million people infected thought the globe currently. An associated HIV co-infection raises the risk of developing active visceral infection by between 100 and 2320 times as per WHO – 2014. As many as 35 countries throughout the world have reported cases of visceral leishmaniasis/ HIV (VL/HIV) co-infection. Majority of the cases of VL/HIV co-infections are reported from countries of southern Europe. As much as up to 70% of cases of VL in adults are associated with HIV infection. This co-infection further complicates the treatment as both infections jointly destroy the immune system of the patient. Geographical distribution of visceral leishmaniasis has been shown in Figure 1.4A as per WHO 2013 report.

1.6 Present Status of Current Chemotherapeutics

In the current scenario, the treatment of leishmaniasis depends on the available chemotherapeutics that include pentavalent antimonials, amphotericin B and its liposomal formulations; miltefosine, paromomycin and pentamidine as shown in Figure 1.4B. Once the patient is confirmed positive for leishmaniasis, generic pentavalent antimonials was the first choice of chemotherapy in leishmaniasis endemic nations particularly in Indian subcontinent (Croft et al., 2006) which has been shifted to miltefosine in recent years (Pinto-Martinez et al., 2018). The pentavalent antimoniate (SbV) was thought to be a pro-drug, and later changed to active trivalent antimonite (SbIII) form of the drug which is highly effective against the parasite as compared to SbV (Ephros et al., 1999). Miltefosine was developed as an oral drug and showed an early promise; however, there are now increasing incidences of relapse in patients

treated with this drug. Combination therapy of drugs has also started gaining interest in the global market. Combined chemotherapy of sodium stibogluconate and paromomycin are highly effective, curing approximately 95% of the patients suffering from *L. donovani* infection (Thakur, 2000). Though all pharmaceutical industries and scientific community are trying hard to find different potential drug target against *Leishmaniasis*, the parasites have started gaining resistance against most of the available drugs including miltefosine. In Bihar, India, 40–60% of patients suffering from VL do not respond to pentavalent antimony treatment (Jha, 2006). Similar results, with 25% unresponsiveness, were recorded with pentamidine (Mishra et al., 1992). Resistance of *Leishmania donovani* promastigotes to miltefosine was shown *in-vitro*, and resistance of both *Leishmania major* and *Leishmania tropica* promastigotes to paromomycin was induced *in-vitro* by repeated exposure of the parasites while gradually increasing the drug concentrations. The main reason of acquired resistance is due to extensive misuse of drugs in endemic regions where they are made widely available, causing the loss of drug activation by the parasite. The disability of the SbV resistant strain to reduce SbV to SbIII was reported following an *in-vitro* study (2011). Moreover, in clinical isolates of resistance parasites many genes have been identified suggesting the various factors responsible for drug resistance (Singh and Dhiman, 2003, Decuypere et al., 2005, Choudhury et al., 2008). The rise of drug resistance has a devastating outcome in terms of treatment failure, and understanding its causes, spread, and impact will help us manage the risks it imposes. Molecular mechanisms causing resistance to anti-leishmanials (Ponte-Sucre et al., 2017) has been deciphered along with the urgent need to consolidate strategies to monitor drug efficacy, epidemiological surveillance, and local policies. These strategies will act as a successful guide and help in further research and prevention approaches in terms of better and efficient drug discovery.

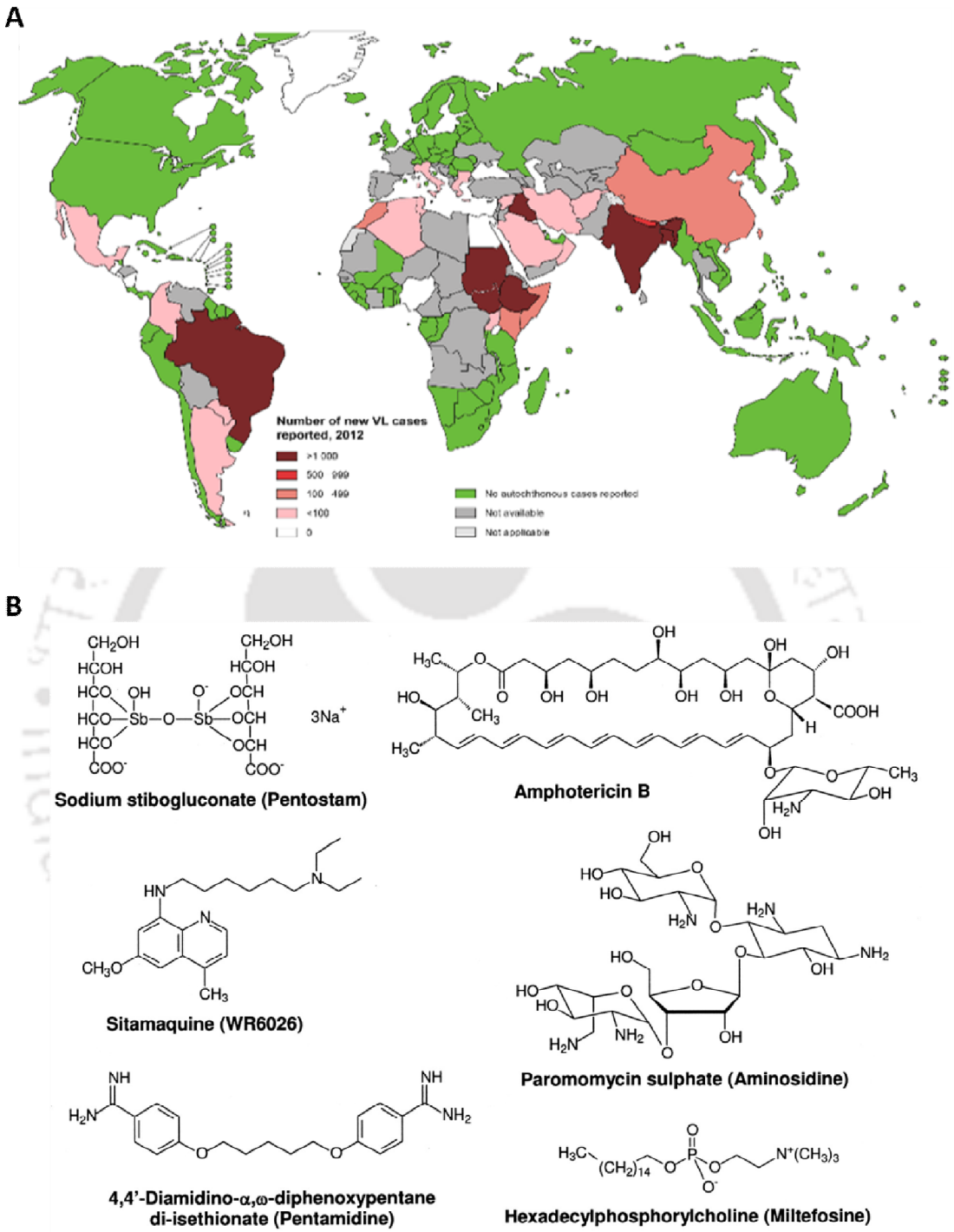

Figure 1.4: (A) According to WHO 2013 report, new number of VL cases has been reported worldwide [Adapted from http://www.stopleishmania.org/leishmaniosis-humans.php], (B) Current drugs that are used for treatment of leishmaniasis. Among the commercially available drugs such as pentavalent antimonials, amphotericin B, miltefosine, paromomycin, pentamidine and sitamaquine, pentavalent antimonials were first drugs used against leishmaniasis and the latest and most successful drug is amphotericin B. Theses available drugs suffer severe drawbacks in terms of cost, efficacy, administration mode and toxicity

1.7 Vaccines against Leishmaniasis

Despite different *Leishmania* species causing a range of clinical manifestations, genomic analysis indicates a large degree of sequence homology between species, suggesting it may be possible to generate broadly effective vaccines against different clinical diseases. Vaccination is the most cost-effective way of controlling infectious diseases. The success of vaccine development depends upon under-standing the immuno-biology of pathogen/host interactions, selection of appropriate vaccine candidates and choosing the right adjuvant or delivery vehicle. In addition, the vaccine must be able to generate long-lasting immunity so that the transition from preclinical testing to human trials can be made. Till date, numerous attempts have been made to develop a successful vaccine candidate against Leishmaniasis as a prophylactic measure. More than 12 million peoples are affected worldwide by these protozoa's, increasing the risk of infection every day. The various complications and side effects with recent chemotherapies have urged the scientific community to shift toward vaccine development but unfortunately no vaccines are available in the market against any form of *Leishmania* infection till now. Host – pathogen interactions are the key areas to successfully understand the immunological paradigm between the host and the parasite. Out of various categories of vaccines like live attenuated, killed parasite vaccine, leishmania fractionated vaccine, etc., recombinant vaccines are the primary focus. Recombinant vaccines are the protein vaccines produced by genetically engineered cells to produce antigenic proteins. The first candidate to reach phases I and II clinical trials was LEISH-F1 from Infectious Disease Research Institute, Seattle, WA (Beaumier et al., 2013). LEISH-F1, is a fusion protein made up of three polypeptides in tandem, formulated with monophosphoryl lipid A-stable emulsion (MPL-SE). The immunogenicity of this vaccine were further assessed in animal models like hamsters and showed efficient reduction of parasitic loads. Initially vaccine development against Leishmaniasis was started with first generation vaccines which includes live attenuated parasite and killed parasites. Gradually second and third generation vaccine came into existence. In spite of various problems, the quest for safe and practical vaccine is becoming possible with the help of newer technological advances and developments in the field of vaccinology, which evolved from whole live attenuated parasites to the use of defined antigens such as LEISH-F1. Today, DNA vaccines and chimeric vaccines are also the hotspots for research for the development

of vaccine candidates against Leishmaniasis (Dumonteil, 2007, Fiers et al., 2009). Some of the vaccine candidates' currently undergoing clinical trials against visceral leishmaniasis have been presented in Table 1.2.

1.7.1 First Generation Vaccines

First-generation vaccines include both killed and live-attenuated parasites. These vaccines were based on the practice of leishmanization (common practice in Israel and Iran during 1970 – 1980) whereby individuals are inoculated with parasites in a hidden body location, to protect against the formation of cutaneous lesions in visible locations. Clinical trials of first-generation vaccines were conducted in humans, focusing mainly on cutaneous leishmaniasis (Noazin et al., 2009). Live-attenuated or killed whole parasite vaccines are advantageous due to the presence of many different immunogenic antigens, unlike defined protein or DNA vaccines. Live-attenuated vaccines persist longer in the host and are especially important in the case of leishmaniasis since antigen persistence is involved in vaccine efficacy. Side-effects of first generation vaccines don't pose any serious threats to the treatment. In case of cutaneous leishmaniasis, mixing killed *L. major* with live *L. major* was associated with delayed lesion development. Addition of CpG oligodeoxynucleotides to live *L. major* was also associated with lower parasite burden and significantly smaller lesion size, most likely due to increased IFN-γ production (Badiee et al., 2008). These mitigation steps make leishmanization more acceptable. Use of live-attenuated vaccines is restricted to HIV – positive patients since persistent parasites could lead to disease. Thus, defined protein or DNA vaccines may represent the only suitable option for these patients.

1.7.2 Second and Third Generation Vaccines

Second- and third-generation vaccines are composed of parasite fractions or recombinant antigens, either delivered directly or by DNA vaccination. A large number of antigens have been investigated mainly in mice models, either singly or in combination. Defined antigens used for protein or DNA immunization include for instance gp29, A2, gp63, LACK, cysteine peptidases, histone proteins. Among them, gp29 is important in inducing splenic macrophages to release nitric oxide (NO) and reactive oxygen species (ROS) in appreciable amounts that resulted in effective parasite clearance from macrophages (Paul et al., 2012)

whereas LACK antigen is a key target of the immune response in susceptible BALB/c mice and remains a viable vaccine candidate for human leishmaniasis (Kelly et al., 2003). DNA vaccines are often very immunogenic and stable, making them suitable for tropical regions due to enhanced humoral and cell mediated immune responses against leishmaniasis (Nagill and Kaur, 2011). Till date, two vaccine candidates have been in clinical trials which include Leish-F1 (previously known as Leish 111f) and Leish-F3. Leish-F1 is a fusion of the thiol-specific-antioxidant antigen (TSA), *L. major* stress-inducible 1 (LmSTI1), and *Leishmania* elongation initiation factor (LeIF) adjuvanted with MPL-SE (Modabber, 2010). On the other hand, Leish-F3 is a fusion peptide of nonspecific nucleoside hydrolase (NH) and sterol 24-c-methyltransferase adjuvanted with glucopyranosyl lipid A-stable oil-in-water nanoemulsion (GLA-SE) (Coler et al., 2015). Both of them are safe as compared to other live-attenuated vaccines and also standardization procedure is much simpler. But the heightened immune response generated by live-attenuated vaccines is being remedied in case of second and third generation vaccines by the use of adjuvant. Finally, second- and third-generation vaccines are more complicated to produce and require more sophisticated equipment to generate them than first-generation vaccines, making them a more expensive but safe option.

1.7.3 Peptide Vaccines

Amino acid sequences in proteins are currently being used as vaccines to elicit the immune response. Thus synthetic polyvalent peptide vaccines have been developed. Epitope interactions and epitope mapping have been utilized for the development of such vaccines. There are many epitopes from gp63 that have been identified which induces Th1 response and has established gp63 as a potential vaccine candidate. In a similar way, 30 peptides from kinetoplast membrane protein 11 (KMP 11) protein were analyzed for their ability to induce IFN-γ production by CD8+T cells (Basu et al., 2005). Enzymes involved in redox metabolism of the parasite have always been targeted as drug candidates, but their use as vaccine candidates has been shown in case of enzymes like pVAXγGCS (gamma-glutamyl cysteine synthetase) and UBQ-ORFF which also elicited IL-12 and IFN-γ production indicating Th1 response (Carter et al., 2007). Recently, a hypothetical protein (LiHyp1) elicited IFN-γ production by CD4+ T cells via IL-12 has also been shown (Martins et al., 2013).

Table 1.2: Some vaccine candidates against visceral leishmaniasis in clinical trials

Vaccine Details	Trials	Disease	Volunteer	Reference
Leishmune or LeishTec	Field Trial	Zoonotic Canine Visceral leishmaniasis	Dogs	*(de Souza Testasicca et al., 2014)*
Leish F1 + MPL-SE vaccine	Clinical Trial	Visceral leishmaniasis	Human Volunteers	*(Chakravarty et al., 2011)*
Leish-111f + MPL-SE	Open trial and blind trial	Canine Visceral leishmaniasis	Dogs	*(Trigo et al., 2010)*
Alum precipitated autoclaved *L. major* + BCG + SSG	Randomized double blind trial	Post kala-azar dermal leishmaniasis (PKDL)	Human	*(Musa et al., 2008)*
Autoclaved *L. major* with BCG	Trial in healthy Sudanese volunteer	Visceral leishmaniasis	Human	*(Satti et al., 2001)*

1.8 Innate Immune System

The innate immune system is the fundamental and first line of defense against any invading pathogens. They are the major contributors of acute inflammation induced by tissue damage or microbial infection. Innate immunity is comprised of a number of pattern recognition receptors (PRRs) recognizing conserved pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) (Akira et al., 2006). These PRRs activate the downstream signaling pathways that lead to the activation of innate immune response producing inflammatory cytokines, type I interferon (IFN) and other mediator molecules. These processes also prime and orchestrate antigen-specific adaptive immune responses along with triggering immediate host defensive responses such as inflammation. These responses are pivotal in clearance of infecting pathogens as well as crucial in heightening antigen-specific adaptive immune responses. Germ line encoded PRRs have the ability to sense extracellular and intracellular pathogens and are specific for various classes of molecules including proteins, lipids, carbohydrates and nucleic acids (Akira et al., 2006, Jang et al., 2015). PAMPs are molecular patterns specific to invading pathogens while DAMPs are host cell-danger signals elicited by pathogen assault. Currently, four different classes of PRR

families have been identified which include trans-membrane proteins such as the Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs) and Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) (Kawasaki et al., 2011). These PRRs are mostly expressed in macrophages (Mφ), dendritic cells (DCs) and other immune cells along with few non – professional immune cells. The inflammatory response triggered by activation of PRRs is mainly orchestrated by proinflammatory cytokines like interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF). These cytokines regulate the cell death of inflammatory tissues, recruit blood cells to inflamed tissues, modify vascular endothelial permeability, and induce the production of acute-phase proteins. Figure 1.5 shows the timeline of innate and adaptive immunity and how innate immunity provides the initial defense against the pathogens.

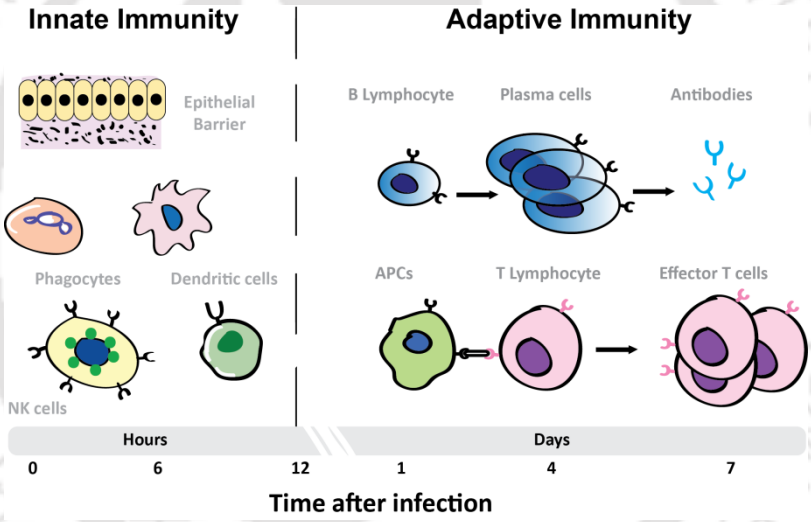

Figure 1.5: Innate and adaptive immunity time line. The mechanisms of innate immunity provide the initial defense against infections. Adaptive immune responses develop later and require the activation of lymphocytes. The kinetics of the innate and adaptive immune responses is approximations and may vary in different infections [Adapted & modified from https://www.creative-diagnostics.com/innate-and-adaptive-immunity.htm]

1.8.1 Toll-like receptors (TLRs)

TLRs are evolutionarily conserved from the worm *Caenorhabditis elegans* to mammals and was initially identified as a gene product essential for the development of embryonic dorso-ventral polarity in *Drosophila* (Hoffmann, 2003). Toll-like receptors (TLRs) play very significant roles in the innate immune system by recognizing PAMPs derived from various

microbes, signaling through the recruitment of specific adaptor molecules, leading to activation of the transcription factors NFκβ and interferon regulatory factors (IRFs) (Honda and Taniguchi, 2006), which dictate the outcome of innate immune responses. The precise mechanisms underlying TLR signaling have been clarified within last decade by various approaches involving genetic, structural, cell biological, biochemical, and bioinformatics studies. TLR signaling appears to be divergent and to play important roles in many aspects of the innate immune responses to given pathogens. Among different PRRs, TLRs were the first to be identified, and are the best characterized. The TLR family comprises 10 members (TLR1–TLR10) in human and 12 (TLR1–TLR9, TLR11–TLR13) in mouse. Few of them localize on the cell surface (like TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10) and some on the surface of intracellular compartments such as endosome, endoplasmic reticulum (ER), lysosome, or endolysosome (like TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13) (Figure 1.6A). All TLRs are synthesized in the ER, traffic to the Golgi, and are recruited to the cell surface or to intracellular compartments such as endosome. They recognize distinct or overlapping PAMPs such as lipid, lipoprotein, protein, and nucleic acid (Akira et al., 2006) (Figure 1.6B) and are mostly expressed in innate immune cells such as DCs and macrophages as well as non-immune cells such as fibroblast cells and epithelial cells (Qian and Cao, 2013).

Each TLR is composed of an outer domain (ectodomain) with leucine-rich repeats (LRRs) that mediate PAMPs recognition, an intermediate transmembrane domain, and an intracellular cytoplasmic Toll/IL-1 receptor (TIR) domain that initiates downstream signaling. TLRs interact with their respective PAMPs or DAMPs as a homo- or heterodimer via horse-shoe structure of ectodomain (Botos et al., 2011, O'Neill et al., 2013). Upon PAMPs and DAMPs recognition, TLRs recruit TIR domain-containing adaptor proteins such as myeloid differentiation factor 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF), which initiate signal transduction pathways culminating the activation of NFκβ, IRFs, or mitogen activated protein kinases (MAPK) to elicit a potent pro-inflammatory milieu (Akira and Takeda, 2004, O'Neill et al., 2013) (Figure 1.7). Recent studies have revealed that proper cellular localization of TLRs is important in the regulation of the signaling and regulate the expression of chemokines, cytokines and type I IFNs that ultimately protect the host from microbial infection.

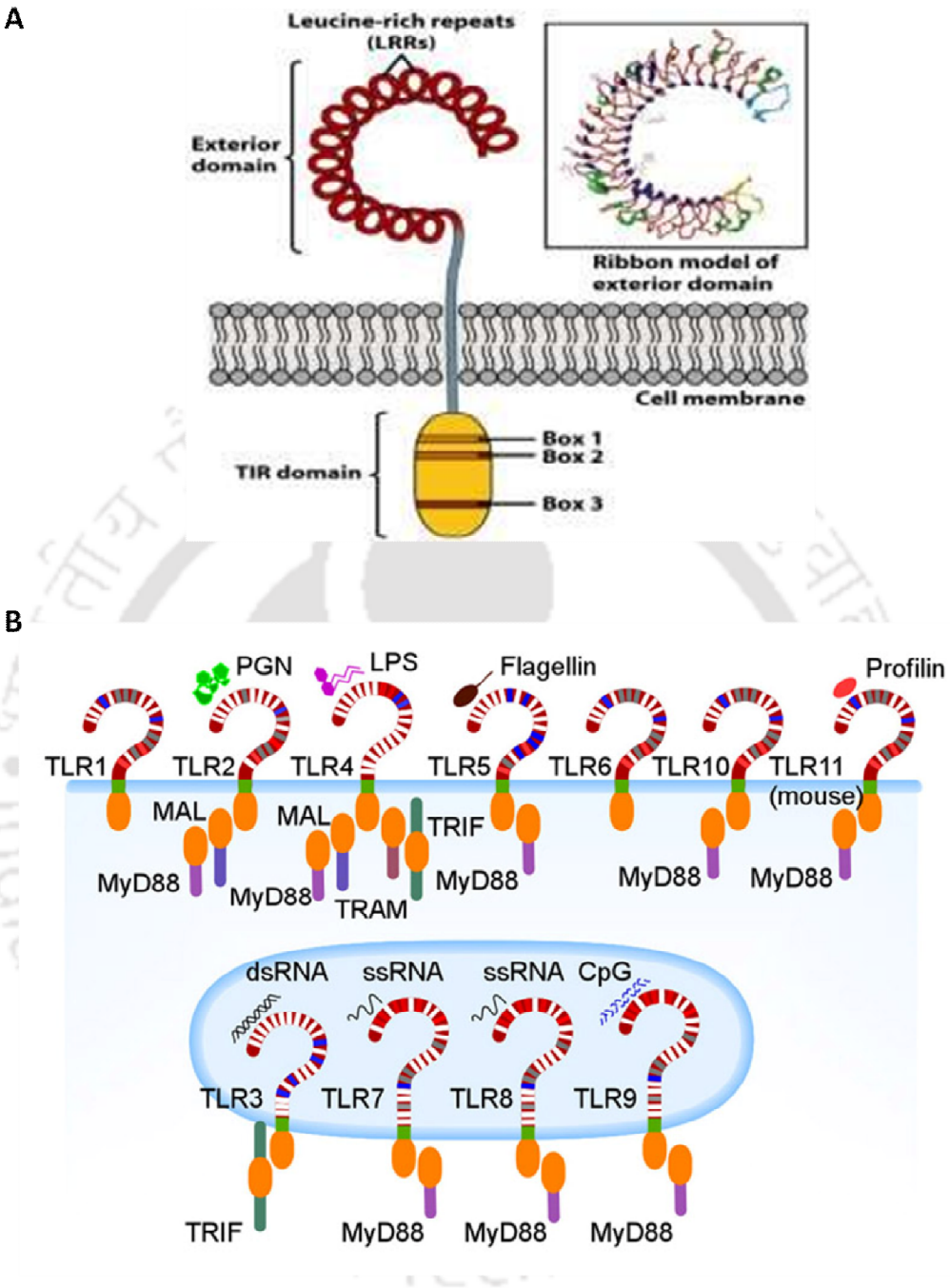

Figure 1.6: Toll-like receptors (A) Structure of TLR showing the exterior LRR domain, transmembrane domain and cytosolic TIR domain [Adapted from *Kuby Immunology 7th Edition* book], (B) Different TLRs location has been shown along with their specific recognition ligands. For example, TLR4 homodimers, present on the plasma membrane, recognize lipopolysaccharides (LPS) from Gram-negative bacteria. TLR2, along with either TLR6 or TLR1 recognize lipopeptides from Mycoplasma or mycobacteria. Interestingly, nucleic acids sensing TLRs, (TLR3, TLR7, TLR8 and TLR9) reside in intracellular membranes as opposed to plasma membrane where most microbial cell wall components are recognized by other TLRs [Adapted from http://labs.mmg.pitt.edu/sarkar/Signaling.htm].

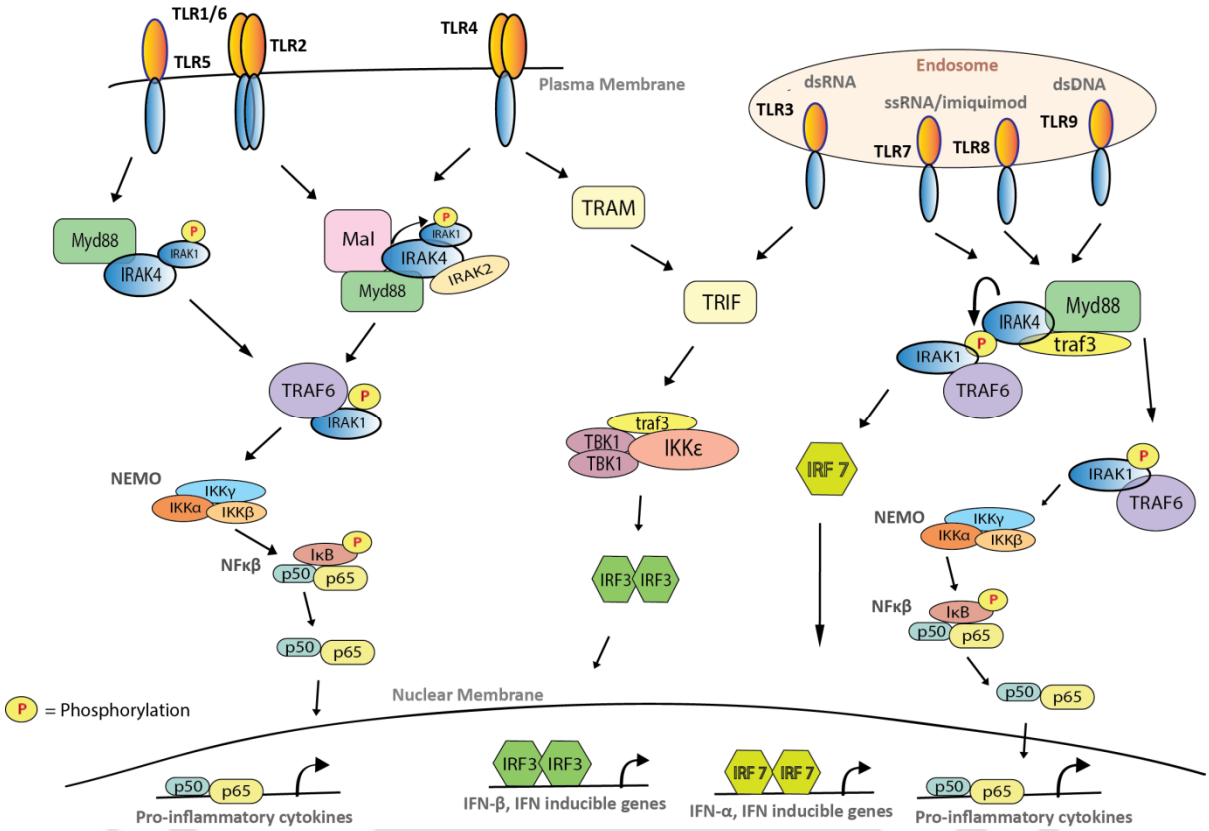

Figure 1.7: TLR signaling pathways. Upon PAMPs and DAMPs recognition, TLRs recruit TIR domain-containing adaptor proteins such as myeloid differentiation factor 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF), which initiate signal transduction pathways culminating the activation of NFκβ, IRFs, or mitogen activated protein kinases (MAPK) to elicit a potent pro-inflammatory milieu. TLR3 and TLR4 both use TRIF to activate the non-canonical IKKs TBK1 and IKK, resulting in the dimerization and activation of IRF3 and the transcription of IFN and IFN-inducible genes [Adapted & modified from (Neill et al., 2009)].

1.8.2 NOD-like receptors (NLRs)

The NLR family of proteins has emerged recently to the PRR super family as important and necessary receptors in pathogen detection and innate immune activation, and much evidence has been provided to indicate and clarify the roles for the NLR family members in host inflammatory disorders. Nucleotide-binding and oligomerization domain containing receptors (NOD)-like receptors (NLRs) contain a central NACHT domain that facilitates oligomerization, and bear multiple leucine-rich repeats (LRRs) on their C-terminal for ligand sensing (Yeretssian, 2012). They have been shown to complement and synergize with TLR signals arising from pathogen recognition, and are not necessarily separate pathways (Fukata et al., 2009). Evolutionarily, NLR orthologs have been found in plants (R-genes / R-proteins)

too which are required for anti-pathogen responses (Ronald and Beutler, 2010). The NLR family activates NFκβ and MAPK-dependent responses and also signals through pro-inflammatory caspases to activate pro-inflammatory cytokines which is termed as inflammasome-forming NLRs. To date, the NLR family is comprised of 23 human genes and 34 in the mouse.

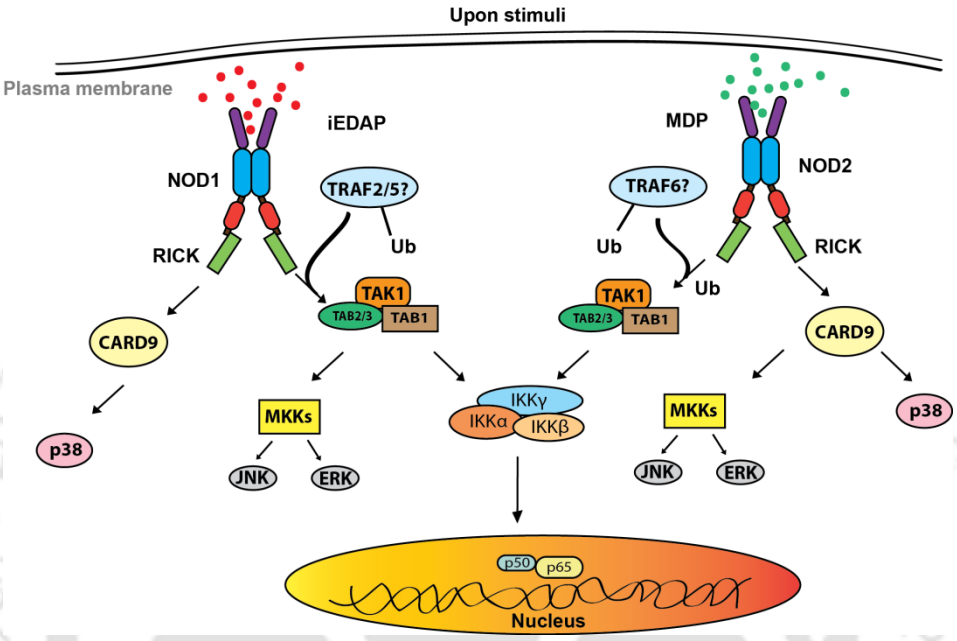

Figure 1.8: NOD1/NOD2 activation and signaling where they sense the peptidoglycan motifs in the cytoplasm and form a complex with RICK leading to the recruitment of TAK1. The interaction between RICK and NEMO ultimately results in the activation of IKKs and MKK by TAK1.

Although these are primarily expressed in immune cells, a few are expressed in other tissues, such as epithelial cells (Shaw et al., 2008). The NOD domain was first reported in apoptotic protease activating factor 1 (APAF1) and its nematode homolog CED-4, which are regulators of p53-dependent programmed cell death and developmental studies (Derry et al., 2001). Through database screening of APAF1/CED-4 homolog's, two NOD-containing molecules were discovered namely NOD1 (caspase recruitment domain 4, CARD4) and NOD2 (CARD15) (Shaw et al., 2008). These NOD1 and NOD2 are the most studied and characterized members of the NLR family (Inohara et al., 2001, Ogura et al., 2001). NOD1 is ubiquitously expressed in almost all the cells, while NOD2 expression is restricted to monocytes, macrophages, dendritic cells, and intestinal Paneth cells (Inohara et al., 2005).

Subsequent analyses and research shows different activation signals for both the NLRs. NOD1 is triggered by γ-d-glutamyl-meso-diaminopimelic acid (meso-DAP) which is unique to peptidoglycan (PGN) structures from all Gram-negative bacteria and certain Gram-positive bacteria, including the genus *Bacillus* and *Listeria* (Chamaillard et al., 2003) whereas NOD2 is activated by muramyl dipeptide (MDP), a peptidoglycan motif present in all Gram-positive and Gram-negative bacteria (Girardin et al., 2003). Both undergo conformational changes and self-oligomerization upon ligand recognition and thus activation of the serine – threonine kinase RICK (RIP2), which is essential for the activation of NFκβ and MAPKs. They induce similar K63-linked ubiquitination of RICK for NFκβ activation but they differ in utilizing the E3 ligases. TNF receptor associated factor 6 (TRAF6) is preferentially utilized by NOD2 signaling whereas TRAF2 and TRAF5 were shown to be important for NOD1-mediated signaling. Nevertheless, NOD1 and NOD2-mediated activation of NFκβ results in the upregulated transcription and production of inflammatory mediators (Pashenkov et al., 2018) (Figure 1.8). NLR proteins are sub-classified by the differences in their varying N-terminal domains with largest group shared by an N-terminal PYD, termed NLRP, and contains proteins such as hNLRP1, NLRP3, NLRP6, NLRP7, and NLRP12. There is another group expressing an N-terminal CARD domain which is termed as NLRC containing the proteins NOD1 and NOD2 as well as NLRC4. Other NLR families have emerged containing a baculovirus inhibitor of apoptosis repeat (BIR) domain such as NAIP5 (apoptosis inhibitory protein 5). Different types of stimuli signal inflammasome activation by recruiting various NLRs in order to initiate a cascade of proinflammatory response to get rid of invading pathogens.

1.8.3 C-type lectin receptors (CLRs)

C-type lectin receptors (CLRs) are one of the important PRRs involved in recognition and induction of adaptive immunity to pathogens. They are reported to play an important role in viral infections as they efficiently interact with viruses. However, it has become clear that deadly viruses evade the role of CLRs to escape antiviral immunity and promote infection. In particular, viruses mostly target CLRs to suppress or modulate type I interferons that play a central role in the innate and adaptive defense against viruses. CLRs comprises of a large family of receptors that bind to carbohydrates moieties in a calcium-dependent manner to

independently induce immunity or provide protective signals via crosstalk to modulate responses that are triggered by other PRRs (Zelensky and Gready, 2005). The lectin activity of these receptors is mediated by conserved carbohydrate-recognition domains (CRDs). Depending upon their molecular structure, two groups of membrane-bound CLRs and a group of soluble CLRs have been identified. DEC-205 and the macrophage mannose receptor (MMR) are type I transmembrane proteins containing several CRDs or CRD-like domains which are important in antigen uptake. Type II transmembrane CLRs typically carry a single CRD domain and include Dectin-1, Dectin-2, macrophage-inducible C-type lectin (Mincle), the dendritic cell-specific ICAM3-grabbing nonintegrin (DC-SIGN), and DC NK lectin group receptor-1 (DNGR-1). These receptors are involved in fungal ligand recognition and thereby modulate the innate immune response (Sancho and Sousa, 2012). Soluble CLRs include mannose binding lectin (MBL), an oligermeric protein that binds an array of carbohydrate patterns on pathogen surfaces (Takahashi and Ezekowitz, 2005). CLRs are expressed mostly in macrophages and dendritic cells (DCs), which phagocytose microbes for the purposes of clearance and antigen presentation to T-lymphocytes. However, there are many informations still remains to be understood about CLRs in general, their ligands and and cooperation with other molecules. As the immune system is in a never-ending arms race with viruses, many viruses have devised strategies to evade recognition and antiviral immune responses to successfully infect the host.

1.8.4 RIG-like receptors (RLRs)

RIG-like receptors (RLRs) constitute a family of cytoplasmic RNA helicases that are critical for host antiviral responses. To date, three receptors have been identified, including retinoic acid-inducible receptor, RIG-I, melanoma differentiation-associated factor 5 (MDA5), and laboratory of genetics and physiology 2 (LPG2) (Loo and Gale, 2011). RIG-I and MDA-5 contain a DExD/H box RNA helicase and two caspase recruiting domain (CARD)-like domains. The helicase domain interacts with dsRNA, whereas the CARD domains are required to relay the signal. These receptors detect viral or self-RNA in the cytoplasm to initiate a signaling cascade involving type 1 IFN and downstream innate immune genes, ultimately controlling an infection. Importantly, they are also involved in signaling crosstalk networks with TLRs and other factors to induce innate immunity and further translate it into

adaptive immune response (Kawai and Akira, 2009). RLR regulation occurs at a variety of levels ranging from autoregulation to ligand and cofactor interactions and posttranslational modifications (Sun et al., 2011, Kubota et al., 2008). Various studies have reported that RIG-I is associated with recognition of the hepaciviruses and members of the *Paramyxoviridae*, *Rhabdoviridae*, and *Orthomyxoviridae* virus genera, whereas MDA5 is associated with the detection of members of the *Picornaviridae* (Kato et al., 2006). RLR signaling activation by ligand interaction serves to initiate the immune response to virus infection, so an increased understanding of the molecular features underlying these processes could offer new strategies to consider for immune and antiviral therapy by targeting the RLR pathway for the therapeutic control of virus infection and enhancement of the immune response, and strategies of immune suppression to control inflammation or specific autoimmune diseases.

1.9 Inflammasomes: A platform to sense pathogens

The term "inflammation" is often used during any pathological disturbances in our body and in response to any unwanted stimuli, a protective immune response is mounted by our innate immune system which is tightly regulated by the host. A well balanced inflammation can help us to get rid of any invading pathogens or else insufficient inflammation can lead to persistent infection while excessive inflammation can cause chronic or systemic inflammatory diseases (Takeuchi and Akira, 2010). Numerous advances have been made in the role and regulation of inflammasomes during pathogenic insults in the context of inflammation. The inflammasome is a multiprotein supramolecular complex that detects PAMPs and DAMPs, and activates the highly pro-inflammatory cytokines IL-1β and IL-18 (Schroder and Tschopp, 2010). So in simple terms, we can say that these inflammasomes are innate immune system receptors and sensors that regulate the activation of caspase-1 and induce inflammation in response to infectious microbes and molecules derived from host proteins. Caspases are cysteine proteases which are synthesized as inactive zymogens, and their potent cellular activities are tightly controlled by proteolytic activation that initiate or execute cellular programs, leading to inflammation or cell death. Caspases are categorized as either proinflammatory or proapoptotic, depending upon their participation in these cellular programs. The proinflammatory caspases are comprised of caspase-1, -11 and -12 in mouse and caspase-1, -4, and -5 in human. Of the proinflammatory caspases, caspase-1 is the most

fully characterized which was first identified and characterized as IL-1β converting enzymes (ICE/caspase 1) (Martinon and Tschopp, 2006) . Its catalytic activity is tightly regulated by inflammasome mediating caspase-1-dependent processing of cytokines such as IL-1β (Martinon et al., 2002). IL-1β is an important proinflammatory mediator which is tightly controlled by expression, maturation, and secretion; proinflammatory stimuli induce expression of the inactive IL-1β proform, but cytokine maturation and release are controlled by inflammasomes. Inflammasome components and activation mechanisms depend on the nature of the individual protein scaffolds and are assembled by self-oligomerizing scaffold proteins. Previous studies reported that most of them are formed with one or two NLR family members (Vanaja et al., 2015). There are various types of inflammasomes such as NLRP1, NLRP3, NLRP6, NLRC4, IPAF and AIM2 which play significant role in various inflammatory diseases. Most members of the NLR family have a tripartite structure with a C-terminal LRR domain, a central NACHT domain, and an N-terminal caspase recruitment and activation (CARD) or pyrin (PYD) domain with few exceptions (Kanneganti, 2010). Upon activation, the NLRs oligomerize through their NACHT domains, resulting in recruitment of the adaptor protein, ASC, through interaction between the PYD of ASC and the PYD of the NLR member (de Alba, 2009). Further, ASC recruits procaspase 1 into the complex via its CARD domain. The protein ASC is believed to be a molecular platform for protein–protein interactions during inflammasome assembly and a key mediator in apoptosis and inflammation. ASC interacts with cell death executioners (NLR members and caspase 1) through its two death domains (PYD and CARD) and oligomerizes into functional supramolecular assemblies to form a discrete foci, or “speck,” within the activated cell (Sharma and Kanneganti, 2016). Recruitment of procaspase-1 by ASC undergoes autocatalytic cleavage to produce the active subunits p10 and p20 (mature caspase 1). These active caspase 1 subunits are responsible for processing of pro-forms of IL-1β and IL-18 into their mature counterparts, and induce a specific form of inflammatory cell death termed “pyroptosis” (Fink and Cookson, 2005) (Figure 1.9). This novel form of cell death was first reported during infection with *Salmonella* and *Shigella* species (Boise and Collins, 2001, Sansonetti et al., 2000). Pyroptosis shares some characteristics of both apoptosis and necrosis but has its own unique signature hallmark of caspase 1 activation (Fink and Cookson, 2005) (Figure 1.10). Activated caspase-1 thus provides the host cell with dual defense mechanisms

through the release of mature cytokines (IL-1β and IL-18) and removal of the infected or damaged cell. Inflammasome assembly is thus a coordinated signaling event essential for mounting an appropriate immune response against pathogenic assaults. Depending upon the types of pathogens (like bacteria, fungi, viruses, parasites, etc), different types of inflammsomes come into play to initiate downstream signaling effects (Table 1.3). Although most studies have used *in-vitro* culture systems and reconstitution assays to analyze inflammasome activation, recent advances have allowed visualization of these processes *in-vivo* after an infectious insult (Sagoo et al., 2015). Dysregulation of inflammasomes is associated with a number of autoinflammatory syndromes and autoimmune diseases (Guo et al., 2015).

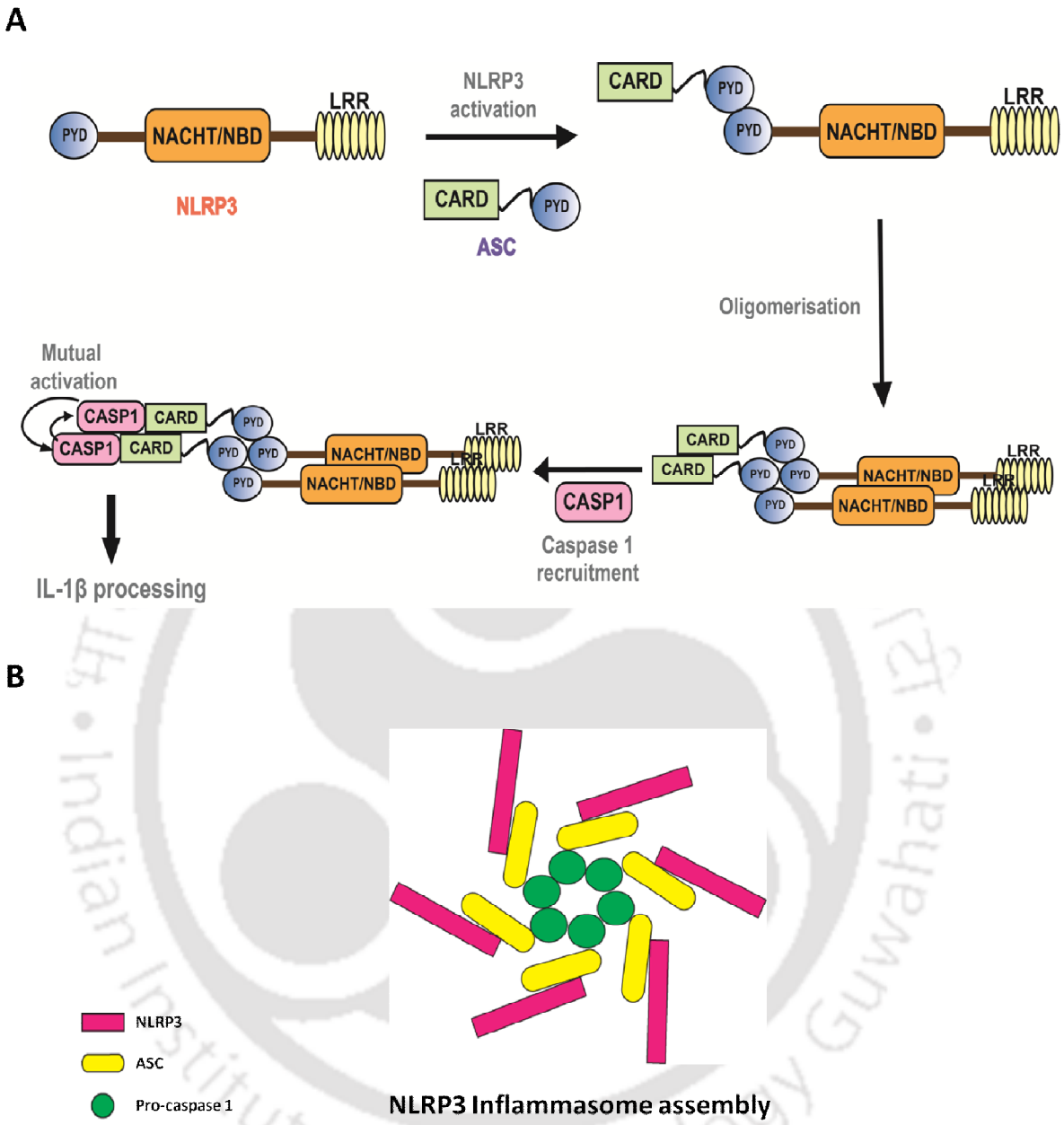

Figure 1.9: Inflammasome formation and assembly. (A) The NLRP3 oligomerize through their NACHT domains, resulting in recruitment of the adaptor protein, ASC, through interaction between the PYD of ASC and the PYD of the NLR member. ASC interacts with cell death executioners (NLR members and caspase 1) through its two death domains (PYD and CARD) and oligomerizes into functional supramolecular assemblies to form a discrete foci, or "speck," within the activated cell [Adapted & modified from (Abderrazak et al., 2015)], (B) A schematic diagram of full assembly of inflammasome with all the components ready for activation.

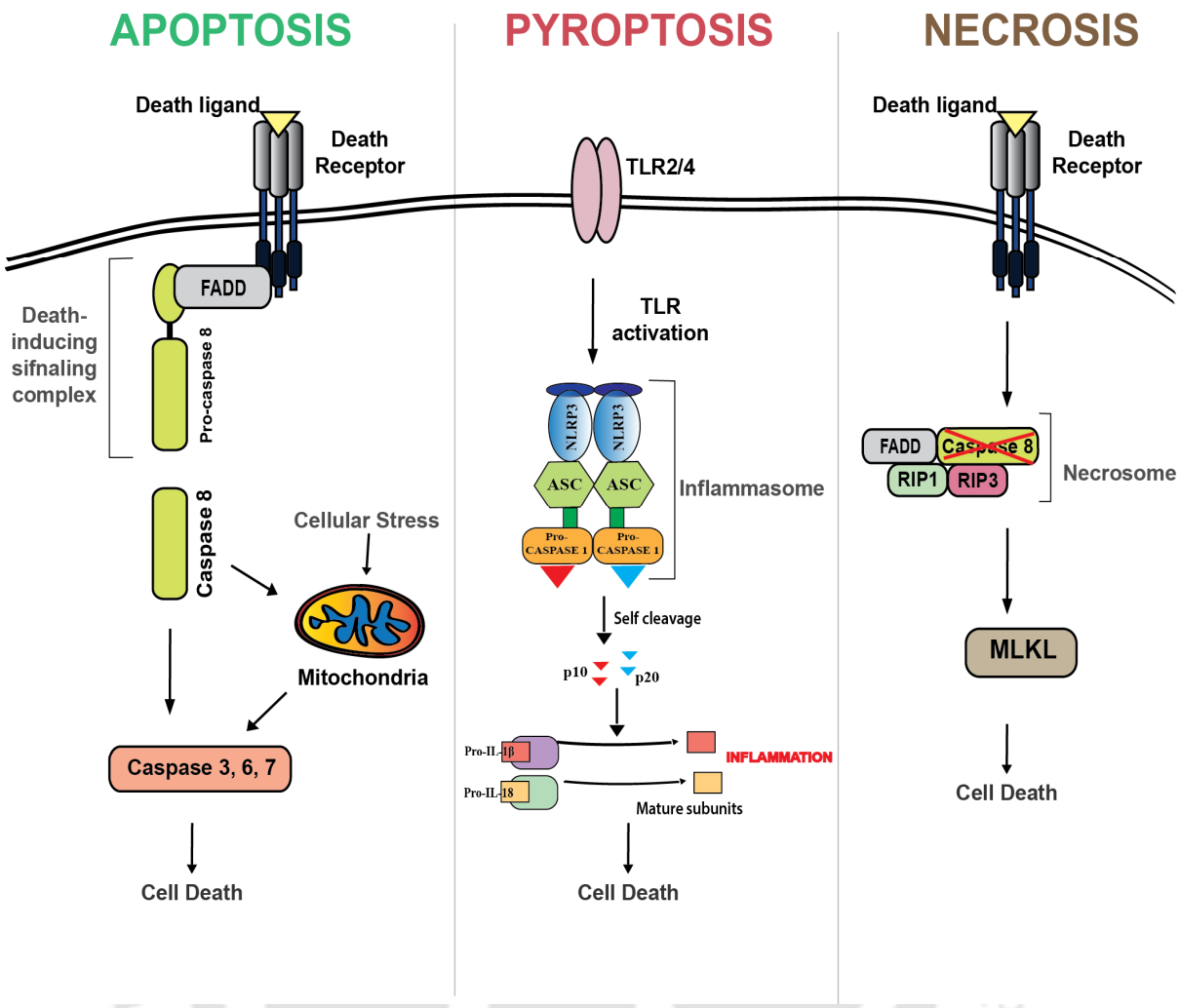

Figure 1.10: Different modes of cell death pathways showing the usage of different mediator molecules and caspases for the execution of cellular death. Pyroptosis has its own unique signature hallmark of caspase 1 activation along with few similar characteristics as compared to apoptosis and necroptosis (or necrosis) [Adapted and modified from (Hirsova and Gores, 2015)]

Table 1.3: Inflammasome-mediated pathogen recognition

S. No.	Pathogens	PRR	PAMPs, Activators	References
BACTERIA				
1.	*Mycobacterium tuberculosis*	NLRP3	ESAT-6, Ag85	*(Mishra et al., 2010, Koo et al., 2008)*
2.	*Listeria monocytogenes*	NLRC4	Flagellin	*(Kim et al., 2010)*
3.	*Legionella pneumophila*	NLRC4	Flagellin	*(Silveira and Zamboni, 2010)*
4.	*Fransicella tularensis*	AIM2	DNA	*(Rathinam et al., 2010)*
VIRUSES				
5.	*Influenza*	NLRP3	M2 ion channel, ssRNA	*(Ichinohe et al., 2010)*
6.	*Vescicular Stomatitis Virus*	RIG-I	5' – triphosphate on RNA	*(Poeck et al., 2009)*
FUNGI				
7.	*Candida albicans*	NLRP3	SYK, ROS	*(Rogiers et al., 2019)*
PARASITES				
8.	*Plasmodium*	NLRP3	Hemozoin	*(Labbé et al., 2010, Dostert et al., 2009)*
9.	*Leishmania donovani*	NLRP3, non-canonical inflammasome	LPG or gp63?	*(Saha et al., 2019, de Carvalho et al., 2019)*

1.9.1 NLRP3 inflammasome

The NLRP3 inflammasome is one of the fully characterized inflammasome and contain a NLRP3 protein scaffold, ASC adaptor molecule and caspase 1. This inflammasome has been reported to get activated during bacterial, fungal and viral infection and help the host to get rid of those pathogens (Schroder and Tschopp, 2010). Macrophages when stimulated with NLRP3 activators show no or very minimal level of activation but priming/pretreatment with microbial components like TLR ligands or TNF through NFκβ activation induce the activation to a large extent and considered as the first signal for NLRP3 inflammasome activation (Bauernfeind et al., 2009). Therefore, priming positively regulates the NLRP3 inflammasome at the trancriptional level through the induction of NLRP3 expression by using adaptor MyD88 and the downstream kinases IL-1 receptor-associated kinase 1 (IRAK1) and IRAK4 molecules (Bauernfeind et al., 2009). A prototypical example of such a priming event is the binding of bacterial lipopolysaccharide (LPS) to TLR4 and initiating NFκβ activation. In addition, recent study showed that priming also regulates NLRP3 activation at the post-transcriptional level by using adaptor TRIF and IRAK1 (Lin et al.,

2014). Next comes the second signal for NLRP3 inflammasome activation which are supported by various mechanisms including potassium efflux out of the cell, the generation of mitochondrial ROS, the translocation of NLRP3 to the mitochondria, the release of mitochondrial DNA or cardiolipin, increases in intracellular calcium and the release of cathepsins into the cytosol after lysosomal destabilization (Vanaja et al., 2015, Lee et al., 2012). The NLRP3 inflammasome can also be activated by host/self molecules, such as ATP, amyloid-β, cholesterol crystals and monosodium urate (MSU), all of which acts as second signal and are associated with cell death, tissue damage, or danger (Swanson et al., 2019). The overall downstream effect of priming and activation of NLRP3 inflammsome is induction of pyroptosis mediated by IL-1β signaling (Figure 1.11). A recent report has also highlighted the existence of an NLRP3 inflammasome pathway, mediated by caspase-11 (Kayagaki et al., 2011).

1.9.2 NLRC4 inflammasome

The NLRC4 inflammasome responds to a more limited set of stimuli in constrast to NLRP3 inflammasome activation. NLRC4 is mainly expressed in macrophages and hematopoietic tissues and cells, and its expression is not upregulated by TLR activation. It contains a CARD domain instead of PYD (necessary for ASC recruitment) that can interact directly with pro-caspase-1 (Figure 1.12). NLRC4 is very specific in activation by forming a complex with various neuronal apoptosis inhibitor proteins (NAIP) and NLRC4 activating ligands using a functional bacterial type-three or -four secretion system (T3SS/T4SS) or flagellin (Zhao et al., 2011, Yang et al., 2013). *Salmonella typhimurium* was one of the first bacterial species shown to activate caspase-1 via the NLRC4-inflammasome (Miao et al., 2010). This model of ligand binding and differentiation by NAIPs is interesting, however one NAIP has been identified in human till date which recognize the *Chromobacterium violaceum* T3SS needle protein CprI (Zhao et al., 2011). Increased release of signaling lipids like prostaglandins and leukotrienes causes rapid inflammation due to the activation of NLRC4 inflammasome by flagellin and are often called as “eicosanoid storm” (von Moltke et al., 2012). The response were not yet confirmed during *in-vivo* infection and its crosstalk with antimicrobial response is still warranted.

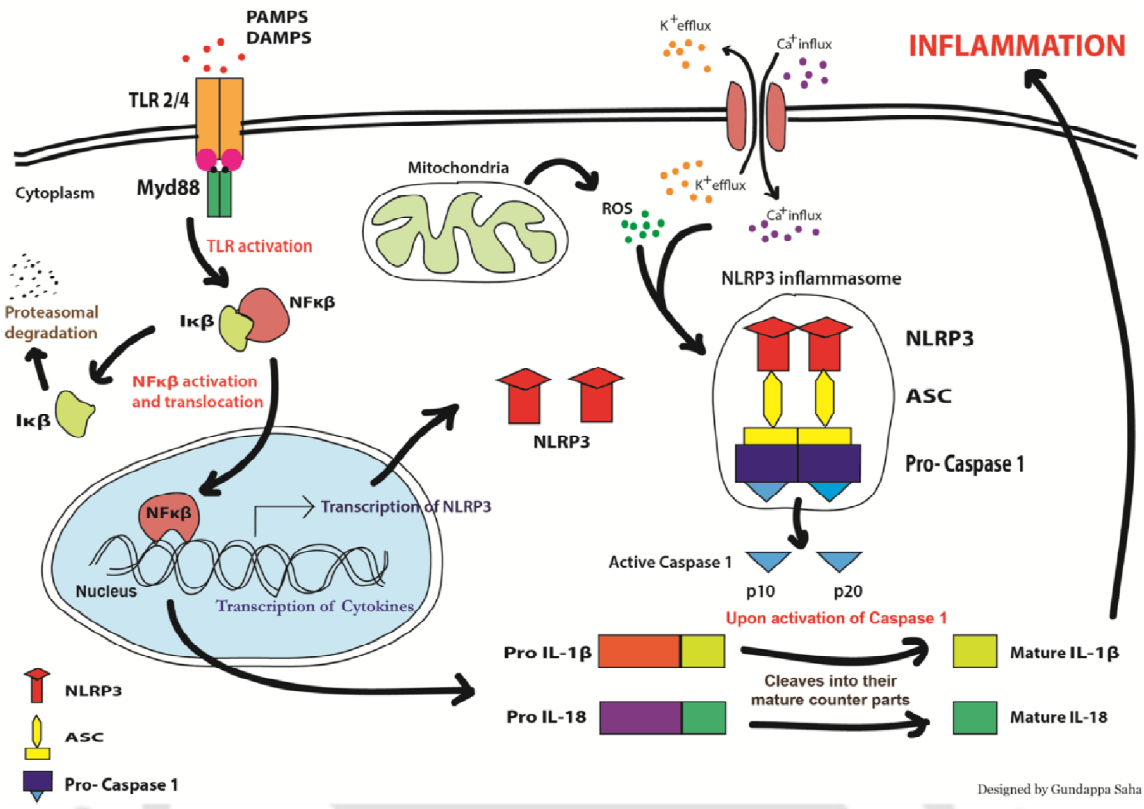

Figure 1.11: NLRP3 can be primed by a TLR-dependent,but transcription-independent signalling event. Various stimuli can activate NLRP3 which presumably forms multiprotein aggregates with the adaptor protein ASC and recruits caspase-1to process pro-IL-1β to an active and secretable form. A plausible downstream mechanism or signalling mediator that integrates these processes induced by a plethora of canonical NLRP3 stimuli including ROS production or Ca^{2+} release from the endoplasmatic reticulum were proposed but many of them were still not identified.

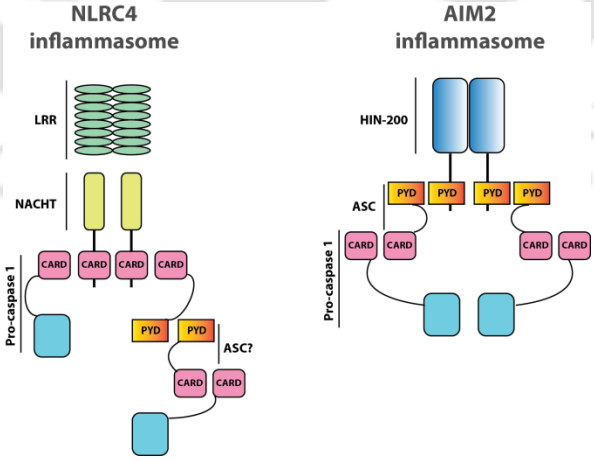

Figure 1.12: Oligomerization of NLRC4 and AIM2 inflammasome. NLRC4 directly interact with procaspase-1 but might require ASC for maximal activation. The AIM2 inflammasome is composed of AIM2, ASC, and caspase-1 where the PYD domain of AIM2 interacts with the PYD of ASC, so that the ASC CARD domain can recruit procaspase 1 to the complex [Adapted & modified from (Eitel et al., 2011)].

1.9.3 AIM2 inflammasome

AIM2 (Absent in Melanoma 2) is a non-NLR member, but capable of forming a caspase 1 containing inflammasome in the presence of bacteria and viruses (Hornung et al., 2009). It is a cytosolic receptor for double-stranded DNA and is known to form caspase-1 activating inflammasome (Rathinam et al., 2010). Unlike NLRs, the C-terminal DNA binding HIN-200 domain bind directly to its stimulus (cytosolic dsDNA) during any pathogenic infection along with an N-terminal pyrin domain (Hornung et al., 2009). Like NLRP3, AIM2 oligomerizes and nucleates ASC through PYD-PYD interactions and converts ASC to its prion form, leading to the development of long PYD-PYD ASC filaments (Cai et al., 2014) (Figure 1.12). The importance of AIM2 inflammasome to prevent bacteria replication intracellularly has been shown by AIM2 knock-out (KO) mice which fails to activate caspase 1 maturation and thus impaired pro-IL-1β and pro-IL-18 processing after *Listeria monocytogenes* and *Francisella tularensis* infection (Rathinam et al., 2010, Fernandes-Alnemri et al., 2010). In addition, IL-18 and the NK cell-dependent IFN-γ secretion were reduced and correlated to increased viral titers in mice deficient in AIM2 during murine cytomegalovirus (MCMV) infection (Rathinam et al., 2010). Recently, a noncanonical AIM2 inflammasome was shown to mediate protection against *Francisella novicida*, inducing the expression of the transcription factor IRF1 which in turn increases the expression of guanylate-binding proteins, killing the intracellular bacteria (Man et al., 2015).

1.10 Role of inflammasome in innate immunity

Inflammasome activation is a key event in the response to pathogens. Nowadays, scientists have delved inside to decipher its role in other disorders from metabolic to neurological to various infectious diseases. Previous reports and recent developments in the field of inflammasomes have greatly enhanced our understanding of mechanisms by which different inflammasomes are activated. Inflammasome is a double-edged sword where dysfunction and dysregulation can drive human inflammatory diseases, and thus more in-depth understanding about the balance between resolution of infection and excessive inflammation is warranted. Different inflammasome-forming NLRs are playing different roles varying from protection against colitis and bowel-inflammation to anti-pathogenic processes, and their co-ordinated relationship is very interesting to study. Some of the NLRs are involved in

common activation and regulatory pathways whereas some pathogens are capable of activating multiple inflammasomes, such as *Y. pestis*, *Salmonella*, and *L. monocytogenes* (Vladimer et al., 2012, Broz et al., 2010, Kim et al., 2010). In a nutshell, synergistic relationship between multiple NLRs or AIM2 and specific caspases during infections is likely to be the focus of future research. The shaping up of adaptive immune response is mostly dependent on innate immunity orchestrated by inflammasomes by initiating optimal response to vaccine adjuvants and thereby prodcuing inflammatory cytokines. Thus, not all inflammasome activation can be considered harmful, and the therapeutic inhibition of this pathway has to be balanced against its beneficial contribution. With the advent of more knowledge and understanding about the inflammasome mechanism of actions, many opportunities to create new therapies for patients with inflammatory diseases are expected to increase proportionately.

1.11 Role of inflammasomes during pathogenic infections

The host innate immune system plays a significant role in the recognition and elimination of invading microorganisms by expressing a range of receptors that act as microbial sensors. These receptors sense microorganisms and transduce signals that activate immune responses by adopting several strategies to recognize PAMPs and DAMPs (Skeldon and Saleh, 2011). Multiple inflammasomes have been characterized like NLRP1, NLRP3, NLRC4, NAIP5 and more recently described AIM2 and RIG-I inflammasomes that are stimulated by cytosolic DNA and viral RNA, respectively. Inflammasomes are mainly activated by a variety of signals and depending upon the associated PRRs, it may have redundant functions during infection (Skeldon and Saleh, 2011) (Figure 1.13). Dysregulation and/or variations in genes encoding inflammasome components have been linked to variety of genetic disorders with autoinflammatory syndromes in humans, including familial cold urticaria, Crohn's disease, Muckle–Wells syndrome, sepsis as well as susceptibility to infection with certain pathogens (Hoffman and Brydges, 2011). Recently, we have gained significant insights into the understanding of how the inflammasomes sense various microbes and respond by triggering signaling cascades.

Salmonella enterica is the best characterized intracellular bacterium studied in term of inflammasome activation by inducing caspase-1-dependent cell death in infected

macrophages (Hersh et al., 1999). NLRP3 deficient mice showed no caspase 1 activation and thereby impaired IL-1β processing was observed *in-vitro*. In addition, the importance of caspase 1 in resisting *S. typhimurium* replication *in-vivo* has also been justified using caspase 1 knock out mice (Mariathasan et al., 2006). Homologous rod proteins from *Shigella flexneri*, *Escherichia coli* and *Pseudomonas aeruginosa* are sensed by NLRC4 inflammasome, showcasing the conserved mechanism of caspase-1 activation found in a number of bacterial species (Sutterwala et al., 2007, Suzuki et al., 2007). *Legionella pneumophila* is another bacteria which survive and replicate inside the macrophage and studies have shown that caspase-1 and NLRC4, but not IL-1β are required to restrict *Legionella* growth in macrophages *in-vitro* and in the lungs of mice *in-vivo* (Zamboni et al., 2006). Macrophage pyroptosis has also been reported during *Legionella* infection through pore formation mediated by NLRC4 dependent caspase 1 activation (Silveira and Zamboni, 2010). Similarly, a putative Zn^{2+} metalloprotease (zmp1) of *Mycobacterium tuberculosis* is a virulence factor in mice and was found to inhibit NLRP3 inflammasome activation and IL-1β secretion in macrophages to evade the host inflammatory immune response (Master et al., 2008). Overall multiple inflammasomes have been implicated in response to intracellular and extracellular bacteria.

Viruses infect and replicate within host cells, utilizing the host cellular machinery to manufacture viral particles. With reference to an earlier study, it has been reported that influenza infected macrophages induces IL-1 production in mice (Hennet et al., 1992). In the context of inflammasome, NLRP3 has been identified to play critical role in influenza and Sendai virus infected macrophages *in-vitro* whereas conflicting results were observed in case of *in-vivo* studies. DNA viruses are also capable of activating caspase-1 through the AIM2 inflammasome is engaged in response to vaccinia virus and mouse cytomegalovirus (mCMV), but not herpes simplex virus type 1 (HSV-1) (Rathinam et al., 2010). Interestingly, infection with vesicular stomatitis virus (VSV – RNA virus) also triggers IL-1β secretion in a RIG-I-dependent manner where ASC interacts with RIG-I, activating the NF-κB and caspase-1 pro-inflammatory pathways (Poeck et al., 2009).

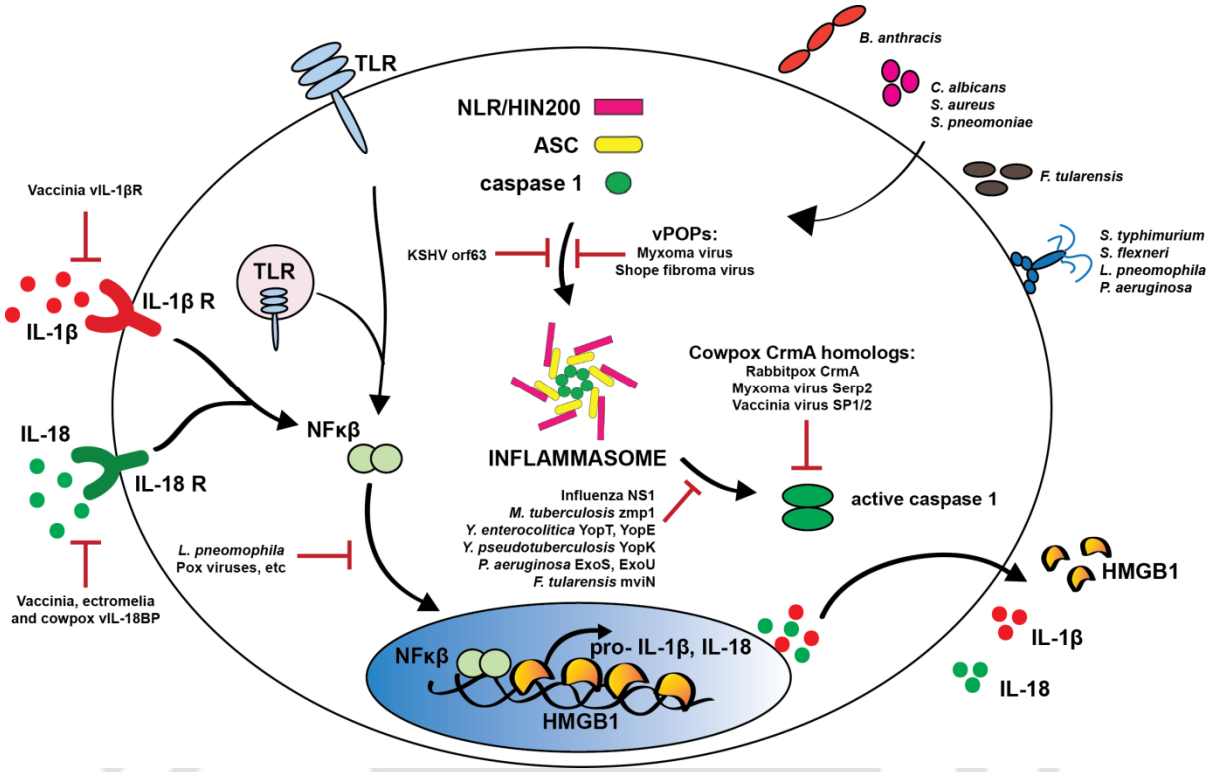

Figure 1.13: Alteration of inflammasome pathways by bacterial, fungal and viral effectors inducing the assembly of inflammasome complexes. These complexes drive the proximity-induced activation of caspase-1, resulting in extracellular release of IL-1β, IL-18, and induction of pyroptosis. These pathogens have evolved a number of mechanisms (shown in red) to interfere with inflammasome assembly and activity [Adapted & modified from (Lamkanfi and Dixit, 2011)].

Recent work in the field of parasitology has suggested that the NLRP3 inflammasome might play a role in the recognition of hemozoin (breakdown product of haemoglobin) during *Plasmodium* infection. This recognition was found to activate caspase-1 and thereby inducing IL-1β secretion *in-vitro*, in an ASC and NLRP3, but not NLRC4, dependent manner (Dostert et al., 2009). Moreover, enhanced inflammatory and immune response to the *Plasmodium* parasites was found in mice deficient in caspase-12, an inhibitor of caspase-1 and NFκβ, showing the susceptibility to cerebral malaria (Labbé et al., 2010). Some studies on the involvement of NLRP3 inflammasome in few species of *Leishmania* like in *L. amazonensis, L. major, L. mexicana, L. braziliensis* and *L. infantum chagasi* have been reported with in the IL-1β production without much mechanistic insight (Reiner et al., 1990, Gupta et al., 2013). This thesis work will shade light on the mechanistic details about how NLRP3 inflammasomes are getting modulated during *L. donovani* infection.

1.12 Immune responses during Leishmaniasis

Human infection with *Leishmania* results in diverse clinical and immunopathological situations. The capacity of the parasites to cause this wide range of disease manifestations depends upon their ability to evade the immune defense mechanisms by performing a well-tuned orchestra of host-parasite interactions inside the macrophages. The activation of the NLRP3 inflammasome during *L. amazonensis* (Lima-Junior DS. *et al.,* 2013) contradicts the inhibition of NLRP3 inflammasome during *L. mexicana* and *L. major* (Shio MT. *et al.*, 2015). Neutrophils are the first immune cells to internalize the parasites from the site of infection. These neutrophils secrete MIP-2 (macrophage inflammatory protein-2) which acts as a chemo attractant for the macrophages, which in turn phagocytose infected neutrophils, thus making a silent entry of the parasite into the host cells (Teixeira et al., 2006). Host immunity is the key determinant of the establishment and progression of the *Leishmania* infection in mammals. The promastigote form of the parasites execute a survival strategy against the host's immune system by inhibiting the secretion of pro-inflammatory cytokines (IL-12, IFN-γ, etc.) and up-regulating the level of anti-inflammatory cytokines (IL-10, IL-4, etc.). In a nutshell, the parasite polarizes the Th1:Th2 balance towards Th2 response leading to the progression of the disease (Nylén and Kumar, 2012) (Figure 1.14A). Innate immune response is the first line of defence against any invading pathogens including bacteria, virus, fungi, protozoa, etc. In addition, the pattern recognition receptors (PRRs) play a significant role in the recognition of pathogen including Toll-like receptors (TLRs) (Takeda and Akira, 2005) and Nod-like receptors (NLRs) (Wilmanski et al., 2008). Activation of the above receptors initiates an array of signaling network that culminates in phagocytes either to aggravate the disease by up regulation of Th2 immune response or to protect the host by up regulation of Th1 response.

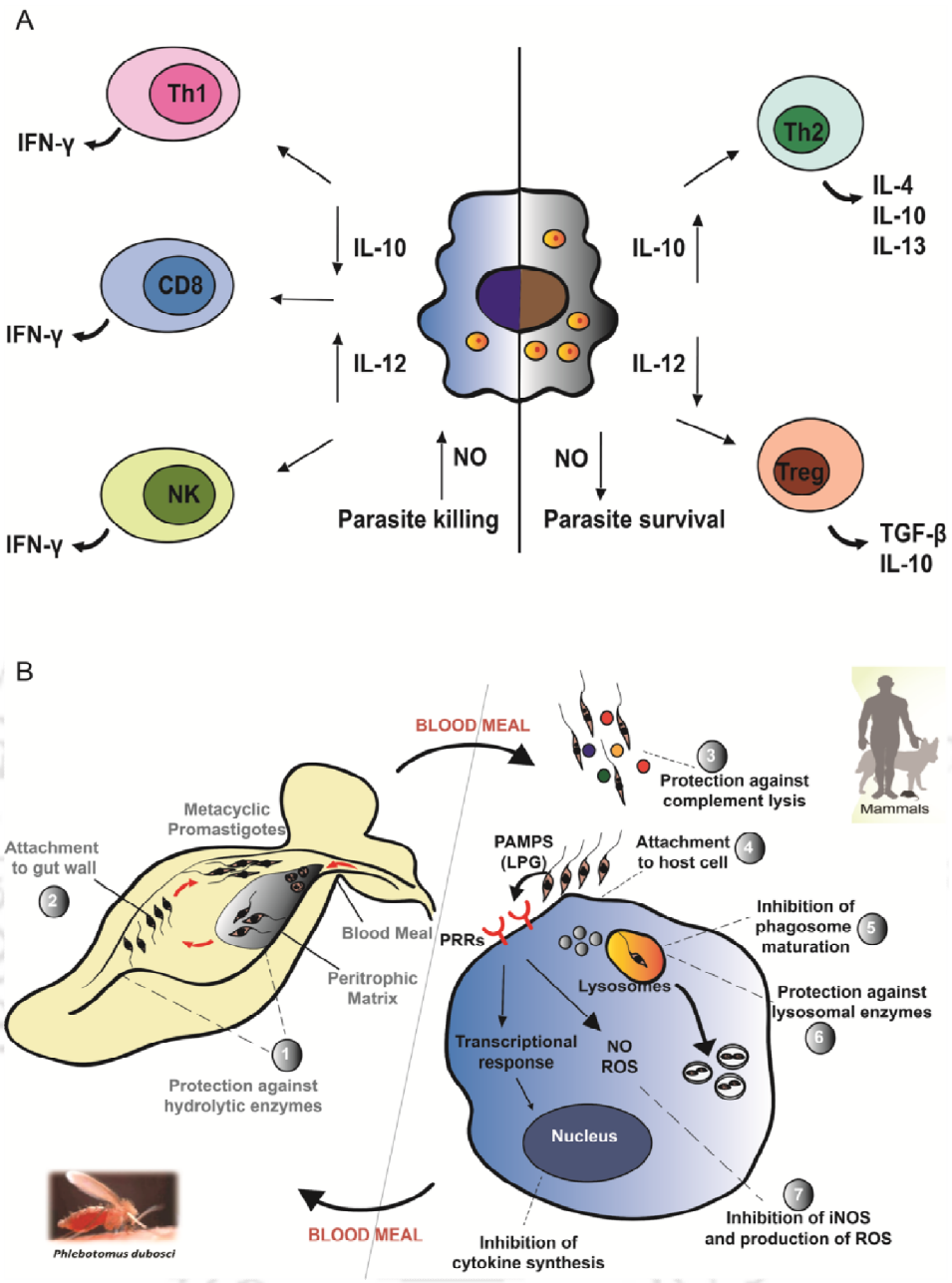

Figure 1.14: Immune response and immune evasion by *Leishmania* parasite. (A) Cytokine profile regulates the type of immune response during infection by balancing between IL-10 and IL-12 produced by macrophages. The whole Th1/Th2 dichotomy depends upon the immune response developed and thereby controls the parasitic burden [Adapted & modified from (Rodrigues et al., 2015)], (B) This schematic represents how the parasite evade the immune response of two host (sandfly and mammals). Inside sandfly, the parasites (1) gets protected against hydrolytic enzymes in the digestive tract of insect; (2) by attachment of promastigotes to the gut wall; while in the mammalian host (3) they escape the complement lysis; (4) gets attached to the cell membranes of neutrophils, dendritic cells and perhaps others; (5) inhibit phagosomal maturation; (6) protection against degradation by lysosomal enzymes; (7) impair the synthesis of nitrogen species and cytokines related to the control of infection and protection from ROS [Adapted & modified from (Franco et al., 2012)].

Increasingly, the inflammatory response to infection appears to be coupled with the induction of cell death as an important mediator of host defense mechanism (Fink and Cookson, 2005). Therefore, it is very crucial to understand different modalities of cell death to elucidate pathogenic mechanism for immunosuppression of the host during *Leishmania* infection. Thus there is an urgent need to decipher the novel immunosuppressive mechanism so as to design vaccine candidates against the parasites.

1.13 Immune evasion by *Leishmania* parasites

Within the mammalian host, *Leishmania* resides as amastigotes in phagocytic cells such as macrophages, dendritic cells (DCs) and neutrophils. The parasite binds to the complement protein, C3b which results in the uptake by the macrophages. These parasites have a very special surface glycoprotein gp63 which convert C3b into iC3b (inactive) evading the phagocytic clearance. After being engulfed, the *Leishmania* must endure harsh conditions inside the phagosomes like the oxidative burst used by the macrophages to kill foreign materials. This process consists of an attack by superoxide and hydroxyl radicals on the parasite and to evade the attack, *Leishmania* produces acid phosphatases on its surface which inhibits its burst. In addition to this, macrophages attempt to degrade the parasites with acidic enzymes but the *Leishmania* resists this attack through blocking a proton pump present on the surface and allowing the intracellular pH to be close to neutral. The lipophosphoglycan (LPG) present on the surface of the parasites plays an important role in inhibiting lysosomal enzymes. The parasites perform a complex host-parasite interaction inside the severe environment of phagolysosome and eventually evade this defense mechanism (Alexander and Russell, 1992). In addition to repressing the microbicidal activities of the host macrophage, *Leishmania* inhibits the ability of the host cell to display parasite antigens to other components of the immune system. Some studies have also shown that *Leishmania* inhibits antigen presentation by repressing major histocompatibility complex (MHC) class II gene expression, both basal and particularly following stimulation with IFN-γ (Almeida et al., 2003, Reiner et al., 1988). *Leishmania* also found to prevent the activation of an effective immune response by inhibiting production of a number of cytokines, particularly those involved in the inflammatory response (IL-1) and tumor necrosis factor alpha (TNF-α) or in T-lymphocyte activation (IL-12). *Leishmania* parasites can induce the production and/or

secretion of various immunosuppressive signaling molecules, such as arachidonic acid metabolites and the cytokines TGF-β and IL-10. These affect numerous different cell types, directly and indirectly, thus distorting the normal immune response and favoring parasite survival (Bogdan and Röllinghoff, 1998). The parasites have also evolved strategies to exploit host cell signaling regulatory mechanisms by distorting the balance between positive and negative influences. The most common pathways that have been studied in regard to *Leishmania* infection are Ca^{2+} and Protein Kinase C dependent pathways (Eilam et al., 1985, Olivier et al., 1992b), JAK/STAT and IFN-γ mediated pathways (Blanchette et al., 1999, Nandan and Reiner, 1995), MAP kinase pathway (Martiny et al., 1999), etc (Figure 1.14B). One of the most common mechanisms employed by the parasites to block these pathways is dephosphorylation of the tyrosine residues of the signaling protein molecule and thus inhibition of downstream cascade of events. It is therefore clear that *Leishmania* parasites are capable of modulating numerous macrophage functions in order to promote survival within the host cells.

CHAPTER 2

Leishmania donovani evades Caspase 1 dependent host defense mechanism during infection

2.1 Abstract

Numerous advances have been made in highlighting the regulation of inflammasomes and its involvement in innate immunity during any pathogenic infections. Inflammasome activation is a tightly regulated process in providing defense against pathogenic insults and thereby balancing the inflammatory response and resolution. Very few studies on the involvement of NLRP3 inflammasome have been reported in leishmanial infections with contradictory results and without much mechanistic insights. However, the role of NLRP3 inflammasome and its components has not been well deciphered in *Leishmania donovani* infection. Here we report for the first time a detailed mechanism and plausible impairment of caspase 1 activation during *L. donovani* infection leading to the survival of these parasites inside the host cells. Low mRNA expression of pro-caspase 1 and lack of caspase 1 maturation were observed after infection, hindering the processing of pro-IL-1β and pro-IL-18 into their mature counter parts. Further, siRNA mediated knock-down of caspase 1 in macrophage cells (THP-1) resulted in significantly higher parasitic burden validating the importance of caspase 1 in the host defense mechanism. Taken together, our data suggests that the parasite inhibits caspase 1 activation to evade the inflammatory nature of pyroptosis. The detailed understanding of this novel mechanism will play a vital role in designing future immunotherapeutics to combat Leishmaniasis.

2.2 Introduction

Leishmaniasis is a group of vector born neglected tropical disease caused by protozoan parasites of the genus *Leishmania* (Murray et al., 2005). A diverse clinical and immuno-pathological manifestation of *Leishmania* infection depends upon the ability of the parasite to evade the host immune response. After a silent entry into the host macrophage cells, the parasites executes a survival strategy to escape the surveillances posed by vast array of host immune cells like monocytes, macrophages, neutrophils, dendritic cells, antigen presenting cells (Gupta et al., 2013, Gurung and Kanneganti, 2015a). Neutrophils and macrophages are the major innate immune cells acting against *Leishmania* infection due to the presence of pattern recognition receptors (PRRs) on the cell surface including Toll-like receptors (Takeda and Akira, 2005) and Nod-like receptors (Wilmanski et al., 2008). The modulation of these receptors initiates an array of signaling network that culminates in phagocytes either to aggravate the disease by up-regulation of Th2 immune response or to protect the host by up-regulation of Th1 response in any parasitic infection. *Leishmania* parasite display immense adaptability to survive under harsh condition and resides as amastigotes in the host phagocytic cells such as macrophages, dendritic cells (DCs) and neutrophils (Gurung and Kanneganti, 2015a). There are various reports on the evasion of host immune response by the parasites for maintaining their replicative niche. They mostly target the cell signaling pathways of the host cells for evasion mechanism like Ca^{2+} and Protein Kinase C dependent pathways (Olivier et al., 1992b, Olivier et al., 1992a), JAK/STAT and IFN-γ mediated pathways (Nandan and Reiner, 1995, Blanchette et al., 1999), MAP kinase pathway (Nandan et al., 1999), etc. One of the most common mechanisms employed by the parasites to block these pathways is dephosphorylation of the tyrosine residues of the signaling protein molecule and thus inhibition of down-stream cascade of events. It is therefore clear that *Leishmania* parasites promote their survival within the host cells by tampering the activation of host immune response.

The inflammasome complex of the immune cells plays a crucial role in the host defense mechanism of various pathogenic infections. Amongst different inflammasomes discovered till date, NLRP3 is the most studied and characterized inflammasomes which consists of NLRP3 (scaffold), ASC adaptor molecule (PYCARD) and pro-caspase 1 (Schroder and Tschopp, 2010). This inflammasome gets activated upon whole pathogen interactions or

diverse pathogen associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs). The activated NLRP3 inflammasome is formed by oligomerisation of three components where the ASC molecule acts as an adaptor molecule to connect NLRP3 and pro-caspase 1 together. This assembly of NLRP3 inflammasome acts as a defense system inside the host cells to fight against various invading pathogens like virus, fungi, bacteria, protozoan, etc. by initiating the processing of proinflammatory cytokines via self – cleavage of pro-caspase 1 into their mature subunits (p10/p20) (Skeldon and Saleh, 2011, Zamboni and Lima-Junior, 2015). The downstream effect of caspase 1 maturation triggers the conversion of pro-inflammatory IL-1β and IL-18 into their mature forms causing inflammation. This process is also called "pyroptosis" (In Greek, *pyro* means fire or inflammation and *ptosis* means falling), an inflammatory form of cell death (Bergsbaken et al., 2009, Fink and Cookson, 2005). Unlike apoptosis, pyroptosis utilizes caspase 1 as a hallmark signature molecule resulting in inflammatory cell death which helps to get rid of various invading pathogens (Miao et al., 2011). The triggering of NLRP3 inflammasome gets substantiated by various activators including ATP, amyloid-β, silica crystals, asbestos, calcium influx, potassium efflux, etc. ATP actually triggers the P2X7 receptors (Dubyak, 2012) and increases the reactive oxygen species (ROS) whereas the amyloid-β, silica crystals, and asbestos leads to lysosomal degradation and ruptures releasing the lysosomal content to the cytosol that gets sensed by NLRP3 inflammasome (Halle et al., 2008). The intracellular ROS plays a pivotal role in favoring the formation of NLRP3 inflammasome followed by its activation and pyroptotic cell death during any pathogenic infection (M. et al., 2015). The inflammatory response to infection appears to be coupled with the induction of cell death pathway as an important mediator of host defense mechanism (Fink and Cookson, 2005). Therefore, it is crucial to understand different modalities of cell death to elucidate the pathogenic mechanism for immunosuppression of the host during *Leishmania* infection.

Plethora of reports, in relation to the altered inflammasome complex and their importance in infections like cholera, tuberculosis, typhoid, etc are well established. In this study, we conjecture that *L. donovani* infection disturbs the caspase 1 mediated activation of pyroptosis and its downstream effect as an immune evasion mechanism. Some studies on the involvement of NLRP3 inflammasome in other species of *Leishmania* like in *L. amazonensis*,

L. major, L. mexicana, L. braziliensis and *L. infantum chagasi* has been reported with conflicting data (Lima-Junior et al., 2013, Shio et al., 2015). The NLRP3 inflammasome gets activated during *L. amazonensis* (Lima-Junior et al., 2013) infection whereas on the contrary, *L. mexicana* and *L. major* (Shio et al., 2015) inhibits NLRP3 inflammasome. Recently, it has also been shown that during *L. major* infection, the NLRP3 inflammasome activation helps in recruiting more neutrophils cells to the site of infection and a Th2 biased immune response to promote disease progression (Charmoy et al., 2016, Gurung et al., 2015). Some preliminary reports of *L. donovani* and *L. tropica* infection show a decrease in the IL-1β production without much mechanistic insight (Reiner et al., 1990, Reiner, 1987). Our current work unravels the role of host caspase 1 in resisting the progression of *L. donovani* infection. The data supports that the parasites evades the inflammatory reaction of the host cells by circumventing the pyroptotic cell death pathway mediated by caspase 1.

2.3 Materials & Methods

2.3.1 Parasite, Cell Culture and Chemicals: *L. donovani* culture (BHU-1081 strain) was a kind gift from Prof. Shyam Sundar, Banaras Hindu University. Procedure for *L. donovani* culture has been optimized in our laboratory and followed as per protocol (Das et al., 2015, Das et al., 2013). In brief, *Leishmania* promastigotes are cultured at 28^{o}C in M199 liquid media supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Human macrophage cell lines (U937 and THP-1) were procured from National Centre for Cell Sciences, Pune and cultured at 37^{o}C and 5% CO_2 in Roswell Park Memorial Institute (RPMI-1640) media supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Caspase 1 substrate, Bacterial Lipopolysaccharides (LPS), Phorbol 12-myristate 13-acetate (PMA), Protease Inhibitor Cocktail, N-acetyl cysteine (NAC) were purchased from Sigma-Aldrich. The antibodies for α-tubulin, Caspase 1, IL-1β and IL-18 were purchased from Sigma Aldrich. The secondary anti-rabbit IgG antibody conjugated with HRP and Novex HRP chemiluminescent substrate reagent kit were purchased from Invitrogen. Rabbit anti-mouse IgG conjugated with HRP, mouse monoclonal β-actin and CM-H_2DCFDA dye were purchased from Thermo Scientific.

2.3.2 Preparation of adherent cells, lipopolysaccharide (LPS/ATP) treatment and infection of adherent macrophage: Monocytic suspension cell lines U937 and THP1 (~1 x

10^6 cells) in RPMI-1640 media were treated with 100ng/ml of phorbol 12-myristate 13-acetate (PMA) and incubated for 24 hr at 37°C, 5% CO_2 to make them adherent macrophages. *L. donovani* (BHU-1081 strain) was used to infect the adherent macrophage with the multiplication of infection (MOI) of 1:5 (macrophage: parasites). For positive control, adherent macrophage cells were treated with bacterial LPS (10µg/ml) followed by 5 mM of ATP stimulation for 30 minutes prior to any study. The control (without any treatment or infection), LPS/ATP treated (positive control) and infected macrophages were incubated at 37°C, 5% CO_2 for 24 hr for further studies.

2.3.3 Clinical PBMC sample isolation and processing: Blood samples of *L. donovani* infected patients have been isolated in the Kala Azar Medical Research Centre, Muzaffarpur, Bihar and PBMCs were processed followed by cDNA preparations in the Institute of Medical Science, Banaras Hindu University. Blood samples of healthy human volunteers were isolated in the institute's hospital of IIT Guwahati and PBMCs were processed in the Department of Biosciences & Bioengineering, IIT Guwahati as per reported protocol (Fuss et al., 2009). The viable cells (~1 x 10^6 cells) for all samples were grown for 24 hr in RPMI-1640 media at 37°C, 5% CO_2 in a 35 mm petri-dishes (Thermo-NUNC) followed by washing with 1x PBS to remove all non-adherent cells including lymphocytes and rare polymorphonuclear cells. The attached monocyte cells were grown for another 24 hr in fresh RPMI-1640 media at 37°C, 5% CO_2. This was followed by total RNA isolation using RNeasy Mini Kit (QIAGEN) protocol. All the clinical samples were isolated under the guidelines of the Ethics Committee of the respective institutes and also with prior consent from all the blood donors. Four random healthy volunteers were paired with four random infected patients' samples and gene expression analyses were done using Real Time PCR.

2.3.4 Gene expression analysis using Real Time PCR: Total RNA was isolated after 24 hr using RNeasy Mini Kit (QIAGEN) protocol followed by cDNA synthesis using ProtoScript® First Strand cDNA Synthesis Kit, (New England Biolab). The mRNA expression levels of *casp1, asc, nlrp3, nlrc4, nlrp6* were evaluated using qRT-PCR with their respective primers (Table 2.1). The *cyclophilin A* and *β-actin* genes were used as housekeeping controls to normalize the relative fluorescence signal of the target genes.

Table 2.1: Primer sequences used for Real Time PCR experiments (Chapter 2)

Primer Names	**Sequences (5' – 3')**
*nlrp3*_FP	TCTATATCCACTGTCGGGAGGT
*nlrp3*_RP	CATGAGGAAGAGGATTCTGGAG
*nlrc4*_FP	CTCTGGAGACAGGAATCTTTGC
*nlrc4*_RP	GGGCTGATTTGGATGTACTCTC
*nlrp6*_FP	AAGGAACTGGAGCAACTGGA
*nlrp6*_RP	TCTGGTCGATGAACTGGTAGGT
*caspase 1*_FP	GACTACAGAGCTGGAGGCATTT
*caspase 1*_RP	GGGACTTGCTCAGAGTGTTTCT
*asc*_FP	GCACTTTATAGACCAGCACCG
*asc*_RP	GGCTGGTGTGAAACTGAAGA
*cyclophilin A*_FP	GGGCCGCGTCTCCTTTGAGC
*cyclophilin A*_RP	GGCGTGTGAAGTCACCACCC
*β -actin*_FP	CTGGCACCCAGCACAATG
*β -actin*_RP	GCCGATCCACACGGAGTACT

2.3.5 Fluorescent based Caspase 1 activity assay: Total cellular protein was extracted after 24 hr in PBS buffer with mild sonication for 2 min at 4^{o}C and quantified using standard Bradford assay (Bradford, 1976). Caspase 1 substrate (Ac-WVAD-AMC) was used for measuring the caspase 1 activity at different time periods like 3 hr, 6 hr, 9 hr and 12 hr in PBS. The cleavage of the substrate will generate fluorescence proportionately and then relative fluorescence intensity was measured fluorometrically at E_{ex}/E_{em} - 380/460 nm.

2.3.6 Western Blotting: The cells were lysed after 24 hr in RIPA buffer containing 1x Protease Inhibitor Cocktail, 10 mM EDTA and 1 mM PMSF. Total cell lysates of equal amount were used to detect Caspase 1, IL-1β and IL-18 using Western Blotting technique (Towbin et al., 1979). Briefly, an equal amount of cell lysates of different samples were run on a 12% SDS-PAGE followed by transfer into a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked using 5% skimmed milk for 2 hr at room temperature followed by washing with 1x TBST buffer thrice. Then primary antibodies for Caspase 1, IL-18 and IL-1β produced in rabbit were used (1:1000 dilution) for incubating the membrane at 4^{o}C overnight. Next, the membrane was washed again with 1x TBST and

incubated with secondary anti-rabbit IgG antibody conjugated with HRP at 1:5000 dilution for 45 min at room temperature. The membrane was washed with 1x TBST and chemiluminescent HRP substrate was used for band detection under a Chemi-Doc. Rabbit α-tubulin or mouse β-actin was used as endogenous controls at 1:1000 dilution. The secondary anti-mouse IgG antibody conjugated with HRP was used at a dilution 1:10000.

2.3.7 Sandwich ELISA: Cultured macrophage supernatants of control (untreated), 10 µg/ml of bacterial LPS treated followed by 5 mM of ATP stimulation for 30 minutes (positive control) and infected samples were used after 24 hr to quantify the release of mature subunits of IL-1β and IL-18 using self-sandwich ELISA. Briefly, capture antibody coating was done in 96-well plates at dilution of 1:1000 for both IL-1β and IL-18 antibodies and incubated overnight at 4^{o}C. The coating buffer used was carbonate-bicarbonate buffer (pH 9.5). Then washing was done thoroughly with 1x TBST followed by 1x TBS twice. Blocking was done using 3% BSA in coating buffer for 2 hrs followed by washing thoroughly. Equal volume of culture supernatants were added in all the wells and incubated for 6 hrs at room temperature (RT). Subsequently washing was done followed by detection antibody coating at dilution of 1: 3000 for both the antibodies (IL-1β or IL-18). Next secondary antibody conjugated to HRP was added at a dilution of 1:8000 after washing thoroughly and incubated for another 1 hr at RT. Final washing was done and TMB substrate was used for colorimetric detection. H_2SO_4 (2M) was used as a stop solution which changes the color from blue to yellow. Absorbance at 460 nm was taken in multi-reader ELISA plate. Secondary antibody controls were also made to nullify the background signals. The quantification of IL-18 and IL-1β were done using a standard graph for the above cytokines. Here capture and detection antibodies for IL-1β or IL-18 used were same. The TMB substrate was purchased from Thermo Scientific.

2.3.8 Lactate Dehydrogenase (LDH) assay: PMA differentiated adherent macrophage (U937 cells) were infected with *L. donovani* using MOI of 1:5 (macrophage: parasites) along with control setups. The cultured supernatants were harvested after 24 hr to quantify the release of LDH out of the cells. The released LDH were detected as per the manufacturer's protocol of Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific).

2.3.9 siRNA mediated knock-down assay: Briefly ~2 x 10^5 cells (THP-1) were seeded in RPMI-1640 media in 35 mm petri-dishes and incubated with 100 ng/ml of PMA at 37^{o}C, 5%

CO_2 for 24 hrs. The media was replaced with siRNA-lipid mix prepared in serum free Opti-MEM media followed by addition of complete RPMI-1640 media after 6 hr of incubation and finally incubated at 37°C, 5% CO_2 for another 18 hr. The knock-down was confirmed by western blot analysis and β-actin antibody was used as an endogenous control (Towbin et al., 1979). The choice of using THP-1 cells for this experiment is due to its easy transfection property which is difficult in case of U937. Both U937 and THP-1 are human monocytic cell lines of the same lineage. Scrambled and Caspase 1 siRNAs were purchased from Dharmacon, GE Healthcare.

2.3.10 Macrophage infectivity assay: The infectivity assay was done on both the wild type and the Caspase 1 knocked-down THP-1 cells at different multiplication of infection as per the method reported earlier with some modifications (Saudagar et al., 2013a). Briefly, ~ 0.5 x 10^5 cells (both wild type and Caspase 1 knock down THP-1) were seeded in 12 well plates and incubated with 100 ng/ml of PMA at 37°C, 5% CO_2 for 24 hrs to produce monocyte differentiated macrophages. The cells were washed carefully with 1x PBS thrice and infected with *L. donovani* promastigotes at a different MOIs like 1:2, 1:5 and 1:10 (macrophage: parasites) for another 24 hrs at 37°C, 5% CO_2. The cells were washed again carefully with 1x PBS thrice to remove the residual parasites in the culture media. The cells were fixed with methanol for 1-2 minutes followed by removal of excess methanol. The cells were nucleus stained using Giemsa stain (5 times diluted) for 30 minutes at room temperature (RT) and subsequently washed thoroughly with 1x PBS to remove the residual stains and air dried. Then Giemsa stained amastigotes were counted inside the infected macrophages. The cells were counted randomly (both uninfected and infected) in an inverted microscope under 60x magnification and infectivity index was calculated according to the formula mentioned below:

Infectivity index = Percentage of infected macrophages x Average number of amastigotes per infected macrophages.

2.3.11 ROS measurement using fluorescence microscopy and flow cytometry: Intracellular ROS was measured in PMA differentiated adherent macrophage (U937 cells) after 24 hr of following treatments. Four types of samples were used for the analysis, control (without treatment), LPS treated, LPS followed by NAC (20 mM) treatment for 45 min and

L. donovani infected macrophages. CM-H_2DCFDA dye (10μM) was used for the detection of ROS via fluorescence microscopy (EVOS FL Auto from Life Technologies) with 40x magnification at E_{ex}/E_{em} - 365/440nm and by flow cytometry using BD FACS Calibur.

2.3.12 Statistical and Densitometry analyses: Sigma-Plot 10 has been used for all statistical data analysis. Data are represented by mean ± SEM. Statistical significance was determined by unpaired t-test due to different biological replicates. A "p" value of less than 0.05 has been considered significant. The densitometry analyses of all the western blot data was performed by calculating a normalization factor for the loading control (α-tubulin) and then normalized the signals of the target proteins relative to α-tubulin.

2.4 Results

2.4.1 Altered NLRP3 inflammasome components during *L. donovani* infection: The infectivity of *L. donovani* (BHU-1081 strain) was checked and assessed quantitatively by *in-vitro* infection in PMA differentiated U937 cells at different time points (Figure 2.1A). Further, the gene expression analysis of various inflammasomes like *nlrp3, nlrc4* and *nlrp6* were investigated during infection as compared to the control samples using Real Time PCR (Figure 2.1B). The mRNA expression for *nlrp3* was found to be significantly higher as compared to other inflammasomes in the infected sample prompting plausible involvement of NLRP3 inflammasome during *L. donovani* infection. Gene expression analyses of other components of NLRP3 inflammasome like *casp1* and *asc* (apoptosis-associated speck-like protein containing a carboxy-terminal caspase recruitment domain) were also investigated (Figure 2.1C). Unlike *nlrp3*, the mRNA level of *asc* was only marginally higher but interestingly the *casp1* mRNA level was significantly reduced in the infected sample. Moreover, the gene expression levels of all the NLRP3 inflammasome components were also analyzed in clinical samples. Peripheral blood mononuclear cells (PBMCs) from four random healthy human volunteers were paired with four random infected human PBMC samples and assessed the gene expression level of *casp1, nlrp3* and *asc* using Real Time PCR. In line with our hypothesis, the mRNA levels of caspase 1 were significantly reduced in infected patient's samples (Figure 2.1D). In contrast to higher *nlrp3* in infected U937 cells, reduced *nlrp3* mRNA levels in clinical samples of infected patient were found (Figure 2.1E). There were only marginal increase observed in the *asc* mRNA levels in infected patient as compared to

healthy volunteers which were randomly paired with infected patients (Figure 2.1F) or as shown by comparative analysis of all healthy volunteers and infected patients (Figure 2.1G). The data indicates less production of inflammasome components during infection which might not be sufficient enough for the formation of NLRP3 inflammasome complex.

2.4.2 Maturation of caspase 1 is hindered during *L. donovani* infection: The low mRNA expression level of *casp1* was further validated by studying the protein level expression and maturation of caspase 1. The maturation of pro-caspase 1 to active caspase 1 was assessed by caspase 1 activity assay and western blot in different conditions using U937 cells. In the infected macrophage cell, the cleavage of caspase 1 specific substrate (Ac-WVAD-AMC) was significantly reduced with substrate as compared to the control samples (uninfected macrophage and LPS/ATP treated macrophages) samples at different time intervals after incubation (Figure 2.2). Likewise, western blot analyses clearly show no mature caspase 1 in the infected sample in contrast to LPS/ATP treated and untreated samples (Figure 2.3A). The data indicate plausible inhibition of caspase 1 maturation which may lead to impaired NLRP3 inflammasome assembly formation during infection. Interestingly, the impaired self-cleavage of pro-caspase 1 to active caspase 1 was found to be paradoxical to that of other infections like, *L. monocytogenes* (Wu et al., 2010), *A. veronii* (McCoy et al., 2010), *S. typhimurium* (Lara-Tejero et al., 2006) and *S. flexneri* infection (Sansonetti et al., 2000). Among other *Leishmania* species, activation or inhibition of caspase 1 *via* regulated NLRP3 inflammasome have conflicting results indicating species dependent modulation of the NLRP3 inflammasome in the *Leishmania* infection.

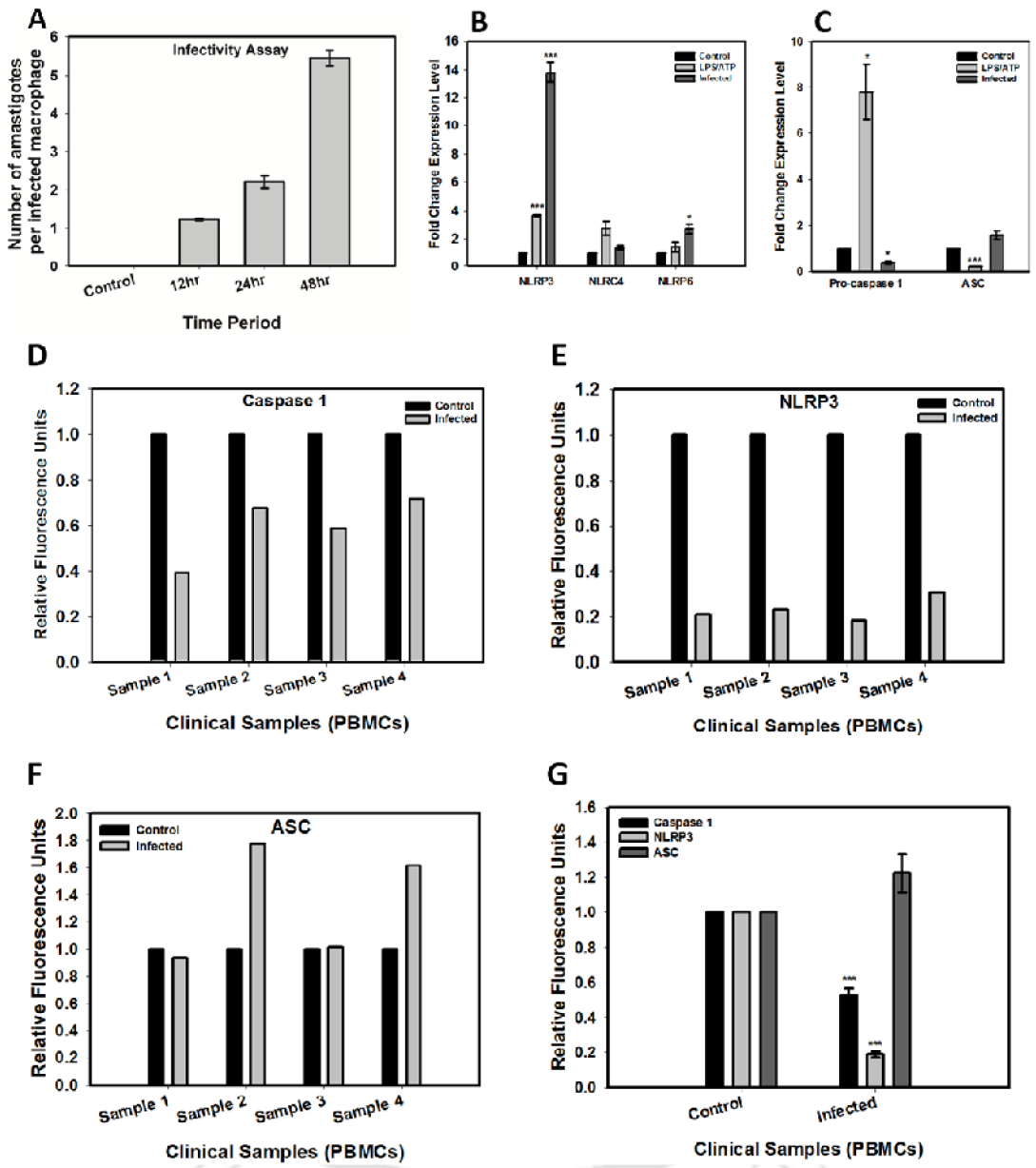

Figure 2.1: (A) Infectivity of *Leishmania donovani* (BHU-1081 strain) was verified at different time period like 12, 24 and 48 hr of incubation as compared to control uninfected PMA differentiated U937 cells; The mRNA expression analyses of (B) *nlrp3, nlrc4,* and *nlrp6* (C) *casp1* and *asc* of different samples have been done in U937 cells. Clinical samples were used to quantify the mRNA expression levels of (D) *casp1,* (E) *nlrp3* and (F) *asc* genes. For the analyses, four PBMC of healthy human volunteers were randomly paired with PBMC of four infected patients and experiments were done six times. (G) A comparative analysis of four healthy and four infected samples (4 pairs of healthy and infected samples) i.e., we have determined the mean of 6 experiments from each donor and then we have calculated the mean of four healthy and four infected individuals with standard deviation and plotted with statistical analyses. Figure 1D, 1E, 1F represents consistent mRNA expression results of respective genes and have been plotted as mean of 6 experiments only without any statistical analysis. (* denotes $p<0.05$ and *** denotes $p<0.001$; x=3 for figure A-C, where x represents three different independent experimental replicates; n=4 for figure G, where n represents different healthy and infected donor pair)

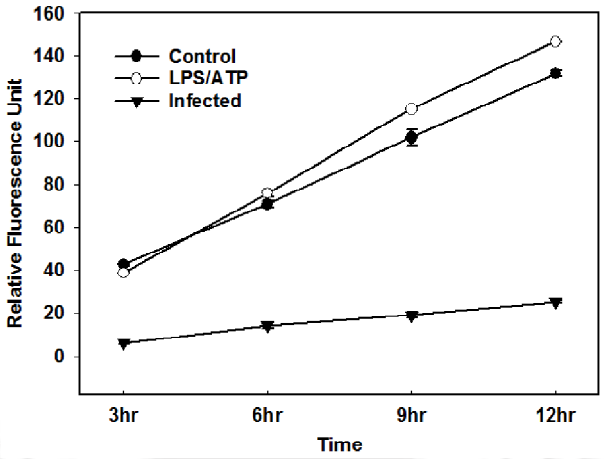

Figure 2.2: Caspase 1 activity assay in infected cells. The caspase 1 activity assay was performed using an equal amount of cell lysates of different samples of U937 cells after 24 hr with a caspase 1 specific substrate (Ac-WVAD-AMC). The caspase 1 activity was found to be reduced in the infected sample using activity assay (Note: n=3, where n represents three different independent experimental replicates)

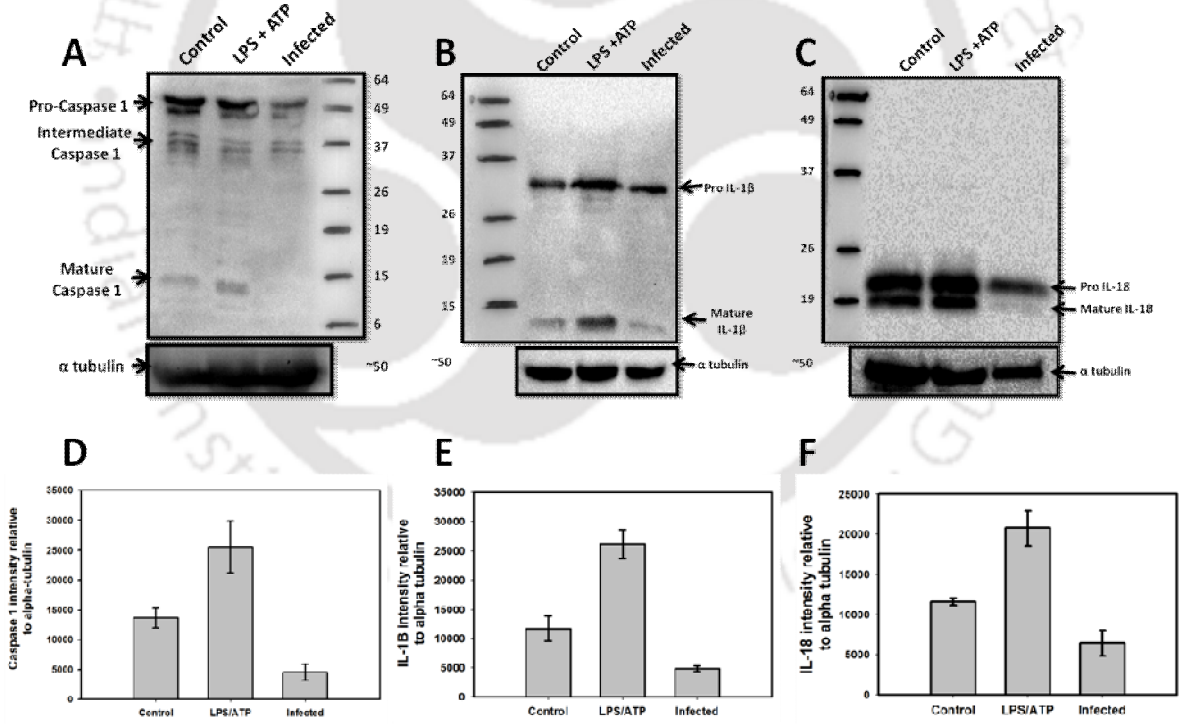

Figure 2.3: Immunoblot of host caspase 1 level upon L. donovani infection (A) Effect of host caspase 1 level was further analyzed by western blot to evaluate the maturation of pro-caspase 1 to active caspase 1. An equal amount of total cell lysate was used for western blotting and α-tubulin was used as an endogenous control. Analysis of pro-inflammatory cytokines after *L. donovani* infection and downstream effects of possibly impaired NLRP3 inflammasome activation was analyzed by showing (B) pro-IL-1β and (C) pro-IL-18 processing into their mature forms by western blot in different samples of U937 cells; (Note: n=3, where n represents three different independent experimental replicates and densitometry analyses have been shown in figure 3D, 3E & 3F)

2.4.3 Decreased IL-1β and IL-18 release during infection: Since mature caspase 1 is solely responsible for the activation of specific pro-inflammatory cytokines, pro-IL-1β and pro-IL-18 and causing inflammation, the maturation of pro-IL-1β (Figure 2.3B) and pro-IL-18 (Figure 2.3C) into their mature forms were analyzed by western blot. The western blot data clearly shows diminished levels of mature IL-1β and IL-18 in the PMA differentiated U937 macrophage cells infected with *L. donovani*. The production of mature IL-1β and IL-18 causes inflammation leading to pore formation in the plasma membrane and ultimately release of the cytokines in the extracellular milieu of the cells (Schroder and Tschopp, 2010, Shio et al., 2015). The release of these cytokines in the culture supernatants was shown by ELISA (Figure 2.4A) and reduced level of IL-1β and IL-18 were found in the infected U937 macrophage culture supernatants compared to the control samples. Inability to mature IL-1β and IL-18 in the infected macrophage, together with low *CASP1* mRNA level, low caspase 1 activity and low/no mature caspase 1 indicates impaired assembly of NLRP3 inflammasome during *L. donovani* infection.

2.4.4 Impairment of pyroptosis process during infection: Pyroptosis is one of the distinct cell death mechanisms apart from apoptosis and necrosis. The caspase 1 dependency is a unique signature for pyroptosis and caspase 1 activation leads to formation of membrane pores through which various cytokines and mediator molecules escapes out of the cells. Amongst the myriads of intracellular contents, LDH is one of the cytosolic protein whose release can be a proxy for cell death due to its easy quantification via enzymatic reactions. Low LDH release in the infected U937 cells was found as compared to the control (or untreated) confirming the impairment of pyroptosis in infected cells. This in turn, prevents membrane pore formations and thereby increasing the survival period of the parasites (Figure 2.4B). Moreover, nitric oxide (NO) release from membrane compromised cells also favors the pyroptosis process. Human monocyte derived macrophage cell lines (like U937) shows undetectable amount of NO release as compared to murine macrophage which releases substantial amount of NO. A similar trend of reduced NO release was observed in infected sample of murine macrophage cells (J774A.1) as compared to control cells (Figure 2.4C). This strongly validates our hypothesis of impaired pyroptosis process in *L. donovani* infected cells.

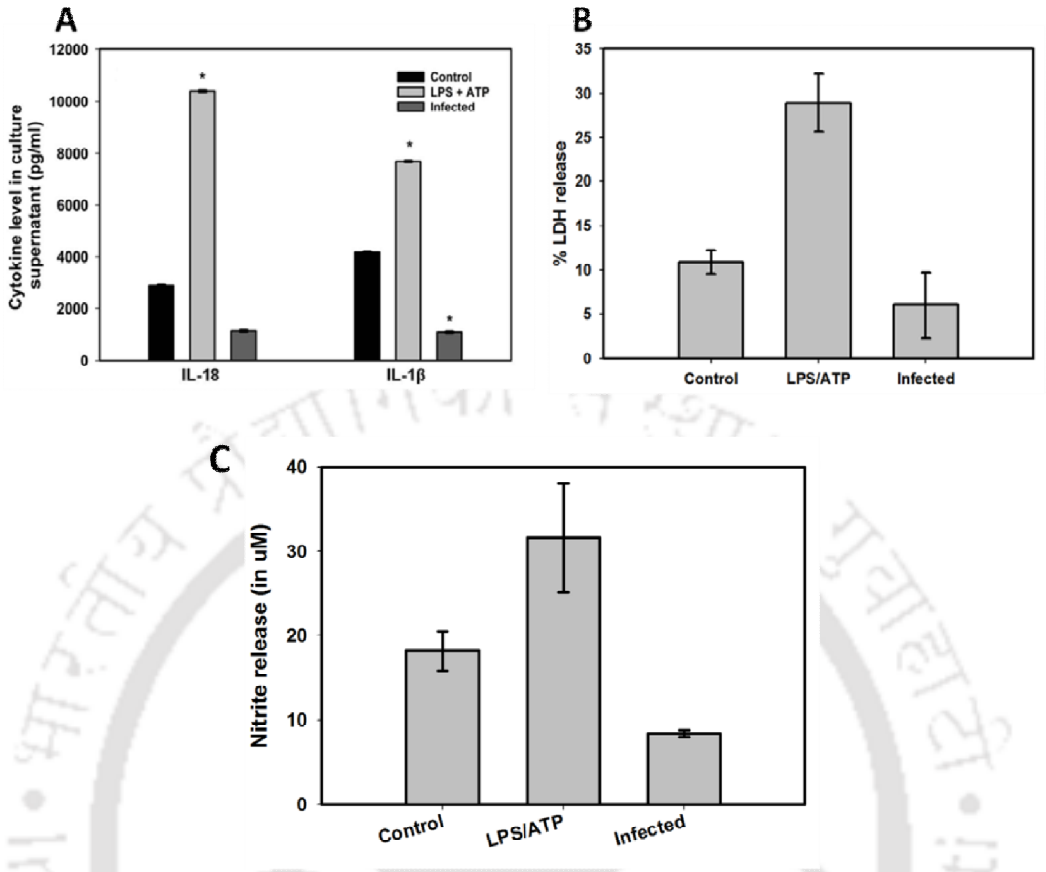

Figure 2.4: Sandwich ELISA showing impaired release of mature IL-1β and IL-18 upon infection. (A**)** Release of the mature forms of IL-1β and IL-18 in culture supernatant has been shown by sandwich ELISA. Impairment of pyroptosis in infected U937 cells was shown by (B) Lactate Dehydrogenase detection. Low LDH release in infected samples validates impairment of pyroptosis process. (C) Estimation of nitrite release has been performed in murine macrophages (J774A.1) cells using Griess Reagent (Invitrogen). Reduced nitrite release in infected sample validates impairment of pyroptosis process. Human monocyte derived macrophage cell lines (like U937 or THP-1) shows undetectable amount of NO release as compared to murine macrophage and thus has been shown in murine macrophages. (* denotes $p<0.05$; n=6 for Figure 4A; n=3 for Figure 4B, where n represents different and truly independent experimental replicates)

2.4.5 Host caspase 1 deficiency increases the susceptibility of infection: Among all the inflammatory caspases, caspase 1 is the most characterized caspase and was originally discovered due to its role in processing inactive IL-1β and IL-18 into its mature subunits (Martinon and Tschopp, 2004, Thornberry et al., 1992, Kostura et al., 1989, Gracie et al., 2003). Since *L. donovani* infection impairs the maturation of caspase 1 followed by non-processing of the pro-IL-1β and pro-IL-18 into their active counterparts, we have assessed the importance of caspase 1 in the progression of the disease. Knock down of caspase 1

expression using siRNA in PMA differentiated THP-1 cell was performed as transfection of U937 cell is difficult to achieve. The knock-down was confirmed by Real time PCR (Figure 2.5A) and western blot analysis (Figure 2.5B). Normal and caspase 1 knocked-down macrophages were infected with *L. donovani* promastigote cells at different multiplicity of infection (MOI) and parasitic infectivity index (Figure 2.5E) was evaluated along with percentage infection (Figure 2.5C) and number of parasites per infected macrophage (Figure 2.5D). The caspase 1 knocked down cells have significant increase in parasite burden at MOI of 1:5 and marginal changes at MOI of 1:2 or 1:10 (Figure 2.5E). This increase in the parasite burden in caspase 1 knock-down macrophage cells substantiate the possible role of host caspase 1 in the resistance to infection.

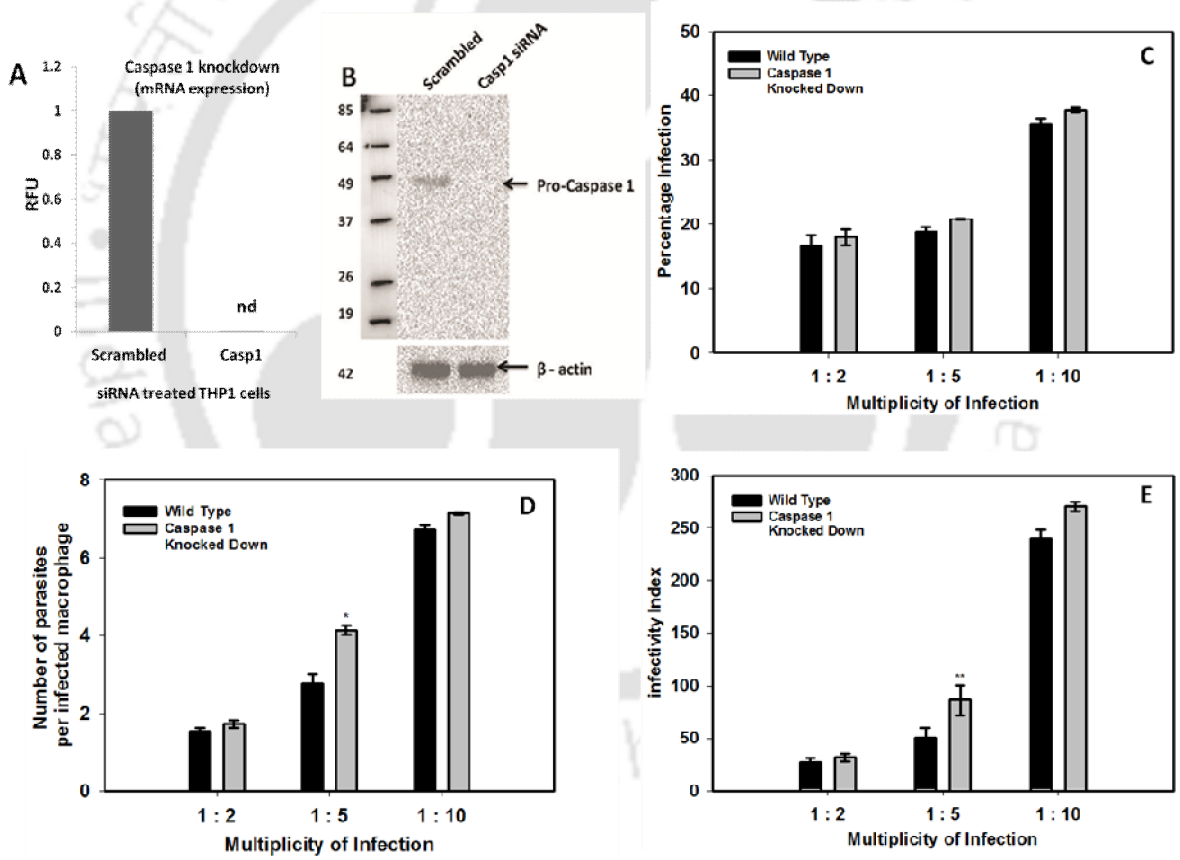

Figure 2.5: Confirmation of caspase 1 knock down using (A) mRNA expression analyses by Real Time PCR and (B) western blot analysis. In the western blot analysis, β – actin was used as an endogenous control; antibody dilution for Caspase 1, IL-18, IL-1β, α-tubulin, β-actin was 1:1000. The dilution for secondary anti-rabbit IgG was 1:5000 and secondary anti-mouse IgG was 1:10000. Effect of host Caspase 1 deficiency on the susceptibility of *L. donovani* infection is shown as (C) percentage of infected macrophages (D) number of parasites per infected macrophages (E) infectivity index were shown. (Note: "nd" denotes not determined, * denotes $p<0.05$ and ** denotes $p<0.005$; n=3 for figure 5C, 5D and 5E, where n represents different independent experimental replicates)

2.4.6 Decreased ROS production impairs NLRP3 inflammasome assembly formation: The NLRP3 inflammasome activation in various pathogenic infections has a major role to play where the host immune system fight the infection by increasing or decreasing the release of pro-inflammatory cytokines depending upon the pathogens (Schroder and Tschopp, 2010, McIntire et al., 2009, Skeldon and Saleh, 2011, Zamboni and Lima-Junior, 2015). The activation of this inflammasome is mainly favored by various secondary factors like intracellular reactive oxygen species, intracellular calcium and potassium levels (Heid et al., 2013, Brough et al., 2003, Hornung et al., 2008). The intracellular ROS was evaluated semi-quantitatively in the infected cells as compared to controls.

The flow cytometric analysis of macrophage cells clearly shows increase in total intracellular ROS in the LPS/ATP treated macrophage cell (positive control) which is reverted back after N-acetyl cysteine (NAC) treatment. Further, *L. donovani* infection did not show any increase in ROS in macrophage which was comparable to untreated macrophage (Figure 2.6A). Moreover, this flow cytometric data is also supported by fluorescence microscopy after CM-H_2DCFDA dye treatment. Fluorescence microscopy data shows similar pattern of ROS levels in different experimental samples consistent with flow cytometric data (Figure 2.6B).

2.5 Discussion & Conclusion

Parasites try to evade the innate immune response and remain immunologically silent in the host environment to stay protected. The role of inflammasomes as a defense system of our body against invading pathogens is critical. The involvement of different inflammasomes like NLRP3, NLRC4, NLRP6, AIM2 etc in different types of pathogenic infections play important role in the activation or inhibition of inflammasome complex and subsequent downstream effects (Skeldon and Saleh, 2011, Zamboni and Lima-Junior, 2015, Sani et al., 2014).

Although, not much work on *L. donovani* has been published, some works on other species of the parasite have been reported (Lima-Junior et al., 2013, Shio et al., 2015). While *L. amazonensis* infection activates NLRP3 inflammasome (Lima-Junior et al., 2013), *L. major* and *L. mexicana* inhibit NLRP3 inflammasome and subsequent IL-1β production (Shio et al., 2015) indicating species dependent modulation of the inflammasome. Even in the case of *L.*

major infection, Sani *et al.* reported caspase 1 activation in murine macrophage cell line J774G8 (Sani et al., 2014) while Shio *et al*, reported attenuated caspase 1 activation (Shio et al., 2015). In addition, some report about *L. donovani* and *L. tropica* infection reports low IL-1β expression without much mechanistic details.

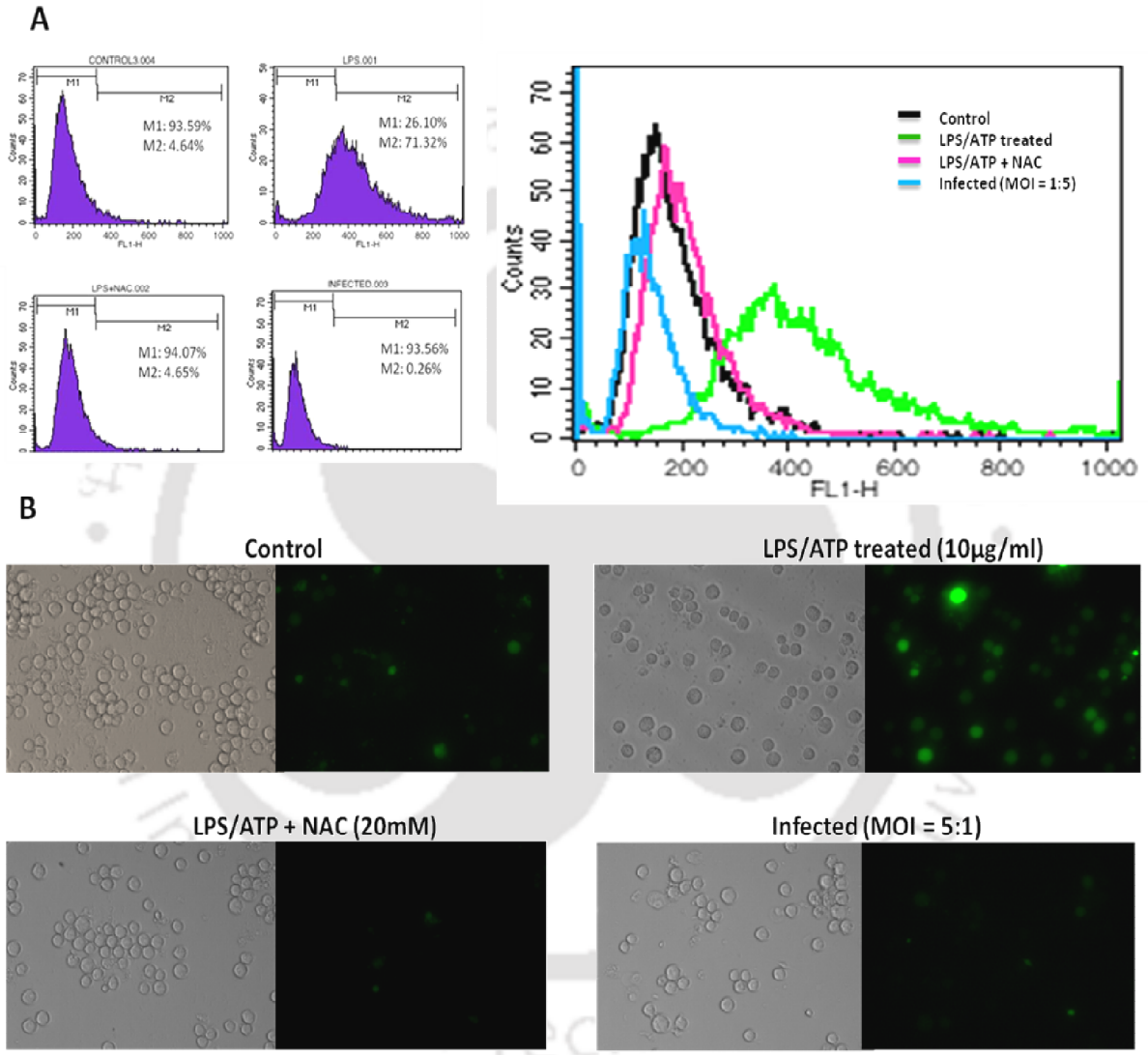

Figure 2.6: Reactive oxygen species (ROS) measured in control (untreated), 10µg/ml bacterial LPS followed by 5mM ATP treated (positive control) and infected macrophage (U937) cells after 24 hr of incubation using CM-H_2DCFDA dye which interacts with the intracellular ROS. Decrease in ROS in infected sample shown by (A) Flow cytometry using BD FACS Calibur and (B) Fluorescence microscopy at E_{ex}/E_{em} 365/440nm using fluorescence inverted microscope (EVOS FL Auto from Life Technologies).

In the supramolecular complex of the NLRP3 inflammasome, the NLRP3 is attached to the pyrin domain of ASC molecule which is attached to the caspase recruitment domain (CARD) of pro-caspase 1 (Sutterwala et al., 2006, Martinon and Tschopp, 2005). The higher mRNA expression of *CASP1* in LPS treated cells followed by 5 mM ATP stimulation (positive control) was in line with the earlier report (Le Feuvre et al., 2002). The significantly higher mRNA expression level of *NLRP3* gene in *L. donovani* infected U937 macrophage cells (Figure 2.1B) point out the possible role of NLRP3 inflammasome but interestingly the lower mRNA expression of pro-caspase 1 in infected macrophages (Figure 2.1C) depicts the inhibition of the pro-caspase 1 transcription. Moreover, significantly reduced levels of *CASP1* mRNA in infected patient's PBMC samples substantiated our hypothesis (Figure 2.1D and Figure 2.1G). Interestingly, contradictory results were found for *NLRP3* mRNA levels in clinical samples. A drastic down-regulation of *NLRP3* expression was observed in infected patient's PBMC samples as compared to healthy volunteers (Figure 2.1E and Figure 2.1G). This reduced *NLRP3* mRNA levels in clinical samples synergistically favors the possible inhibition of NLRP3 inflammasome assembly formation during *L. donovani* infection. Contradictingly higher *NLRP3* mRNA expression in infected U937 cells can be associated to either PMA stimulation prior to experimental setup to differentiate human monocytic cell line into macrophages or some other unknown mechanisms. Also marginal increases in ASC mRNA levels were observed in clinical samples (Figure 2.1F and Figure 2.1G).

To get further insights, the protein level expression of caspase 1 was quantified during infection. Both, activity assay and western blot data indicate inability of caspase 1 maturation in the infected macrophages (Figure 2.2 & 2.3A). Diminished expression of mature caspase 1 (p10/p20) in the infected macrophage sample was observed whereas mature caspase 1 was found in significantly higher level in the LPS/ATP treated sample. This finding advocates the possibility of impaired assembly of NLRP3 inflammasome which is required for maturation process of caspase 1. The downstream effect of caspase 1 activation leads to processing of proinflammatory cytokines, IL-1β and IL-18, into their mature subunits which will finally result in cellular inflammation and bursting of the cells (Martinon et al., 2002). Our results also indicate reduced processing of pro-IL-1β and pro-IL-18 into its mature form (Figure 2.3B & 2.3C). The reduced release of both the cytokines into the extracellular milieu of the

cells were also observed by sandwich ELISA (Figure 2.4A). Overall, our study suggests that the parasite suppresses caspase 1 dependent, IL-1β and IL-18 mediated pyroptotic death of macrophage. Pyroptosis, another form of programmed cell death, is exhibited by various host cells to fight against invading pathogens (Bergsbaken et al., 2009, Fink and Cookson, 2005, Miao et al., 2011). In many pathogenic infections like *Shigella* (Sansonetti et al., 2000), *Salmonella* (Lara-Tejero et al., 2006), *Listeria* (Wu et al., 2010, Tsuji et al., 2004), *Legionella* (Molofsky et al., 2006), the cell death mechanism of the host cells is processed via pyroptosis tailored by caspase 1 activation in response to recognition by the innate immune system and ultimately clearance of pathogens. As mentioned earlier, our study on *L. donovani* infected macrophage also shows insignificant maturation and release of IL-1β and IL-18 subunits. The lack of processing of these proinflammatory cytokines is mainly due to unavailability of mature caspase 1 in the infected cells. This demonstrates the importance of host caspase 1 for host defense mechanism against *L. donovani* infection and its activation needs to be enhanced to curb toward pyroptotic cell death pathway to cause inflammation and release of proinflammatory cytokines. Since caspase 1 activation and its downstream effects finally leads to pyroptosis, estimation of LDH release from the membrane compromised cells were quantified as a proxy to cell death pathway. Reduced LDH release in infected samples of U937 cells were observed as compared to the controls (Figure 2.4B). Moreover, murine macrophages were used to quantify nitric oxide (NO) release as human U937 cells intrinsically releases undetectable NO in the extracellular milieu of cells. Also undetectable NO release in U937 human monocytic cells was observed. Reduced NO release was observed in infected J774A.1 cells as compared to control setups (Figure 2.4C). This validates our hypothesis of impaired pyroptosis process in infected cells, thereby increasing their survival period inside the host cells.

The biological significance of host caspase 1 in the pathogenesis of *L. donovani* infection will unlock the key issue of immunosuppression by the parasite in terms of NLRP3 inflammasome modulation. Utilization of appropriate cell death pathway in healthy or infected cells is vital in initiating the activation of immune responses. The pyroptotic cell death pathway being inflammatory in nature is essential for the host cells to get rid of the pathogens and the parasites trying to replicate inside the host cells overcomes NLRP3 inflammasome activation by abrogating the maturation of caspase 1. siRNA mediated knock

down of caspase 1 (Meister and Tuschl, 2004, Agrawal et al., 2003) in PMA differentiated THP-1 cells was performed and analyzed the parasitic burden at different MOIs. In context with our previous results, the increase in the parasite infectivity index in the caspase 1 knocked down cell as compared to the wild type cells (Figure 2.5) validates the role of caspase 1 in the .host defense mechanism against *L. donovani* infection.

The indulgence of various secondary factors collaboratively initiates the NLRP3 inflammasome assembly formation followed by its activation. Since the initial triggering starts with the binding of PAMPs or DAMPs of the pathogens with various receptors like TLRs or NLRs[46], secondary factors like reactive oxygen species (ROS), calcium influx and potassium efflux also plays a crucial role in enhancing the activation of NLRP3 inflammasome and finally leading to pyroptosis (Heid et al., 2013, Brough et al., 2003, M. et al., 2015). The priming of NLRP3 inflammasome activation is mainly mediated by an increase in the ROS level which in addition also increases the oxidative stress inside the cellular environment. The increase in oxidative stress inside the cells during any pathogenic infection assists inhibition of infection advancement and finally killing the parasite. A significant decrease in the total intracellular ROS level in the infected cells affirms our hypothesis of evading the activation of the NLRP3 inflammasome by the parasites as validated by flow cytometry and fluorescence microscopy experiments (Figure 2.6).

The choice of host cell death pathway during infection is a critical factor which can decide the fate of disease progression. The tampering of caspase 1 activation and its downstream inflammatory effects during *L. donovani* infection is crucial for parasite survival inside their replicative niche. The down-regulation of pro-caspase 1 mRNA expression followed by its hindered maturation blocked the processing of inflammatory pyroptotic cell death pathway (Figure 2.7). This type of mechanism of caspase 1 inactivation has been shown previously by human *Influenza A/PR/8/34* (H1N1) virus (Stasakova et al., 2005) and *Yersinia* bacteria (Schotte et al., 2004) to resist their clearance. The repulsive nature of *L. donovani* infected host macrophage cells toward pyroptotic cell death pathway mediated by the parasites needs more understanding and knowledge as to how these parasites modulate the NLRP3 inflammasome via inhibition of caspase 1 maturation. We conjecture interaction of certain *L. donovani* proteins or host dependent molecules with NLRP3 inflammasome components and

thereby interrupting the assembly formation. Furthermore, post-translational modification of host macrophage NLRP3 inflammasome components during *L. donovani* infection renders it incapable of assembly formation and activation can also not be ruled out. Further, detailed study of evading the host immune response by *L. donovani* in impairing the NLRP3 inflammasome activation is under investigation in our laboratory.

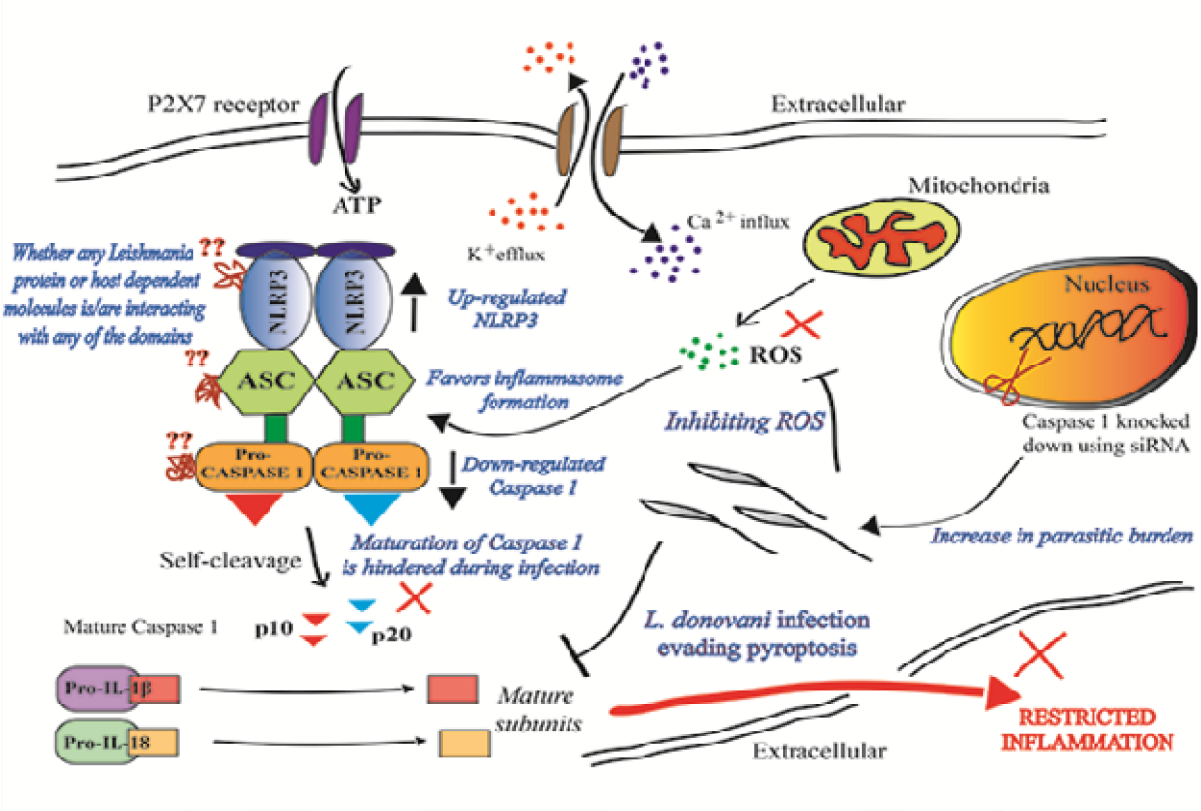

Figure 2.7: Schematic representation summarizing the escape mechanism of Leishmania donovani inhibiting the maturation of caspase 1. This terminates the processing of proinflammatory IL-1β and IL-18 into their mature counterparts and ceases the inflammatory pyroptotic cell death. We have knocked down the caspase 1 expression using siRNA technique in the host cell and shown that there is a 2-fold increase in parasite burden in the caspase 1 knocked down cells compared to wild type cell. This signifies the importance of caspase 1 in resisting the host cells against the parasite. The parasite executes this immunosuppression mechanism for their survival in their replicative niche by overcoming pyroptotic cell death pathway. The parasite also dampens the activation of the inflammasome by inhibiting the production of intracellular ROS and thereby minimizing the oxidative stress inside the cellular environment. This work needs more attention and understanding as to how the parasites down regulate the above NLRP3 inflammasome formation in sub-cellular level.

CHAPTER 3

Leishmania donovani exploit host BLIMP-1 transcription factor for their long term survival

3.1 Abstract

Visceral leishmaniasis, one of the fatal forms of the disease, is caused by *Leishmania donovani* and presents morbid clinical manifestations. The parasite evades the pro-inflammatory immune responses by several reported mechanisms and modulates the host immune system to cause fatal symptoms. A plethora of reports related to the role of BLIMP-1 and its involvement in suppressing the immune response in various infectious diseases have been documented. Higher parasitic burden due to increased BLIMP-1 production has been reported earlier for malaria and leishmaniasis but with no detailed informations. We report for the first time the role of BLIMP-1 in suppressing the macrophage pyroptosis during *L. donovani* infection and thereby tweaking the tight regulation of NFκβ – NLRP3 signaling pathway. Expression analyses of BLIMP-1 and NFκβ have been measured using real-time PCR and western blotting. Importance of BLIMP-1 has been validated using siRNA-mediated experiment along with caspase 1 activity, LDH release assay and infectivity index analyses. An inverse relationship between BLIMP-1 and NFκβ expression has been highlighted during *L. donovani* infections which get reversed in *blimp-1* deficient cells infected with promastigotes. The above fact has been further validated with caspase 1 activity assay, LDH release along with IFN-γ and TNF-α release assay. We conjecture that parasites modulate the NFκβ – NLRP3 signaling pathway by taking advantage of BLIMP-1 dependent IL-10 production. Better understanding of the detailed mechanism behind this modulation will help in developing new immunotherapeutic strategies to combat the disease.

3.2 Introduction

Parasitic protozoa of the genus *Leishmania* are the main causative organisms of the most neglected tropical disease, *leishmaniasis* (Murray et al., 2005). Different species of *Leishmania* are responsible for manifestations of a different form of the disease. *L. donovani,* most common species in India, causes visceral leishmaniasis (VL, commonly called "*kala-azar*" in India). The survival and proliferation of these parasites inside the host macrophages exhibit high tolerance against the defending immune system of the host cells. There are various reports on how these parasites hijack the immune system of the host and tailor it to their convenience or zone of comfort (Gurung and Kanneganti, 2015b). The Th1/Th2 balance of the host immune system gets disturbed upon infection and these parasites attempt to dominate Th2 anti-inflammatory response over Th1 pro-inflammatory response. One of the main pathways involved in the pathogenesis of *Leishmania* infection is the Toll-like receptors (TLRs) pathway where pathogen-associated molecular pattern (PAMPS) plays a vital role in initiating downstream cascades of reactions (Faria et al., 2012, Takeda and Akira, 2005). As per reported facts, TLR4 and TLR9 play a pivotal role in recognizing and responding against *Leishmania*. Interestingly, *Leishmania* hijacks TLR2, which evolved similarly as TLR4 and TLR9 to respond against them. Indeed, recognition of *Leishmania* LPG seems to promote their growth by inhibiting IL-12 production. Blockade of TLR2/4 signaling by anti-TLR2/TLR4 antibodies prevent growth of the parasites during *L. donovani* infection, reducing intracellular parasitic burden (Murray et al., 2013). NFκβ activation and its translocation to the nucleus for the transcription of various pro-inflammatory genes are an important part of TLR activation whose inflammatory reaction is very crucial to get rid of any infecting pathogens (Kawai and Akira, 2007). A significant role of NFκβ in innate and adoptive immunity has been established on the basis of its regulation of inducible transcription of various genes whose products are important components of the immune response such as cytokines, chemokines, and adhesion molecules. Several studies have reported that *Leishmania* and other parasites such as *Toxoplasma gondii* interfere with the activation of the NFκβ signaling pathways (Shapira et al., 2002, Kumar et al., 2018). In addition, NFκβ is a central mediator in the regulation of NLRP3 inflammasome activation and acts by inducing the transcriptional expression of NLRP3 and pro-IL-1β and pro-IL-18 in response to various pathogen recognition receptors (PRR) ligands and cytokines (Liu et al.,

2017, Zamboni and Lima-Junior, 2015). The NLRP3 inflammasome is believed to sense the disturbance of cellular homeostasis caused by pathogenic encounters rather than directly recognizing a common motif present in its activators, and multiple cellular signals have been proposed to trigger its activation, including K+ efflux, Ca^{2+} signaling, mitochondrial dysfunction, and lysosomal rupture. This NLRP3 inflammasome is a complex multi-protein assembly and forms an integral part of innate immunity against any pathogenic infection including *L. amazonensis* infection (Gupta et al., 2019, de Carvalho et al.). Down-regulation of NFκβ expression and induction of IL-10 cytokine has been already reported during the progress of *L. donovani* infection (Saha et al., 2019). Another recent report demonstrated altered NLRP3 inflammasome during *Leishmania* infection due to caspase 1 inactivation and non-processing of pro-inflammatory cytokines (IL-1β and IL-18), thereby increasing the survival period of these parasites inside the replicative niche (Saha et al., 2019). These reports validate the hijacking processes of host NFκβ – NLRP3 signaling system by the notorious parasite. The dependency of NFκβ expression on NLRP3 inflammasome formation is pivotal and its regulation by IL-10 dependent B lymphocyte-induced maturation protein 1 (BLIMP 1), might also provide new insight in the regulation and management of this disease

BLIMP-1 is a zinc-finger motif containing transcription factor and acts as a repressor of interferon β (IFN-β) (Kallies et al., 2006). As per previous reports, we found BLIMP-1 serving as the master regulator of antibody-producing B-cells and also orchestrates the T-cell homeostasis (Fu et al., 2017, Savage et al., 2017, Cretney et al., 2018). It is also essential for the production of IL-10 cytokine and promotes T helper 2 (Th2) lineage commitments limiting Th1 and Th17 cells differentiation. In addition, it also attenuates the IFN-γ production in non-polarizing T cells (Cimmino et al., 2008, Salehi et al., 2012). BLIMP-1 has been involved in the molecular signaling directing IL-10 production and acts as a key transcription factor in effector T cells (Kallies et al., 2006, Martins and Calame, 2008). In this regard, the limitation of Th1 immune reactions upon parasitic infection like VL can also be attributed to its conversion to Type I regulatory T-cells (Tr1 cells) which prevent tissue damage during excessive inflammation. A previous study reports that these Tr1 cells are mainly responsible and dependent on BLIMP-1 driven IL-10 production in experimental VL, controlling parasite killing and increasing its burden inside the host cells (Montes de Oca et al., 2016). Recently, a group has explored the role of B-cells dysfunctioning during VL with

altered BLIMP-1 expression (Singh et al., 2019). This finding instigated us to evaluate the role of BLIMP-1 in the regulation of macrophage immune response during *L. donovani* infection. It is worth mentioning that the parasite resides and multiplies in macrophages. This work unravels the importance of BLIMP-1 in controlling the proinflammatory response during infection by tweaking NFκβ – NLRP3 signaling pathway. This hostile response by BLIMP-1 favors the replication of parasite inside the cells. The data supports the fact that BLIMP-1 can be a potential host molecule exploited by the parasites in the pathogenesis of *kala-azar*.

3.3 Materials & Methods

3.3.1 Parasite and other materials: *L. donovani* culture (BHU-1081 strain) was a kind gift from Prof. Shyam Sundar, Institute of Medical Sciences, Banaras Hindu University. Procedure for *L. donovani* culture has been done and followed as per protocol (Das et al., 2015). In brief, *Leishmania* promastigotes are cultured at 28^{o}C in M199 liquid media supplemented with 10% fetal bovine serum (FBS), 10,000 U/ml penicillin and 10 mg/ml streptomycin. Human monocytic cell line (THP-1) and mouse macrophage cell line (J774A.1) were procured from National Centre for Cell Sciences, Pune and cultured at 37^{o}C and 5% CO_2 in Roswell Park Memorial Institute (RPMI-1640) media and Dulbecco's Modified Eagle's Medium (DMEM) respectively supplemented with 10% FBS, 2 mM glutamine, 10,000 U/ml penicillin, 10 mg/ml streptomycin and 25 µg/ml amphotericin B. Phorbol 12-myristate 13-acetate (PMA) was used at a concentration of 100 ng/ml to differentiate monocytic THP-1 cells into macrophages at 37^{o}C and 5% CO_2 overnight. Scrambled/Negative control and *blimp-1* siRNAs were purchased from Invitrogen. Antibodies like IFN-γ, TNF-α, IL-10, IL-4 and BLIMP-1 were purchased from Abcam whereas other antibodies like α-tubulin, IL-1β, IL-18 and, NFκβ were procured from Sigma Aldrich.

3.3.2 Gene expression analysis using qRT-PCR: RNeasy Mini Kit (QIAGEN) protocol was used for isolating total RNA and quantified using Nano-Drop after DNase treatment for 1 hr at 37^{o}C. An equal amount of RNA was used to synthesize cDNA using ProtoScript® First Strand cDNA Synthesis Kit (New England Biolabs). THP-1 and J774A.1 cells were used for the analysis of *blimp-1* mRNA expression in control and infected cells with specific

primers using qRT-PCR. The mRNA levels of IL-1β and IL-18 were also analyzed with specific primers using qRT-PCR. For all the qRT-PCR experiments, *β-actin* gene was used as endogenous housekeeping control to normalize the relative fluorescence signal of the target genes. The sequences of all the primers used are listed in Table 3.1.

Table 3.1: Primer sequences used for Real Time PCR experiments (Chapter 3)

Primers	**Sequences (5' – 3')**
*IL-1β*_FP	GCTGGAGAGTGTAGATCCCAAA
*IL-1β*_RP	GCTGGAGAGTGTAGATCCCAAA
*IL-18*_FP	GCTGCTGAACCAGTAGAAGACA
*IL-18*_RP	ATAGAGGCCGATTTCCTTGG
*blimp-1*_FP	CCCCTCGTACAACGCTCACTA
*blimp-1*_RP	AGCGCTCAGGCCATTACAAT
*β-actin*_FP	CTGGCACCCAGCACAATG
*β-actin*_RP	GCCGATCCACACGGAGTACT

3.3.3 Immunoblotting: Briefly, 2 x 10^5 cells/well were used to seed in 6 well plates for the experiment. The cells were lysed in RIPA buffer containing 1x Protease Inhibitor Cocktail, 1 mM PMSF and 10 mM EDTA. BLIMP-1 and NFκβ expressions were detected using total cell lysates by a standard western blotting technique (Saha et al., 2019). An equal amount (25µg/lane) of cell lysates of different samples were run on a 12% SDS-PAGE followed by transfer into a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked using 5% skimmed milk for 2 hr at room temperature followed by washing with 1x TBST buffer thrice. Then primary antibodies for BLIMP-1 (1:500 dilution) and NFκβ (1:1000 dilution) produced in goat and rabbit respectively were used for incubating the membrane at 4^{o}C overnight. Next, the membrane was washed again with 1x TBST and incubated with secondary anti-goat and anti-rabbit IgG antibody conjugated with HRP at 1:3000 dilution for 45minutes at room temperature. The membrane was washed with 1x TBST and chemiluminescent HRP substrate was used for band detection under a Chemi-Doc. Rabbit α-tubulin was used as endogenous control to normalize the expression level of the target proteins.

3.3.4 Sandwich ELISA: Culture supernatants of THP-1 and J774A.1 cells were used to quantify the release of various cytokines like IFN-γ, TNF-α, IL-10 and IL-4 using sandwich ELISA as per standard protocol (Saha et al., 2019). Briefly, capture antibody coating was done in 96-well plates for IFN-γ, TNF-α, IL-10 and IL-4 antibodies and incubated overnight at 4^{o}C. The coating buffer used was carbonate-bicarbonate buffer (pH 9.5). Then washing was done thoroughly with 1x TBST followed by 1x TBS twice. Blocking was done using 3% BSA in coating buffer for 2 hrs followed by washing thoroughly. Equal volume of culture supernatants were added in all the wells and incubated for 6 hrs at RT. Subsequently washing was done followed by detection antibody coating for IFN-γ, TNF-α, IL-10 and IL-4 antibodies. Next secondary antibody conjugated to HRP was added after washing thoroughly and incubated for another 1 hr at RT. Final washing was done and TMB substrate was used for colorimetric detection. H_2SO_4 (2M) was used as a stop solution which changes the color from blue to yellow. Absorbance at 460 nm was taken in multi-reader ELISA plate. Secondary antibody controls were also made to nullify the background signals. The quantification of IFN-γ, TNF-α, IL-10 and IL-4 antibodies were done using a standard graph for the above cytokines. Here capture and detection antibodies for IFN-γ, TNF-α, IL-10 and IL-4 used were same and thus it is a "self-sandwich" ELISA. The TMB substrate was purchased from Thermo Scientific.

3.3.5 siRNA mediated *blimp-1* knock-down assay: Briefly ~2 x 10^5 cells (THP-1 and J774A.1) were seeded in RPMI-1640 media and DMEM, respectively in 35 mm petri-dishes and incubated at 37^{o}C, 5% CO_2 for 24 hrs. PMA (100 ng/ml) was used for THP-1 cells to differentiate into macrophages. Serum-free Opti-MEM media was used to prepare siRNA-lipid mix and incubated the cells for 6 hrs followed by addition of complete media and finally incubated at 37^{o}C, 5% CO_2 for another 18 hrs. The effect of siRNAs (30 nM) used for knock-down was validated using real-time PCR and western blot analysis. Rabbit α-tubulin antibody was used as an endogenous control to normalize the expression level of the target protein.

3.3.6 Infectivity Assay: Briefly ~0.5 x 10^5 THP-1 and J774A.1 cells (both wild type and *blimp-1* knocked down) were seeded in RPMI-1640 media and DMEM, respectively in 12-well plate and incubated at 37^{o}C, 5% CO_2 for 24 hrs. PMA (100 ng/ml) was used for THP-1 cells to differentiate into macrophages. 1x PBS was used to wash the cells thrice and infected

with *L. donovani* promastigotes at a different MOIs 1:2 and 1:5 (macrophage: parasites) for 24 hrs at 37^{o}C, 5% CO_2. Giemsa stain was used for staining amastigotes and counted randomly inside the macrophages in an inverted microscope under 60x magnification. The infectivity index was calculated according to the formula mentioned below:

Infectivity index = Percentage of infected macrophages x Average number of amastigotes per infected macrophages

3.3.7 Statistical analyses: Different biological replicates were used for all the experiments where statistical data analyses were determined by unpaired t-test in Sigma Plot 10 software. All the data are represented by mean ± SEM and a "p" value of less than 0.05 has been considered significant. The western blot data were analyzed using densitometry where a normalization factor was calculated for the loading control (α-tubulin) to normalize the signals of the target proteins.

3.4 Results

3.4.1 Inverse relationship between BLIMP-1 and NFκβ expression during *L. donovani* infection: In order to determine the effect of a transcriptional repressor, BLIMP-1 on NFκβ dependent NLRP3 inflammasome activation during infection, studies have been formulated to verify any correlation between BLIMP-1 and NFκβ expression pattern. The increased level of *blimp-1* mRNA expression (~1.5 folds in THP-1 cells and ~3 folds in J774A.1 cells) after 24 hr of *L. donovani* infection was found as compared to uninfected cells (Figure 3.1A). This finding was further validated by assessing the BLIMP-1 protein expression in the infected cells. A significant increase in BLIMP-1 expression (~3 folds in THP-1 cells and ~5 folds in J774A.1 cells) in the infected cells as compared to uninfected cells was observed after 24 hr (Figure 3.1B & 3.1C). Here we have observed isoform 1 of BLIMP-1 which is the canonical form out of 3 isoforms. Moreover, decrease in NFκβ (p65) expression in the infected samples (~3.5 folds in THP-1 cells and ~2.5 folds in J774A.1 cells) was observed after 24 hr in contrast to increased BLIMP-1 expression (Figure 3.1D & 3.1E). Thus, we can assume that there is a reciprocal relationship between BLIMP-1 and NFκβ expression during infection and this might shed light on the role of BLIMP-1 in controlling parasite killing and evading the host immune responses.

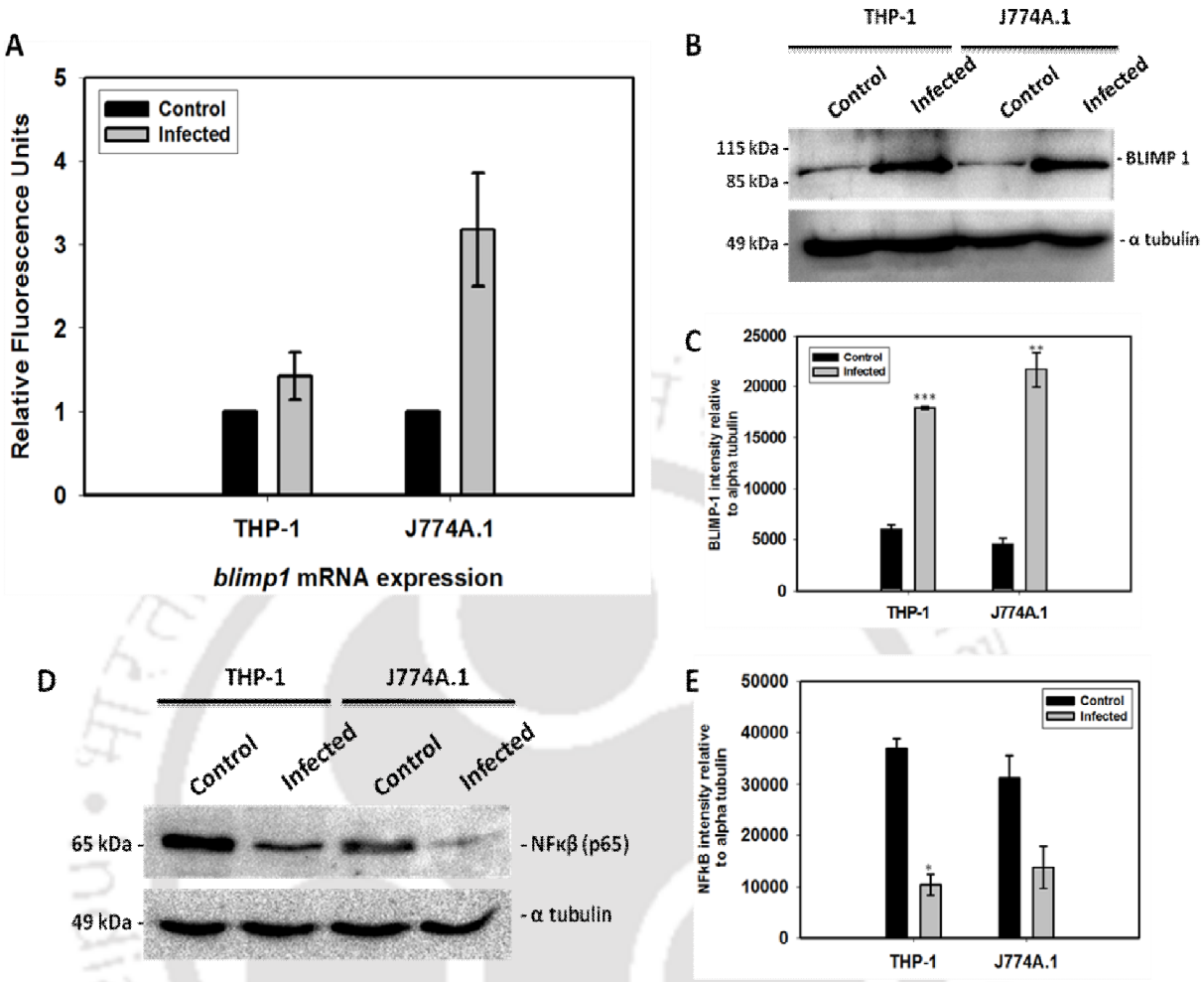

Figure 3.1: Immunoblotting showing inverse relationship between BLIMP-1 and NFκβ expression during *L. donovani* infection. (A) The transcriptional analyses of *blimp-1* in both THP-1 (human) and J774A.1 (mouse) cell lines using Real-Time PCR; (B) The higher expression of BLIMP-1 protein (~95kDa) in both THP-1 and J774A.1 cell lines infected with promastigotes; (C) The densitometry plot for BLIMP-1 expression, normalized by an endogenous control, α-tubulin; (D) Decrease in NFκβ expression in the infected samples has been observed in both the cell lines by western blotting; (E) The densitometry plot for NFκβ expression, normalized by an endogenous control, α-tubulin; (Note: Promastigote infection was incubated for 24 hr; n=3, where n represents three different independent experimental replicates for all the experiments; * denotes $p<0.05$, ** denotes $p<0.005$, *** denotes $p<0.001$).

3.4.2 Impaired Th1/Th2 balance during *L. donovani* infection: We have quantified the release of specific cytokines by the effector cells, Th1 (IFN-γ, TNF-α) and Th2 (IL-10, IL-4) in the culture supernatants during infection and compared with the uninfected cells using sandwich ELISA. A significant decrease in the release of IFN-γ (~8 folds in THP-1 cells and ~20 folds in J774A.1 cells) and TNF-α (~3 folds in THP-1 cells and ~4 folds in J774A.1 cells) was observed in the infected cells as compared to uninfected cells (Figure 3.2A &

3.2B). In contrast, an increase in the release of IL-4 (~2.5 folds in THP-1 cells and ~2 folds in J774A.1 cells) and IL-10 (~4 folds in THP-1 and ~5 folds in J774A.1 cells) was measured in the infected cells as compared to uninfected cells (Figure 3.2C & 3.2D). The data supports the fact that these parasites impair the Th1/Th2 balance in the host cells and curbs toward Th2 driven anti-inflammatory response. This can be further attributed to the fact that up-regulation of BLIMP-1 during infection might be responsible for directing the release of anti-inflammatory cytokines (IL-4, IL-10) in order to protect their replicative niche and enhance their survival period.

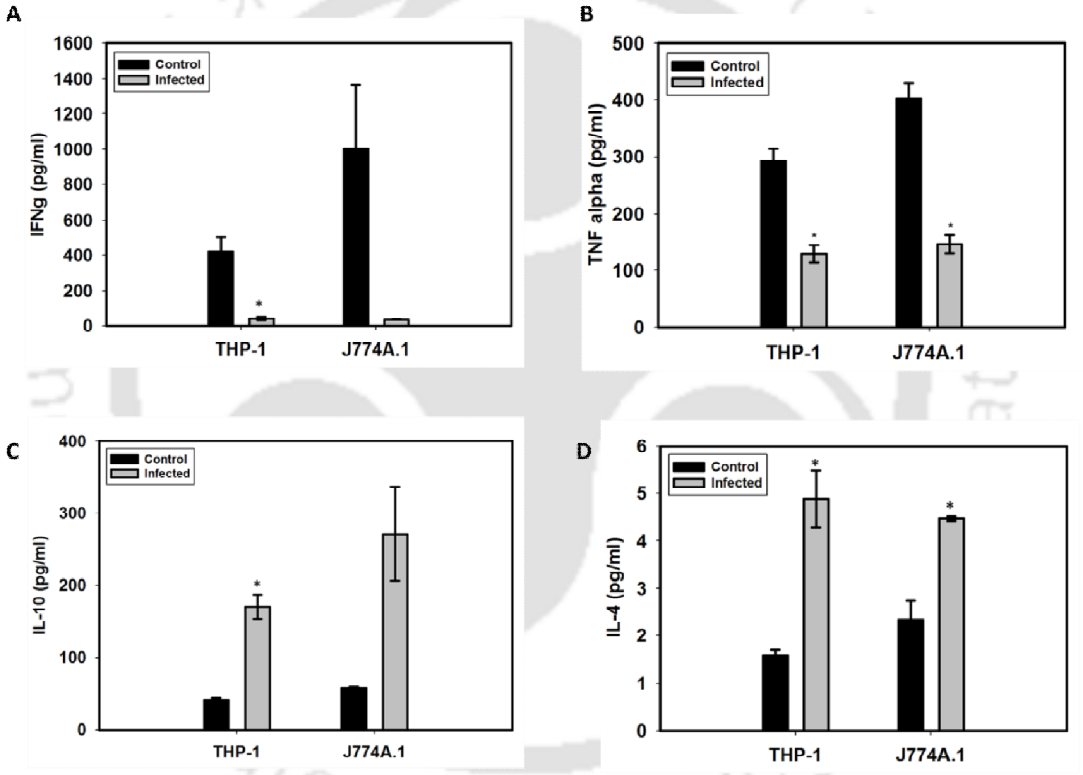

Figure 3.2: Impaired Th1/Th2 dichotomy during *L. donovani* infection has been verified by measuring release of pro-inflammatory (A) IFN-γ, (B) TNF-α, and anti-inflammatory (C) IL-4, (D) IL-10 cytokines using sandwich ELISA. (Note: n=3, where n represents three different independent experimental replicates for all the experiments; * denotes $p<0.05$, ** denotes $p<0.005$, *** denotes $p<0.001$).

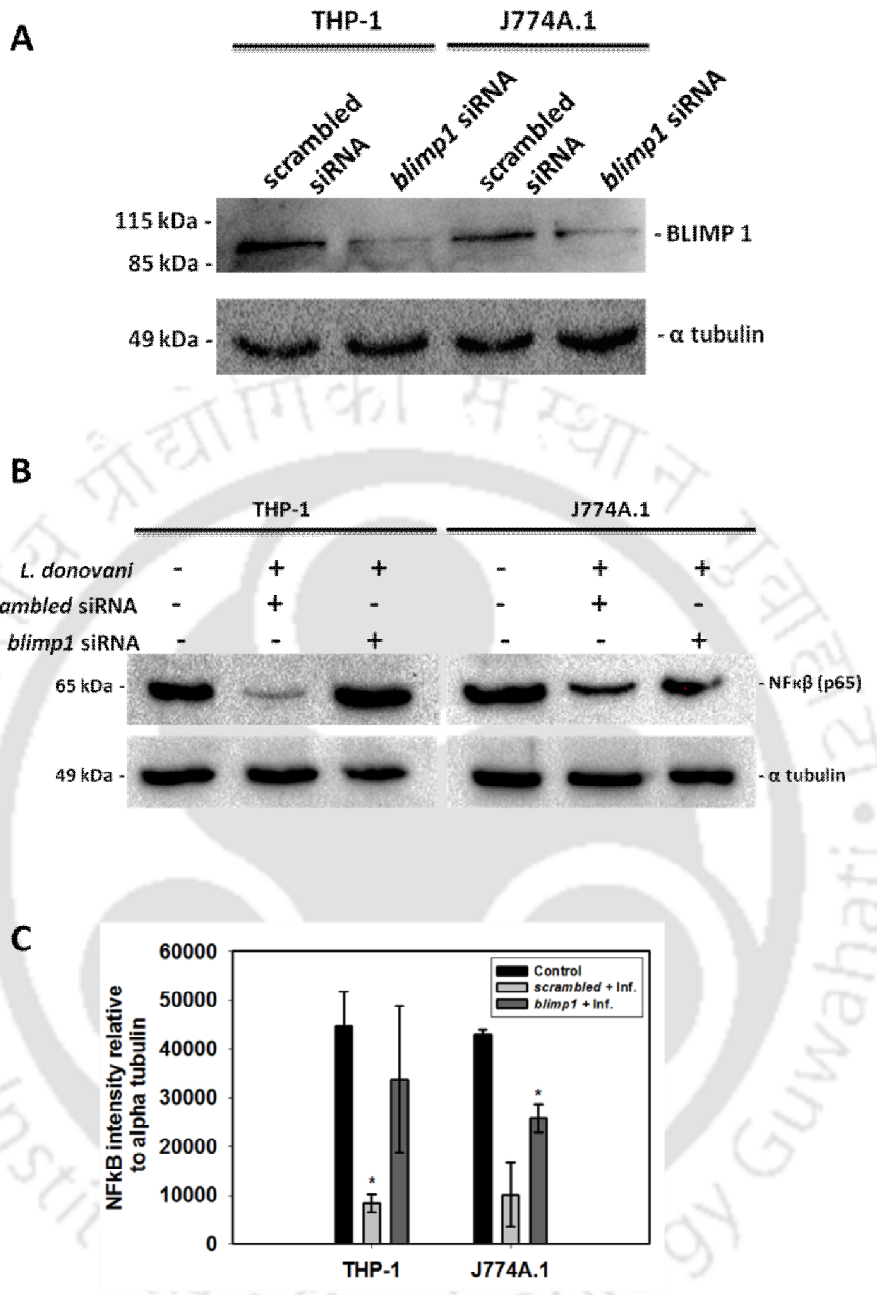

Figure 3.3: *blimp-1* deficiency reverses NFκβ expression using siRNA mediated *blimp1* knock-down in both THP-1 and J774A.1 cell lines. Knock-down has been confirmed by using (A) western blotting; (B) reversal of NFκβ expression at basal level in infected *blimp1* deficient cell as compared to wild type infected cell; (C) The densitometry plot has been shown for NFκβ expression which has been normalized by an endogenous control, α-tubulin; (Note: Promastigote infection was incubated for 24 hr; blimp1 knock down validation was done twice using western blotting; n=3, where n represents three different independent experimental replicates for all the experiments; * denotes $p<0.05$, ** denotes $p<0.005$, *** denotes $p<0.001$)

3.4.3 *blimp-1* deficiency reverses NFκβ expression during infection: In order to validate the importance of BLIMP-1 in the pathogenesis of leishmaniasis, *blimp-1* deficient cell was analyzed to determine the relationship with NFκβ activation pathway. The expression of BLIMP-1 was knocked down using specific *blimp-1* siRNA in both THP-1 and J774A.1 cells. The knock-down of BLIMP-1 expression was confirmed and shown by western blot analyses (Figure 3.3A). The *blimp-1* knocked down cells was infected with promastigotes for 24 hr to study and compare the expression pattern of NFκβ in these cells with that of wild type infected cells. Interestingly, it was found that the expression level of NFκβ was regained in infected *blimp-1* deficient cells (~3 folds in THP-1 cells and ~2.5 folds in J774A.1 cells) as compared to wild type infected cells (Figure 3.3B & 3.3C). The wild type cells were first treated with *scrambled* siRNA followed by infection. This resumption of NFκβ expression in the absence of BLIMP-1 validates their negative feedback regulation during infection and strongly supports our hypothesis on how these parasites over-express BLIMP-1 to dampen the host immune response.

3.4.4 Reduced infectivity index in *blimp-1* deficient cells: Parasite killing was evident from reduced infectivity index in *blimp-1* deficient cells infected with promastigotes at different MOIs and at different time intervals (Figure 3.4A & 3.4B). There is also decrease in the percentage of infected macrophages for both THP-1 and J774A.1 cells compared to controls at all time points (6, 12, 24 hrs) as well as for both MOIs (1:2 and1:5) (Figure 3.4C & 3.4D). There is a decrease in the amastigotes count per infected THP-1 cells for MOI=1:2 at 12 hr and 24 hr as compared to 6 hr, although at MOI=1:5, the count increases slightly (Figure 3.4E). The amastigotes count per infected J774A.1 cells remains almost equal as compared to wild type cells for MOI=1:2 at 12 hr and 24 hr, although at MOI=1:5, the count increases (Figure 3.4F). In the calculation of amastigotes count per infected cells, we are not taking into account the number of uninfected cells whereas for the calculation of % infected cells, we are using the number of uninfected cells in our calculation. This is the reason why we are getting anomalous results from amastigotes count per infected cells. Moreover, when amastigotes count per infected macrophage gets multiplied by % infected macrophages gives the infectivity index which actually measures the infectivity of the parasites.

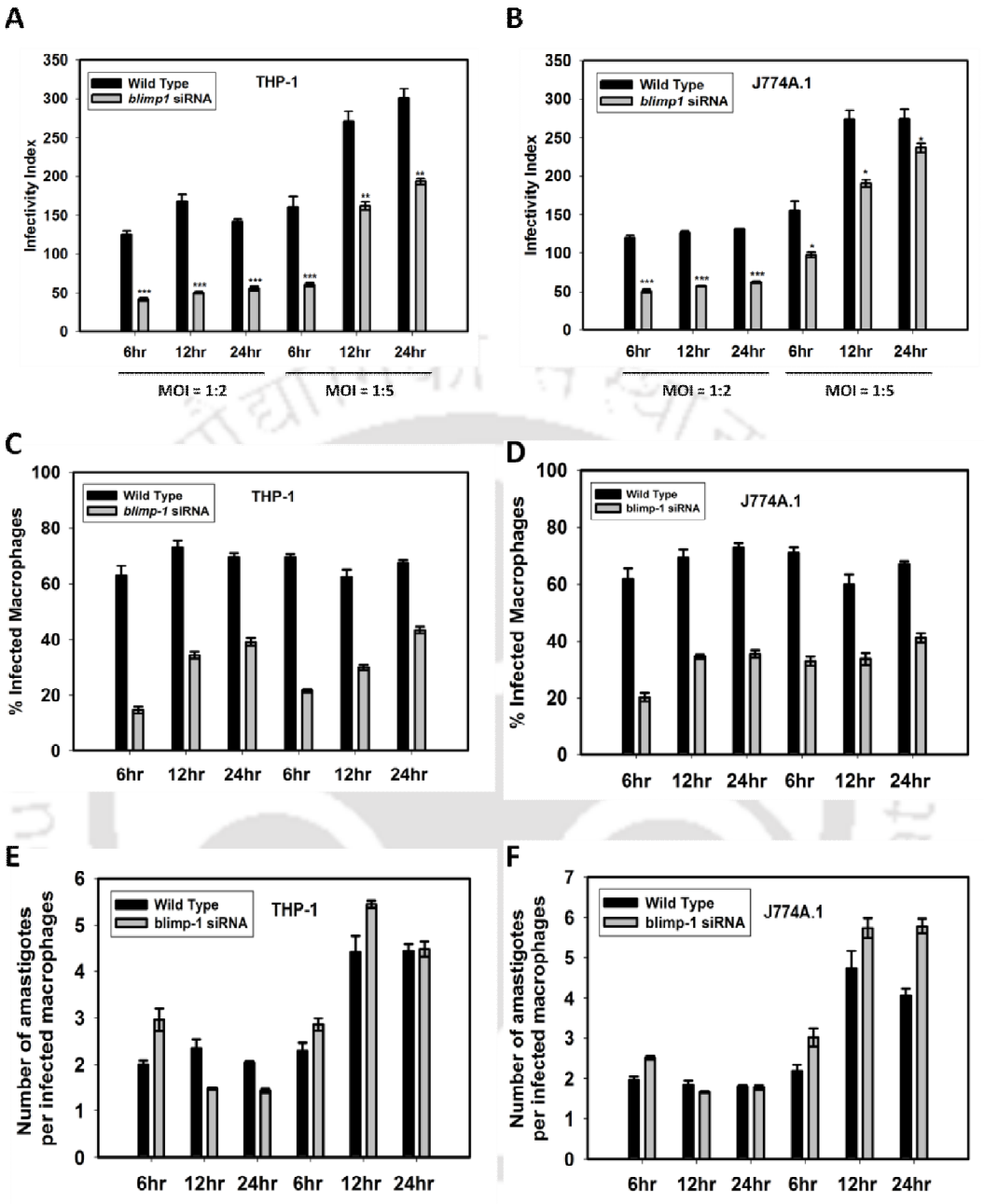

Figure 3.4: Infectivity index in *blimp1* deficient cells infected with promastigotes at different MOIs and at different time intervals was analyzed. (A) Infectivity index of THP-1 cells, (B) Infectivity index of J774A.1 cells, (C) Percentage of infected cells in THP-1 cells, (D) Percentage of infected cells in J774A.1 cells, (E) Number of amastigotes per infected macrophage in THP-1 cells, (F) Number of amastigotes per infected macrophage in J774A.1 cells. (Note: n=3, where n represents three different independent experimental replicates for all the experiments; * denotes $p<0.05$, ** denotes $p<0.005$, *** denotes $p<0.001$)

3.5 Discussion & Conclusion

Pathogenic infections generally persist by evading various immune surveillance of host cells. These immune responses are dodged silently by pathogens for their long term survival inside their replicative niche (Skeldon and Saleh, 2011). Likewise, *Leishmania* parasites overcome these pro-inflammatory attacks by modulating various key signaling pathways and thereby blocking or inhibiting the release of IFN-γ and other pro-inflammatory cytokines (Gurung and Kanneganti, 2015a). NFκβ activation via TLR4/Myd88 pathway is a crucial defense mechanism to kill the invading pathogens including parasites (Reddick and Alto, 2014, Nelson and Nelson, 2018). A plethora of research works has been reported in terms of NFκβ activation defending against intracellular and extracellular pathogens (Rahman and McFadden, 2011, Vitiello et al., 2012).

NFκβ regulates the expression of inflammasome components by activation and translocation to the nucleus, inducing the transcription of NLRP3 and other pro-inflammatory cytokines (IL-1β and IL-18). Finally, it primes the NLRP3 inflammasome activation which in turn initiates various local and systemic inflammatory reactions. The NLRP3 inflammasome is a complex multi-protein assembly and gets activated by various stimuli followed by inflammation (Schroder and Tschopp, 2010). Interestingly, report has been found that the activation of NLRP3 expression is NFκβ dependent after induction with TLR agonists and is mediated through binding of NFκβ to NFκβ binding sequences in the NLRP3 promoter (Qiao et al., 2012). We have previously reported that *Leishmania* parasites evade the caspase 1 dependent host inflammatory response by circumventing the pyroptotic pathway in a effort to favor their survival (Saha et al., 2019). However, it is debatable, how these parasites modulate the inflammatory cell death pathway in addition to caspase 1 inactivation.

In the current study, we have shown that transcriptional repressor molecule, BLIMP-1 regulates macrophage pyroptosis during *Leishmania* parasite's growth within the macrophage. A dependency of NFκβ for NLRP3 signaling pathway is cross-regulated by BLIMP-1, finally leading to the increased parasitic burden upon infection. The down-regulation of NFκβ expression is inversely correlated with the increased production of BLIMP-1 in infected cells. In addition, Th1/Th2 balance has also been compromised during infection leading to tampering of the NFκβ – NLRP3 signaling pathway. The reciprocal relationship between BLIMP-1 and NFκβ expression pattern in infected cells after 24 hr has

been confirmed and validated by siRNA mediated *blimp-1* knock down study. The infected *blimp-1* deficient cells show higher NFκβ expression level as compared to wild type cells treated with scrambled siRNA followed by infection after 24 hr. There is a decrease in infectivity index in *blimp-1* knocked down cells at different time intervals for different MOIs as compared to wild type cells. Although there is an anomaly between the results of percentage of infected macrophages and number of amastigotes count per infected macrophages, their product gives the infectivity index which actually measures the infectivity of the parasites as per previous reports (Saudagar et al., 2013b, Das et al., 2015). There might be a possibility of lack of infectivity or the host cells can control the parasite counts or its multiplicity in a better way in *blimp-1* deficient cells due to some unknown signaling effects. This also highlights the fact that the parasites exploit host protein BLIMP-1 for their survival.

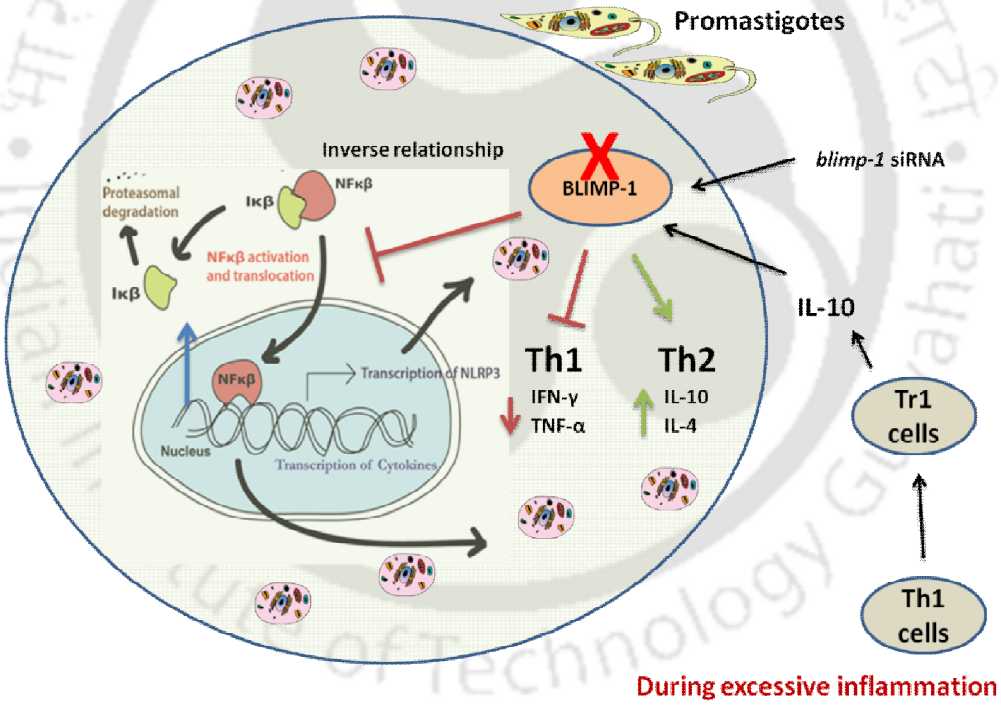

Figure 3.5: Schematic representation of interrupted NFκβ activation followed by NLRP3 inflammasome formation and activation by higher BLIMP-1 production in infected cells

The current findings also advocate a fact that the parasites exploit a fundamental self-defending mechanism of the host cells when excessive inflammation causes host tissue damage as per the previous report. In this situation, host cells direct the Th1 cells to convert

into Tr1 cells which releases IL-10, an anti-inflammatory cytokine which depends on BLIMP-1 production (Montes de Oca et al., 2016, Neumann et al., 2014). Thus, regulation of NFκβ activation followed by NLRP3 inflammasome formation and activation is primarily controlled and orchestrated by higher BLIMP-1 production and finally disrupting an inflammation-mediated pyroptosis cell death pathway in infected cells (Figure 3.5). Whether the parasites can independently inhibit NFκβ and up regulate BLIMP-1 has been shown to be directly linked but exact mechanism behind this phenomenon is still under question. BLIMP-1 has a binding site for NFκβ as per previous report (Morgan et al., 2009) but the reverse is not true suggesting the relationship between the two genes could be quite complex with the opportunity for feedback loops. Therefore, it is critical to know, does BLIMP-1 directly bind to the NFκβ promoter to reduce gene expression, or is this an indirect effect through other signaling pathways. Further studies are needed to get new insight as to how these parasites take advantage of BLIMP-1 induction and tailoring the NFκβ – NLRP3 signaling pathway as per their convenient in the regulation and management of this disease.

CHAPTER 4

BLIMP-1: A negative regulator in the regulation of host macrophage pyroptosis mediated cell death during infection

4.1 Abstract

The immunosuppressive mechanism adopted by the parasite of the genus *Leishmania* is the root of different clinical manifestations. The primary focus of our research is to decode a novel mechanism underlying the inhibition of NLRP3 inflammasome formation mediated by higher BLIMP-1 expression. We have reported for the first time that BLIMP-1 acts as a negative regulator of host pyroptosis mediated cell death during *L. donovani*. The inhibition of cellular death of infected cells by BLIMP-1 has been verified by using siRNA knock down assay. Surprisingly, the pro-inflammatory responses were resumed in *blimp-1* deficient cells infected with promastigotes and thereby increase in caspase 1 activity and LDH release were observed. This resumption of macrophage pyroptosis in *blimp-1* deficient cells infected with promastigotes conclude this host BLIMP-1 protein a key mediator in impairing NLRP3 inflammasome activation.

4.2 Introduction

Pathogens have evolved to circumvent, escape and conquer many of the antimicrobial strategies mounted by macrophages by living inside the cells, obtaining nutrients and proliferate. *Leishmania* parasites, which causes leishmaniasis, has successfully adapted to live inside the host macrophage cells and replicate. These intracellular parasites cause cutaneous, mucocutaneous and visceral pathologies in humans and other animals. *Leishmania*-macrophage interactions are multifaceted and involve a number of pathogenicity factors that allow parasites to use the phagolysosome to obtain nutrients, hijack antimicrobial pathways, and replicate (Podinovskaia and Descoteaux, 2015). With reference to the plethora of reports in last decade, NLRP3 inflammasome is one of the most characterized inflammasome playing a critical role in the pathogenesis of visceral leishmaniasis (VL) (Saha et al., 2019, Shio et al., 2015, Zamboni and Lima-Junior, 2015). Upon NLRP3 inflammasome activation by oligomerization of three components namely NLRP3, ASC and procaspase 1, the processing of proinflammatory cytokines via self – cleavage of pro-caspase 1 into their mature subunits (p10/p20) triggers pyroptosis mediated cell death pathway (Swanson et al., 2019, Schroder and Tschopp, 2010). The transcriptional regulator BLIMP-1 has recently been implicated in the generation of IL-10-producing Tr1 cells (Montes de Oca et al., 2016). Thus, BLIMP-1 expression in T cells enhanced parasite growth and was critical for the generation of IL-10-producing Tr1 cells during experimental malaria. During *Leishmania* infection, the whole NLRP3 inflammasome formation and activation is interrupted due to caspase 1 inactivation (Saha et al., 2019). This interruption has also been further found to be mediated by higher BLIMP-1 expression during infection which helps parasite survival inside their replicative niche. This BLIMP-1 has been shown to downregulate NFκβ activation and thereby tweak the NFκβ – NLRP3 signaling cascade by suppressing pro-inflammatory response (Unpublished). Recently, the role of B-cell dysfunctions in VL pathogenesis in terms of the phenotypic and functional properties of B-cells has been explored during the course of disease (Singh et al., 2019). Moreover, B-cells had compromised abilities of antigen processing and presentation and altered levels of BLIMP-1 ranging from antibody production to antigen presentation and have additional vital roles in immune mechanisms.

Here we report for the first time the role of BLIMP-1 in the suppression of macrophage pyroptosis during *L. donovani* infection *in-vitro* and thereby tweaking the tight regulation of NFκβ – NLRP3 signaling pathway. In *blimp1* deficient cells, we found resumption of pyroptosis in the infected cells and thus validated our findings in evaluating negative role of BLIMP-1 in the regulation of macrophage pyroptosis during *L. donovani* infection. This work unravels the importance of BLIMP-1 in reversing the pro-inflammatory reactions and arresting pyroptosis pathway mediated by NFκβ – NLRP3 signaling pathway.

4.3 Materials & Methods

4.3.1 Parasite and other materials: *L. donovani* culture (BHU-1081 strain) was a kind gift from Prof. Shyam Sundar, Institute of Medical Sciences, Banaras Hindu University. Procedure for *L. donovani* culture has been done and followed as per protocol (Das et al., 2015). In brief, *Leishmania* promastigotes are cultured at 28^{o}C in M199 liquid media supplemented with 10% fetal bovine serum (FBS), 10,000 U/ml penicillin and 10 mg/ml streptomycin. Human monocytic cell line (THP-1) and mouse macrophage cell line (J774A.1) were procured from National Centre for Cell Sciences, Pune and cultured at 37^{o}C and 5% CO_2 in Roswell Park Memorial Institute (RPMI-1640) media and Dulbecco's Modified Eagle's Medium (DMEM) respectively supplemented with 10% FBS, 2 mM glutamine, 10,000 U/ml penicillin, 10 mg/ml streptomycin and 25 µg/ml amphotericin B. Phorbol 12-myristate 13-acetate (PMA) was used at a concentration of 100 ng/ml to differentiate monocytic THP-1 cells into macrophages at 37^{o}C and 5% CO_2 overnight.

4.3.2 Gene expression analysis using qRT-PCR: RNeasy Mini Kit (QIAGEN) protocol was used for isolating total RNA and quantified using Nano-Drop after DNase treatment for 1 hr at 37^{o}C. An equal amount of RNA was used to synthesize cDNA using ProtoScript® First Strand cDNA Synthesis Kit (New England Biolabs). THP-1 and J774A.1 cells were used for the analysis of IL-1β and IL-18 with specific primers using qRT-PCR. For all the qRT-PCR experiments, *β-actin* gene was used as endogenous housekeeping control to normalize the relative fluorescence signal of the target genes. The sequences of all the primers used are listed in Table 4.1.

Table 4.1: Primer sequences used for Real Time PCR experiments (Chapter 4)

Primers	**Sequences (5' – 3')**
*IL-1β*_FP	GCTGGAGAGTGTAGATCCCAAA
*IL-1β*_RP	GCTGGAGAGTGTAGATCCCAAA
*IL-18*_FP	GCTGCTGAACCAGTAGAAGACA
*IL-18*_RP	ATAGAGGCCGATTTCCTTGG
*β-actin*_FP	CTGGCACCCAGCACAATG
*β-actin*_RP	GCCGATCCACACGGAGTACT

4.3.3 Sandwich ELISA: Culture supernatants of THP-1 and J774A.1 cells were used to quantify the release of various cytokines like IFN-γ, TNF-α, IL-1β and IL-18 using sandwich ELISA as per standard protocol (Saha et al., 2019). Briefly, capture antibody coating was done in 96-well plates for IFN-γ, TNF-α, IL-1β and IL-18 antibodies and incubated overnight at 4^{o}C. The coating buffer used was carbonate-bicarbonate buffer (pH 9.5). Then washing was done thoroughly with 1x TBST followed by 1x TBS twice. Blocking was done using 3% BSA in coating buffer for 2 hrs followed by washing thoroughly. Equal volume of culture supernatants were added in all the wells and incubated for 6 hrs at RT. Subsequently washing was done followed by detection antibody coating for IFN-γ, TNF-α, IL-1β and IL-18 antibodies. Next secondary antibody conjugated to HRP was added after washing thoroughly and incubated for another 1 hr at RT. Final washing was done and TMB substrate was used for colorimetric detection. H_2SO_4 (2M) was used as a stop solution which changes the color from blue to yellow. Absorbance at 460 nm was taken in multi-reader ELISA plate. Secondary antibody controls were also made to nullify the background signals. The quantification of IFN-γ, TNF-α, IL-1β and IL-18 antibodies were done using a standard graph for the above cytokines. Here capture and detection antibodies for IFN-γ, TNF-α, IL-1β and IL-18 used were same and thus it is a "self-sandwich" ELISA. The TMB substrate was purchased from Thermo Scientific.

4.3.4 siRNA mediated *blimp-1* knock-down assay: Briefly ~2 x 10^5 cells (THP-1 and J774A.1) were seeded in RPMI-1640 media and DMEM, respectively in 35 mm petri-dishes and incubated at 37^{o}C, 5% CO_2 for 24 hrs. PMA (100 ng/ml) was used for THP-1 cells to differentiate into macrophages. Serum-free Opti-MEM media was used to prepare siRNA-lipid mix and incubated the cells for 6 hr followed by addition of complete media and finally

incubated at 37^{o}C, 5% CO_2 for another 18 hr. The effect of siRNAs (30 nM) used for knock-down was validated using real-time PCR and western blot analysis. Rabbit α-tubulin antibody was used as an endogenous control to normalize the expression level of the target protein.

4.3.5 Lactate Dehydrogenase (LDH) assay: The culture supernatants of THP-1 and J774A.1 cells were harvested to quantify the release of LDH out of the cells. Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific) was used to detect the LDH release as per the manufacturer's protocol.

4.3.6 Fluorescence caspase 1 activity assay: Briefly, ~2 x 10^5 cells (each THP-1 and J774A.1) were seeded in 6 well plates and incubated overnight in 5% CO_2 at 37^{o}C. 100ng/ml of PMA was added to THP-1 cells for differentiation. Next siRNA treatment was given as per manufacturer's protocol followed by promastigote infection. Total cellular protein was used and extracted in PBS buffer with mild sonication for 2 min at 4^{o}C and quantified using standard Bradford assay (Bradford, 1976). Caspase 1 activity was measured at different time intervals (3 hrs, 6 hrs, 12 hrs, and 24 hrs) using commercially available caspase-1 specific substrate (Ac-WVAD-AMC). The cleavage of the substrate generate fluorescence proportionately whose relative fluorescence intensity was measured fluorometrically at E_{ex}/E_{em} - 380/460 nm. Caspase 1 inhibitor (Ac-YVAD-CMK) was also used in the experimental setups after cell lysate preparation.

4.3.7 Statistical analyses: Different biological replicates were used for all the experiments where statistical data analyses were determined by unpaired t-test in Sigma Plot 10 software. All the data are represented by mean ± SEM and a "p" value of less than 0.05 has been considered significant. The western blot data were analyzed using densitometry where a normalization factor was calculated for the loading control (α-tubulin) to normalize the signals of the target proteins.

4.4 Results

4.4.1 Pro-inflammatory reaction drives parasite killing in *blimp-1* deficient cells: Th1 responses are generated from activated macrophages and dendritic cells and help in controlling infection by producing IFN-γ and TNF-α (Spellberg and Edwards, 2001). In order

to dampen pro-inflammatory reactions, parasites induce the over-expression of BLIMP-1 which negatively regulate the IFN-γ and other pro-inflammatory cytokines (Neumann et al., 2014). In order to validate the importance of BLIMP-1 in the pathogenesis of leishmaniasis, *blimp-1* deficient cell was analyzed to determine Th1/Th2 balance during infection. The parasite impairs Th1/Th2 balance in the host cells and curbs towards Th2 mediated anti-inflammatory responses and has been shown with the experiments as well in the previous chapter (Unpublished).). In this regard, we thought of assessing the release of two specific cytokines (IFN-γ and TNF-α) in the culture supernatants of *blimp-1* deficient cells infected with promastigotes. We found significant surge in the level of proinflammatory cytokines IFN-γ (~11 folds in THP-1 cells and ~4 folds in J774A.1 cells) and TNF-α (~5 folds in THP-1 cells and ~6 folds in J774A.1 cells) in *blimp-1* deficient cells infected with promastigote as compared to wild type (THP-1 and J774A.1) cells treated with *scrambled* siRNA and then infected with promastigote (Figure 4.1A & 4.1B). These data substantiate the possibility of BLIMP-1 involvement in protecting the parasites from hostile immune surveillance. The increase in pro-inflammatory response in *blimp-1* deficient cells infected with promastigote also favors parasite killing where there is a significant decrease in parasitic burden in *blimp-1* deficient cells as compared to wild type cells infected with promastigote (discussed in previous chapter – data unpublished).

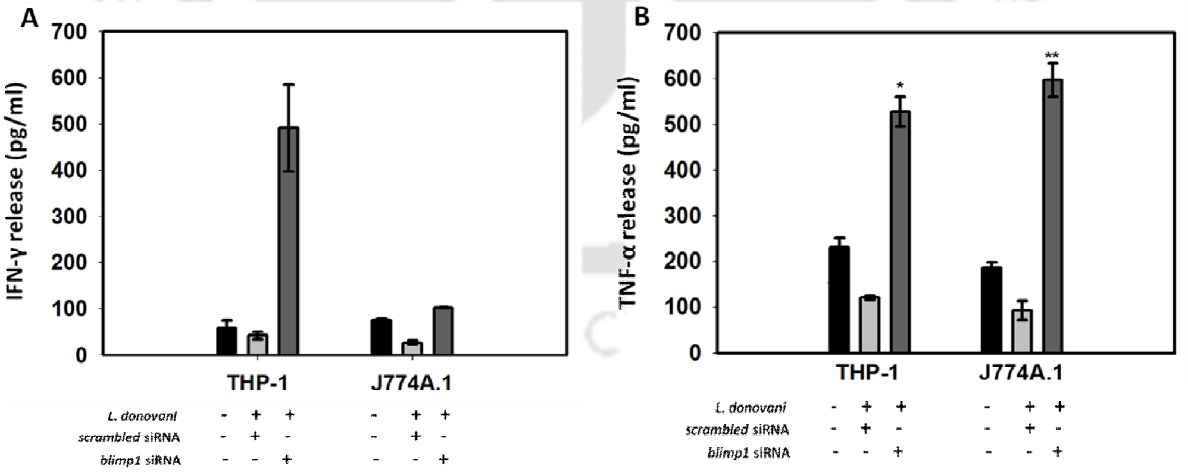

Figure 4.1: There is a resumption of (A) IFN-γ and (B) TNF-α release in *blimp1* deficient cells infected with promastigotes in comparison to wild type cells treated with scrambled siRNA and then infected with promastigote. (Note: n=3, where n represents three different independent experimental replicates for all the experiments; * denotes $p<0.05$, ** denotes $p<0.005$, *** denotes $p<0.001$)

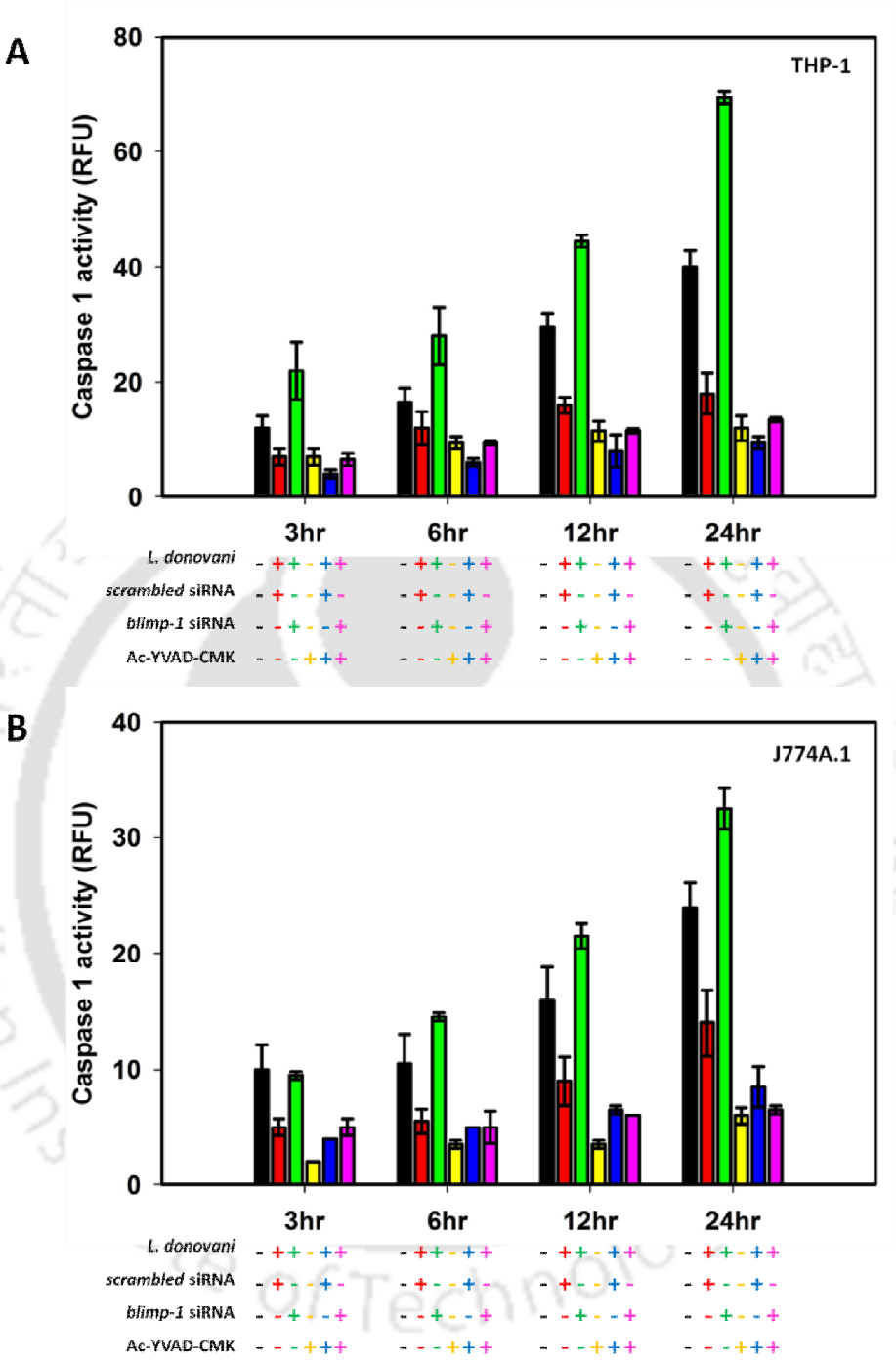

Figure 4.2: Increase in caspase 1 activity in *blimp-1* deficient cells infected with promastigotes in comparison to wild type cells treated with scrambled siRNA followed by infection has been shown in (A) THP-1 cells and (B) J774A.1 cells at different time intervals. Caspase 1 inhibitor (Ac-YVAD-CMK) was also used in the experimental setups to nullify the effect of caspase 1 activity. (Note: RFU = relative fluorescence units; n=2, where n represents two different independent experimental replicates for all the experiments)

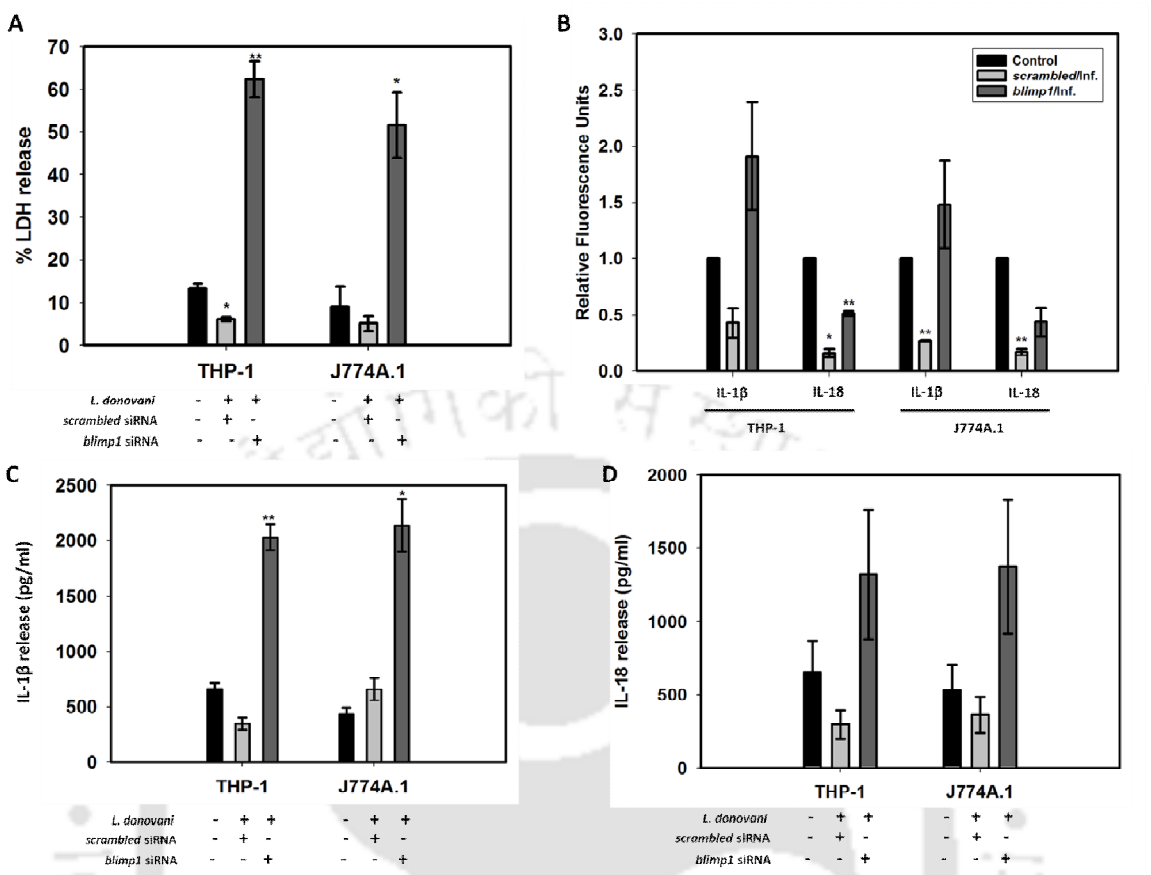

Figure 4.3: Pyroptosis resumes in *blimp-1* deficient cells infected with promastigotes in comparison to wild type cells treated with scrambled siRNA followed by infection after 24 hr have been verified by showing increased **(A)** percentage LDH release. The transcriptional level of **(B)** IL-1β and IL-18 was also found to increase in blimp-1 deficient cells infected with promastigotes in comparison to wild type cells treated with scrambled siRNA followed by infection after 24 hr. Moreover, the downstream pathway of caspase 1 activation has also been assessed by sandwich ELISA showing significantly increased release of **(C)** IL-1β and **(D)** IL-18 from culture supernatants of blimp-1 deficient cells infected with promastigotes in comparison to wild type cells treated with scrambled siRNA followed by infection after 24 hr. (Note: LDH: lactate dehydrogenase; n=2 for figure 5B; n=3 for figure 5A, 5C & 5D, where n represents three different independent experimental replicates for all the experiments; * denotes $p<0.05$, ** denotes $p<0.005$, *** denotes $p<0.001$)

4.4.2 Pyroptosis resumes in infected *blimp-1* deficient cells: The priming of NLRP3 inflammasome via NFκβ activation is the primary defense mechanism of the host cells to get rid of pathogenic infections. Since BLIMP-1 has been found as a key mediator in the pathogenesis of leishmanial infection by dampening the pro-inflammatory responses, experiments were designed to evaluate the effect of pyroptosis in *blimp-1* deficient infected cells. Caspase 1 activation and processing of pro-inflammatory cytokines, IL-1β and IL-18 are the key features of pyroptosis along with release of LDH (lactate dehydrogenase) from compromised cell membranes as a proxy to cell death pathway. We found relatively higher

caspase 1 activity in *blimp-1* deficient cells infected with promastigotes as compared to wild type cells treated with *scrambled* siRNA followed by infection (Figure 4.2A & 4.2B).

This observation was further confirmed by measuring a change in LDH release from the culture supernatants of infected macrophages. There was a significant increase in LDH release from the compromised cellular membranes (~6 folds in THP-1 cells and ~5 folds in J774A.1 cells) of *blimp-1* deficient cells infected with promastigotes for 24 hr as compared to wild type cells treated with *scrambled* siRNA followed by infection (Figure 4.3A). Next we thought of analyzing the level of pro-inflammatory cytokines, IL-1β and IL-18 in *blimp-1* deficient cells infected with promastigotes. To our surprise, the mRNA levels of both the cytokines shoots up in *blimp-1* deficient cells infected with promastigotes as compared to wild type cells treated with *scrambled* siRNA followed by infection after 24 hr (Figure 4.3B). As expected, there were also increased release of IL-1β (~5 folds in THP-1 cells and ~4 folds in J774A.1 cells) and IL-18 (~5 folds in THP-1 cells and ~4.5 folds in J774A.1 cells) in infected *blimp-1* deficient cells as compared to wild type cells treated with *scrambled* siRNA followed by infection after 24 hr (Figure 4.3C & 4.3D).

4.5 Discussions & Conclusion

Cellular death is a fundamental biological phenomenon that is essential for the survival and development of an organism. There are myriads of reports indicating that cell death contributes to immune defense against infectious diseases. All forms of cell death processes like apoptosis, necrosis, autophagy and pyroptosis have been playing a crucial role in the pathogenesis of any infection. The fate of life and death of an infected cell depends on the microenvironment of the cell and how Th1/Th2 immune response gets controlled by the pathogens. Pyroptosis is a form of inflammatory programmed cell death pathway activated by human and mouse caspase-1, human caspase-4 and caspase-5, or mouse caspase-11 (Bergsbaken et al., 2009). These inflammatory caspases are used by the host as a defense tools to control bacterial, viral, fungal or protozoan pathogenic infections. It requires cleavage and activation of the pore-forming effector protein gasdermin D by inflammatory caspases like caspase 1 (Man et al., 2017). The whole process is dependent on the activation of NLRP3 inflammsome mediated by caspase 1 maturation leading to physical rupture of the cell and release of the pro-inflammatory cytokines IL-1β and IL-18, alarmins and

endogenous danger-associated molecular patterns, signifying the inflammatory potential of pyroptosis. Here, we describe the central role of inflammatory caspases and pyroptosis in mediating immunity to infection and clearance of pathogens.

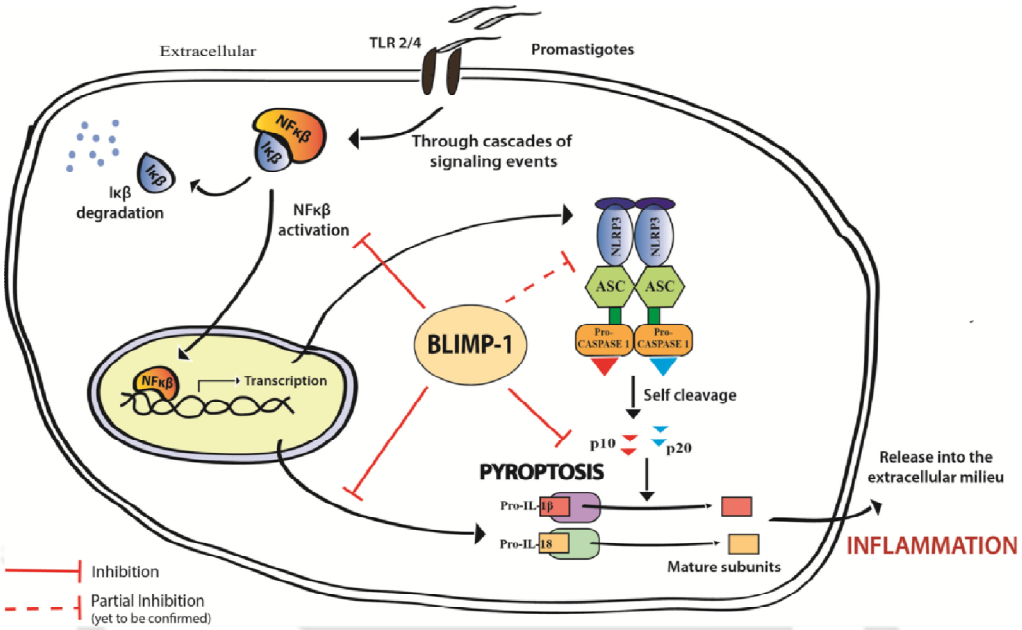

Figure 4.4: Schematic representation of BLIMP-1 regulating pyroptosis during *L. donovani* infection. BLIMP-1 has been shown to involve in inhibiting various steps of NFκβ – NLRP3 signaling pathway. The inhibition of a pyroptosis in infected cells can be attributed to the cumulative effect of all the upstream negative regulations of the molecules in the pathway by BLIMP-1 expression. Further in-depth studies are required to decipher how these parasites take advantage of BLIMP-1 over-expression and tailor the NFκβ – NLRP3 signaling pathway in their favor to increase their survival. This hypothesis will help in designing drug molecules in the regulation and management of *kala-azar*.

Here we have reported an interesting study about how BLIMP-1 protein regulates macrophage pyroptosis during *Leishmania* parasite's growth within the macrophage. We have reported the resurgence of pro-inflammatory responses in *blimp-1* deficient cells infected with promastigotes after 24 hr, driving parasite killing or control and ultimately establishes the role of BLIMP-1 in favoring parasite survival inside the host cells (Figure 4.1). The effect of BLIMP-1 in inhibiting the process of inflammation has been further verified by assessing the quantification of signature molecules of pyroptosis. The downstream effect of dampened NFκβ expression in infected cells on NLRP3 inflammasome activation has been tailored by the parasites in their favor by inducing BLIMP-1 over-expression. Interestingly, we found more caspase 1 activity (Figure 4.2) and a higher

percentage of LDH release in infected *blimp-1* deficient cells as compared to wild type cells treated with scrambled siRNA followed by infection after 24 hr (Figure 4.3A). The transcriptional level (Figure 4.3B) and finally maturation of pro-IL-1β and pro-IL-18 into its active counterpart (Figure 4.3C & 4.3D) was also significantly increased in infected *blimp-1* deficient cells as compared to wild type cells treated with scrambled siRNA followed by infection after 24 hr, validating resumption of pyroptosis. Summarizing all the results, we conjecture that these parasites modulate NFκβ – NLRP3 signaling pathway by prompting BLIMP-1 over-expression to disrupt the Th1/Th2 balance of the host cells and eventually evading pyroptosis of infected cells. Figure 4.4 highlights the current findings and depicts how BLIMP-1 is responsible for cross regulating a tightly regulated pathway crucial for host defense system during pathogenic encounters.

CHAPTER 5

BLIMP-1 mediated downregulation of TAK1 and p53 molecules is responsible for resisting pathogen clearance during *kala-azar*

5.1 Abstract

Precise regulation of inflammasome is critical during any pathogenic encounters and the whole innate immune system comprising of pattern recognition receptors (PRRs) relies on its ability to sense microbes. The fate of cellular death in infected cells depends mostly on the activation of these inflammasome and its dysregulation due to functional manipulation by various pathogens has been the cause of many human diseases as per previous reports. An interesting finding has been observed here related to how *Leishmania donovani* parasites exploit various host mediator molecules to cause immunosuppression. Here we report for the first time that the parasites check pyroptosis in the infected cells *in-vitro* by BLIMP-1 mediated suppression of TAK1 and p53 proteins. This might be one of the reasons how parasite evade the pro-inflammatory response of the host cells. Further understandings and validations are required to come up with better therapeutics approaches against *kala-azar*.

5.2 Introduction

Macrophages are the sentinel immune cells that come in response to pathogenic encounter and direct the innate immune response towards adaptive immune system. The innate immune system relies on the pattern recognition receptors (PRRs) to sense any microbial infection or tissue damage and finally initiate inflammatory reactions by activation of various inflammasomes depending upon the types of pathogen (Newton and Dixit, 2012). Prior to its activation, priming of the signaling cascade is necessary via interaction of pathogenic ligands with host cell surface TLR receptors. Priming leads to the activation of NFκβ and subsequent expression of inflammasome components (Schroder and Tschopp, 2010) and the pro-forms of the inflammatory cytokines, IL-1β and IL-18. Apart from this, priming also modulate the post translational modifications (PTMs) of NLRP3, ASC and pro-caspase 1 to facilitate the assembly of the inflammasome and thus activation (Yang et al., 2016). The NLRP3 inflammasome responds to structurally and chemically diverse stimuli from infections and tissue damage. As per previous report in terms of fungal infections, NLRP3 and ASC has been reported to be phosphorylated by Syk kinase and many other kinases (Gross et al., 2009). Recently, a threonine – serine kinase NEK7 (NIMA Related Kinase 7) was identified by many independent groups to be involved in the activation of NLRP3 inflammasome signaling (He et al., 2016). NEK7 has been reported as NLRP3 binding protein which help in the regulation of NLRP3 oligomerization and activation depending on potassium efflux. Also, intracellular reactive oxygen species (ROS) formation from the mitochondria helps NEK7 to efficiently bind with NLRP3. Whereas through screening of natural products, TAK1 was also identified as a kinase regulating the NLRP3 inflammasome by phosphorylation (Gong et al., 2010). In contrast, recent report says TAK1 control NLRP3 inflammasome homeostasis by restraining inflammation and cell death in myeloid cells (Malireddi et al., 2018). So the role of TAK1 in the regulation of inflammasome is still elusive and needs further investigations. Apart from enzymes involved in the regulation of post-translational modifications of NLRP3 inflammasome, the role of p53 – a tumor suppressor gene in inflammasome regulation cannot be ruled out. There was a report showing that caspase 1 is transcriptionally activated by p53 suggesting its role in inflammation (Gupta et al., 2001). The involvement of p53 gene in various non-canonical cell death pathways have also been discussed apart from apoptosis (Ranjan and Iwakuma, 2016). There is a similar

mechanistic pathway that exist for both p53 and NFκβ activation. The Mdm2 (murine double-minute 2 or human hMdm2) is an E3 ubiquitin ligase that binds to p53 molecule in normal cells and upon stress stimuli, the degradation of Mdm2 allow p53 stabilization, activation and its cellular response (Moll and Petrenko, 2003). On the other hand, NFκβ subunits are held by Iκβ (inhibitor of nuclear - κβ) in the cytoplasm and upon stimulation, Iκβ gets phosphorylated by TAK-1 mediated IKK (inhibitor of nuclear factor - κβ kinase) leading to its proteasomal degradation (Adhikari et al., 2007). This allow NFκβ to translocate into the nucleus for transcription of various pro-inflammatory genes involved in inflammation (Liu et al., 2017). Although p53 and NFκβ have similar activation mechanism, but they cross-talk antagonistically in signaling pathway as per previous report (Huang et al., 2007). But there are reports where both of them co-regulate the pro-inflammatory axis of cell death pathways in macrophages (Ryan et al., 2000, Lowe et al., 2014). So the interaction between p53 and NFκβ are different depending upon the type of cells and cellular microenvironment. Here we have reported for first time the cross talk between NEK7, TAK1, NFκβ and p53 in the context of BLIMP-1 overexpression during *Leishmania donovani* infection. BLIMP-1 is a zinc finger containing DNA binding transcriptional repressor of IFN-β (Huang, 1994) and serves as a master regulator of antibody-producing B-cells, orchestrating T-cell homeostasis (Fu et al., 2017). The role of BLIMP-1 in regulating macrophage pyroptosis during *L. donovani* infection has already been justified (data not published) and how NEK7, TAK1, NFκβ and p53 are cross regulating the pyroptotic cell death pathway has been explored during infection. Our previous report on the impairment of caspase 1 dependent NLRP3 inflammasome activation during *L. donovani* infection (Saha et al., 2019) has been further justified by finding out the roles of how PTM enzymes are pivotal in activating the inflammasome. The data supports the fact that parasites exploit BLIMP-1 upregulation to suppress the expression of TAK1 and p53, thereby tweaking the tightly controlled NFκβ – NLRP3 signaling pathway, inhibiting the intracellular ROS formation and evasion of pyroptosis during infection.

5.3 Materials & Methods

5.3.1 Cell culture and other materials: Briefly, human monocytic cell line (THP-1) and mouse macrophage cell line (J774A.1) were procured from National Centre for Cell

Sciences, Pune and cultured at 37°C and 5% CO_2 in Roswell Park Memorial Institute (RPMI-1640) media and Dulbecco's Modified Eagle's Medium (DMEM) respectively supplemented with 10% FBS, 2 mM glutamine, 10,000 U/ml penicillin, 10 mg/ml streptomycin and 25 µg/ml amphotericin B. Phorbol 12-myristate 13-acetate (PMA) was used at a concentration of 100 ng/ml to differentiate monocytic THP-1 cells into macrophages at 37°C and 5% CO_2 overnight. *L. donovani* promastigotes were received as a kind gift from Prof. Shyam Sundar, Banaras Hindu University, India and cultured at 28°C in M199 liquid media supplemented with 10% fetal bovine serum (FBS), 10,000 U/ml penicillin and 10 mg/ml streptomycin (Das et al., 2015). Antibodies like NEK7 and TAK1 were procured from Santa Cruz while other antibodies like α-tubulin, p53 and Cyt c were procured from Bio-Bharti and Thermo respectively.

5.3.2 Real Time PCR: Total RNA was isolated using RNeasy Mini Kit (QIAGEN) protocol and proceeded with cDNA preparation using ProtoScript® First Strand cDNA Synthesis Kit (New England Biolabs) after DNAse treatment for 1 hr at 37°C. The mRNA levels of *nek7* and *tak1* were analyzed using specific primers in qRT-PCR. For all the qRT-PCR experiments, *β-actin* gene was used as endogenous housekeeping control to normalize the relative fluorescence signal of the target genes. The sequences of all the primers sequences used are listed in Table 5.1.

Table 5.1: Primer sequences used for Real Time PCR experiments (Chapter 5)

S. No.	Primer Name	Sequence (5' – 3')
1	*nek7*_FP	TGGATGAGCAATCACAAGGAAT
2	*nek7*_RP	CCGGTCGTAAGGCCTTCTG
3	*tak1*_FP	GCGTCGGAAACCCTTTGA
4	*tak1*_FP	TGAACAGCCCACATGATTCG
5	*β-actin*_FP	CTGGCACCCAGCACAATG
6	*β-actin*_RP	GCCGATCCACACGGAGTACT

5.3.3 siRNA mediated knock down: BLIMP-1 knock down was carried out using *blimp-1* specific siRNA procured along a *scrambled* siRNA from Invitrogen. Briefly ~2 x 10^5 cells (THP-1 and J774A.1) were seeded in RPMI-1640 media and DMEM, respectively in 35 mm

petri-dishes and incubated at 37°C, 5% CO_2 for 24 hrs. PMA (100 ng/ml) was used for THP-1 cells to differentiate into macrophages. Serum-free Opti-MEM media was used to prepare siRNA-lipid mix and incubated the cells for 6 hrs followed by addition of complete media and finally incubated at 37°C, 5% CO_2 for another 18 hrs. The effect of siRNAs (30 nM) used for knock-down was validated using western blot analysis (shown in Supplementary Figure 1). Rabbit α-tubulin antibody was used as an endogenous control to normalize the expression level of the target protein.

5.3.4 Promastigote infection: Live *L. donovani* promastigotes were used to infect the differentiated macrophage cell lines at MOI of 1:5. The cells were counted in haemocytometer and resuspended in respective media (RPMI-1640 for THP-1 and DMEM for J774A.1 cells) and incubated for 24 hr at 37°C, 5% CO_2. For all siRNA knock down experiments, infection was done after siRNA treatment.

5.3.5 Immunoblotting: Briefly, 2 x 10^5 cells/experimental sample of THP-1 and J774A.1 were used to lyse the cells using RIPA buffer containing 1x Protease Inhibitor Cocktail, 1 mM PMSF and 10 mM EDTA. An equal amount (25µg/lane) of cell lysates of different samples were run on a 12% SDS-PAGE followed by transfer into a polyvinylidene fluoride (PVDF) membrane. Blocking was done using 5% skimmed milk for 2 hr at room temperature (RT) followed by washing with 1x TBST buffer thrice. Then primary antibodies for NEK7 (1:100 dilution), TAK1 (1:100 dilution), p53 (1:1000 dilution) and Cyt c (1:750 dilution) were used for incubating the membrane at 4°C overnight. Next, the membrane was washed again with 1x TBST and incubated with secondary IgG antibody conjugated with HRP at for 45 min at room temperature. The membrane was washed with 1x TBST and chemiluminescent HRP substrate was used for band detection under a Chemi-Doc. Rabbit α-tubulin was used as endogenous control to normalize the expression level of the target proteins.

5.3.6 Immunofluorescence using flow cytometry: Cells were trypsinized using 1x Trypsin, centrifuged and washed with cold 1x PBS twice prior to flow cytometry analyses. Cells were fixed with 4% paraformaldehyde for 5 min at RT and then permealized using 0.01% Triton-X 100 for 5 min at RT. Blocking was done with 3% bovine serum albumin (BSA) for 2 hr at RT and then was incubated with primary antibodies (TAK1 and p53) at dilution of 1:50 at

4^{o}C overnight. Then cells were incubated with secondary antibody conjugated with FITC was used (1:500 dilution) for 1 hr at RT. Cold 1x PBS were used in all the intermediate and final steps to wash the cells thrice. Cells were resuspended finally in 1x PBS to process them in flow cytometer. BD FACS Calibur was used to run the samples and green fluorescence of FITC was measured in FL1-H channel. FCS Express 5 software was used to analyze the data using histogram plots and finally overlaid histogram plot was shown. Median Fluorescence Intensity (MFI) has also been plotted for the samples to statistically justify the mean fluorescence emitted by the stained cells.

5.3.7 Statistics and densitometry analyses: The western blot data were analyzed using densitometry using Image J software where a normalization factor was calculated for the loading control (α-tubulin) to normalize the signals of the target proteins. A statistical data analysis were determined by unpaired t-test in Sigma Plot 10 software using different biological replicates for all the experiments and has been represented by mean ± SEM. A "p" value of less than 0.05 has been considered significant.

5.4 Results

5.4.1 Downregulation of NEK7 and TAK1 during infection: NEK7 is very crucial in phosphorylating the NLRP3 component and thereby activating the inflammasome in the presence of higher intracellular ROS level. On the other hand TAK1 is a significant kinase playing a role in activating NLRP3 inflammasome via NFκβ activation. However, significant decrease in the mRNA level of *nek7* and *tak1* during *L. donovani* infection in both THP-1 and J774A.1 cell lines has been found (Figure 5.1A). The transcriptional downregulation of both *nek7* and *tak1* has been correlated with their decrease in the protein level expression in the infected cells as compared to control uninfected and LPS treated cells (Figure 5.1B – 5.1D). Bacterial LPS was used as a positive control for the experiment. This decrease in NEK7 and TAK1 expression can be attributted to inactivation of caspase 1 activity during infection as per our earlier report (Saha et al., 2019).

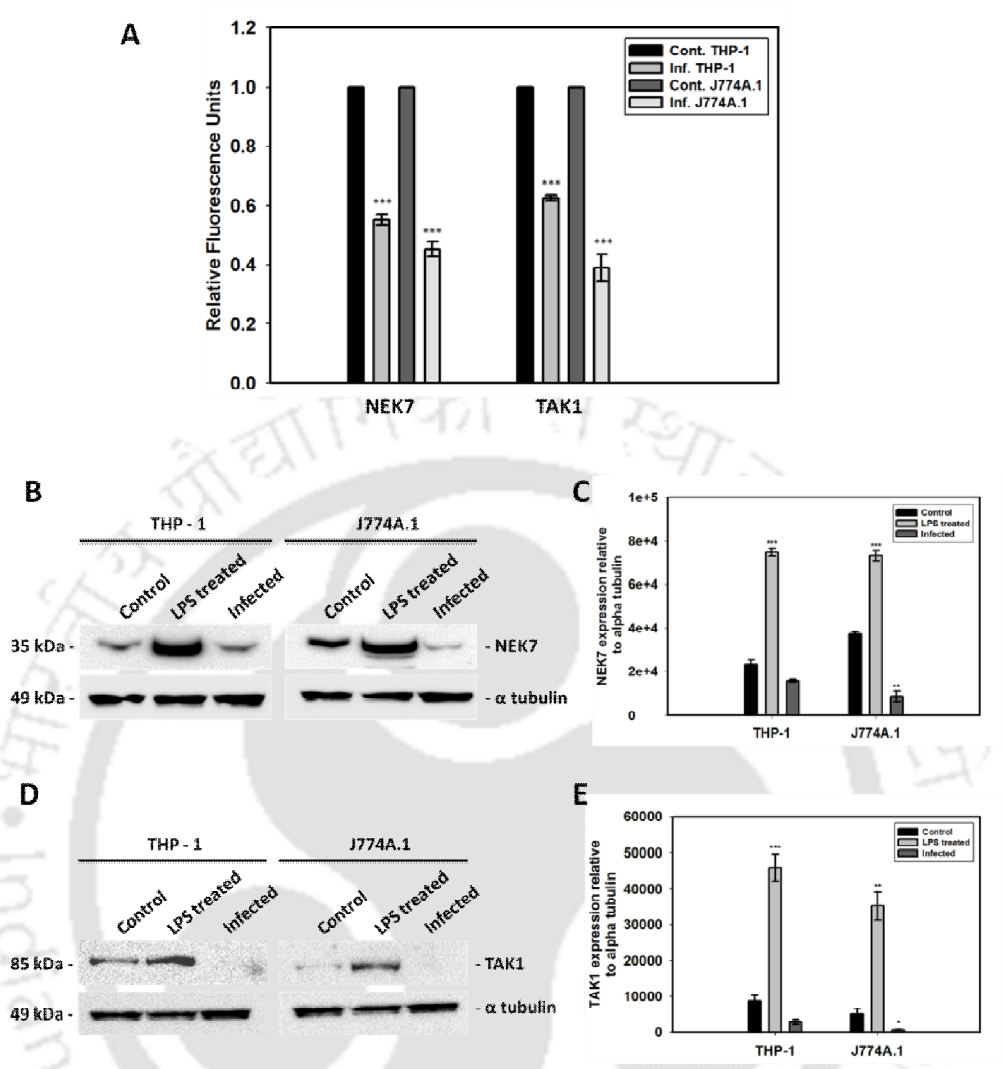

Figure 5.1: Gene expression analyses and immunoblotting of NEK7 and TAK1. (A) Transcriptional analyses of two kinases, *nek7* and *tak1* involved in the PTMs of NLRP3 inflammasome has been shown in both THP-1 and J774A.1 cells infected with promastigotes at MOI of 1:5 for 24 hr; Decrease in (B, C) NEK7 expression and (D, E) TAK1 expression was found in both THP-1 and J774A.1 cells infected with promastigotes at MOI of 1:5 for 24 hr. Bacterial LPS treated cells were used as positive controls for the experiment. (Note: n=3, where n represents three different independent experimental replicates for all the experiments; * denotes $p<0.05$, ** denotes $p<0.005$, *** denotes $p<0.001$)

5.4.2 Resumption of TAK1 expression in *blimp-1* deficient infected cells: We thought of assessing the expression of both NEK7 and TAK1 in *blimp-1* deficient cells infected with promastigotes since BLIMP-1 has been found to be a key player in the pathogenesis of *kala-azar* (discussed in previous chapter). Interestingly, we found BLIMP-1 over-expression to be involved with TAK1 regulation as a repressor because TAK1 expression has been resumed in *blimp-1* deficient cells infected with promastigotes (Figure 5.2). The same has been further

verified by flow cytometry experiment where cells were stained more with TAK1 antibody in *blimp-1* deficient cells infected with promastigotes as compared to infection control (Figure 5.3). This resumed staining of cells is comparable with LPS treated cells and has been shown by median fluorescence intensity (MFI). This finding advocate us to dig deeper into the mechanisms of how BLIMP-1 is regulating the TAK1 mediated pathway of NFκβ activation.

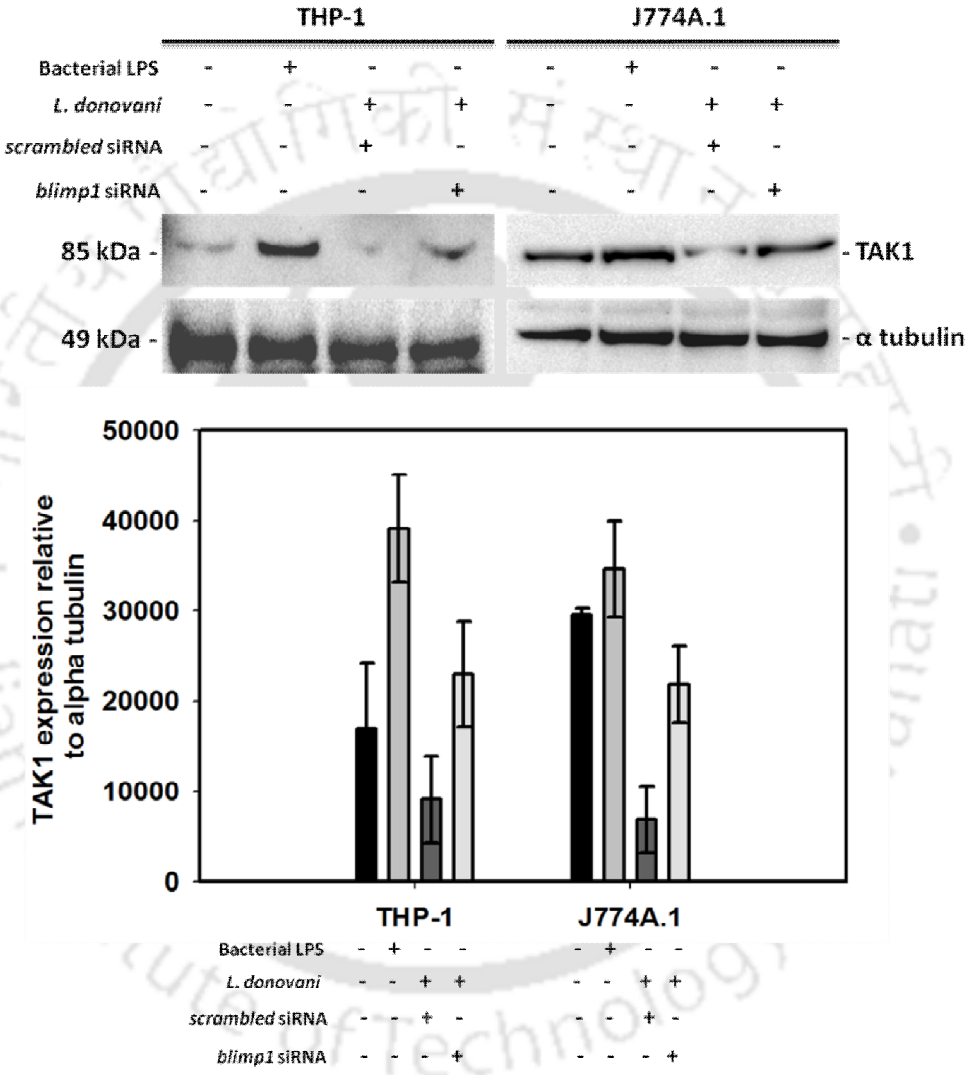

Figure 5.2: Immunoblotting of TAK1 expression in *blimp-1* deficient cells infected with promastigotes at MOI of 1:5 for 24 hr in both THP-1 and J774A.1 cell lines. Bacterial LPS treated cells were used as positive controls for the experiment; **(A)** Western Blot, **(B)** Densitometry analyses of TAK1 expression. (Note: n=2, where n represents three different independent experimental replicates for all the experiments)

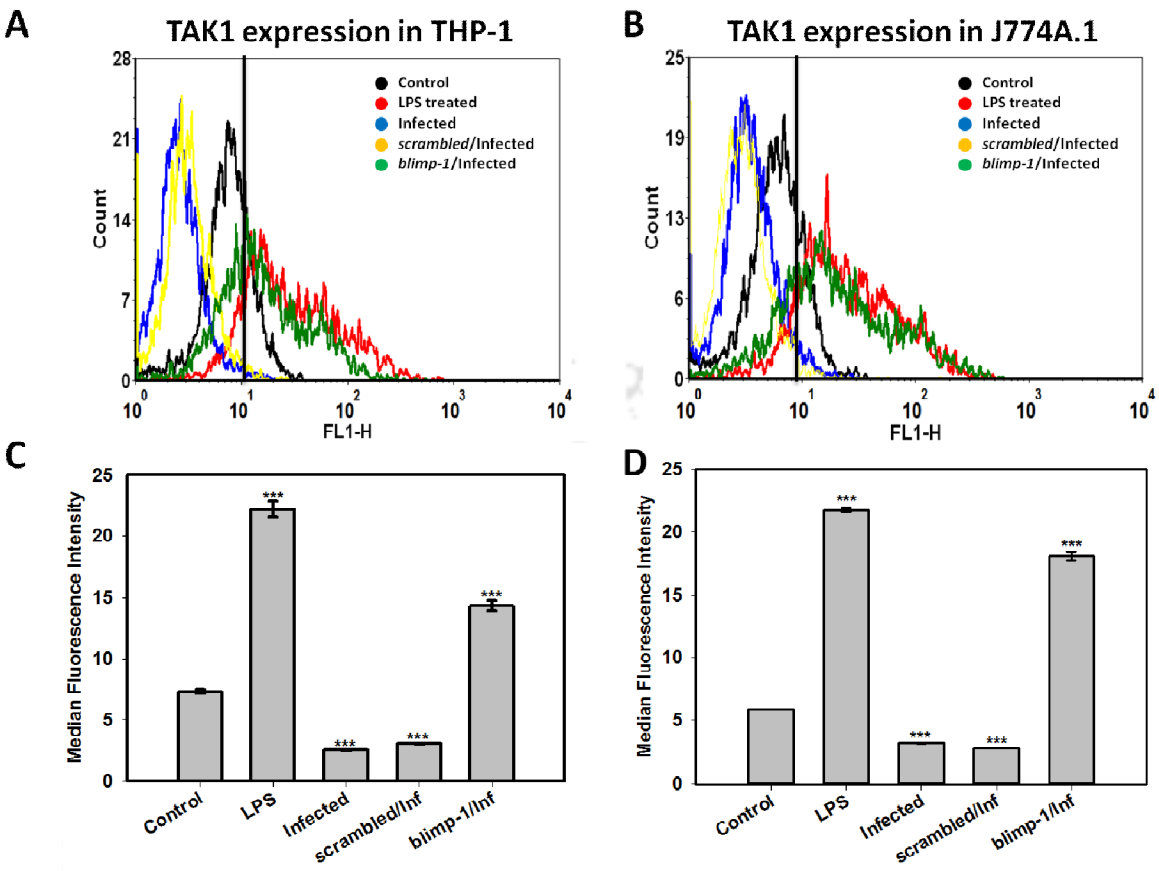

Figure 5.3: Flow cytometry analysis of TAK1 expression in *blimp-1* deficient cells infected with promastigotes. BD FACS Calibur was used to run the samples and green fluorescence of FITC was measured in FL1-H channel. FCS Express 5 software was used to analyze the data. Overlaid histogram plot was shown for (A) THP-1 cells and (B) J774A.1 stained cells. Median Fluorescence Intensity (MFI) has also been plotted for (C) THP-1 and (D) J774A.1 cells. (Note: n=3, where n represents three different independent experimental replicates for all the experiments; *** denotes $p<0.001$)

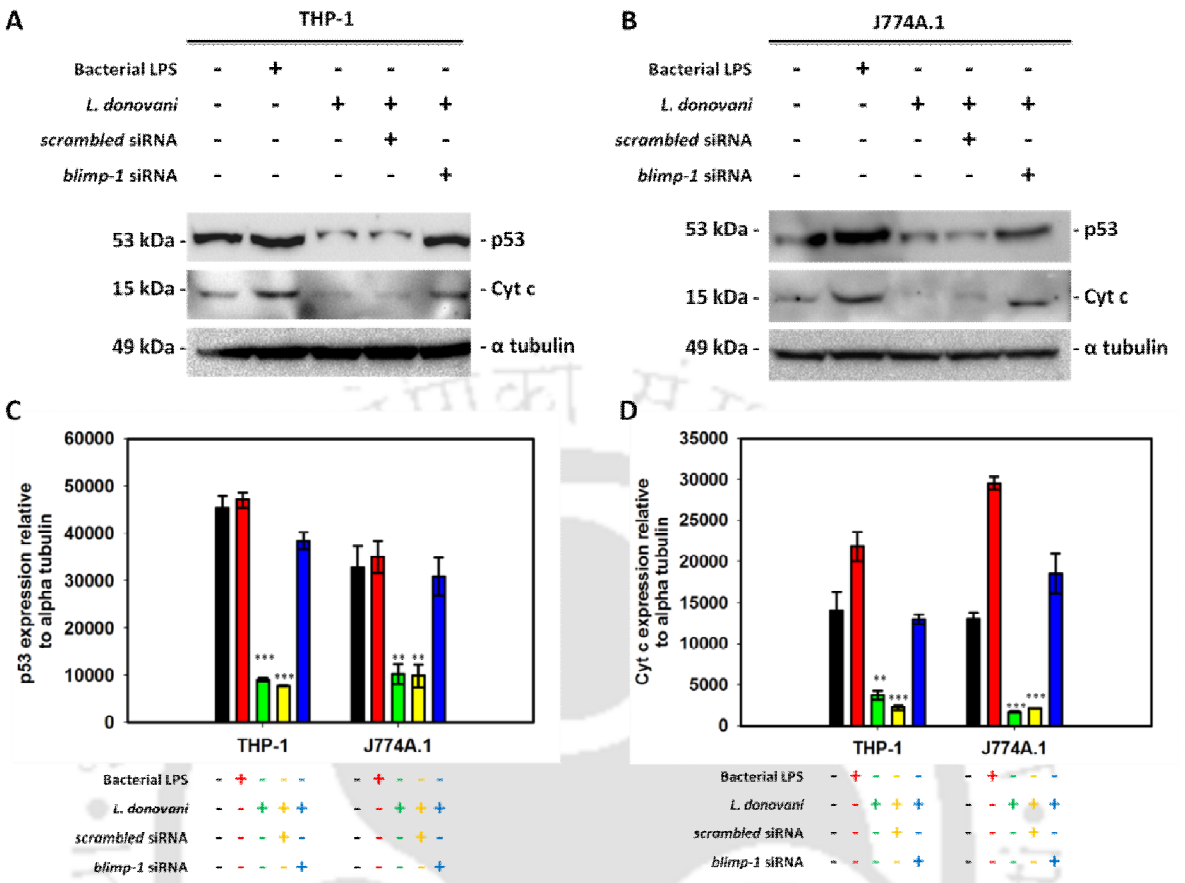

Figure 5.4: Immunoblotting of p53 and Cyt c expression in infected cells. Downregulation of p53 during *L. donovani* infection has been highlighted as compared to control in (A) THP-1 cells and (B) J774A.1 cells along with decreased Cyt c formation in the infected cells, substantiating the mechanism behind impairment of NLRP3 inflammasome since p53 is a direct transcriptional activator of caspase 1. However, the level of p53 and Cyt c has been shown to be resumed in *blimp-1* deficient cells infected with promastigotes suggesting an antagonizing role of BLIMP-1 on p53 level. The densitometry analyses were also shown for (C) THP-1 and (D) J774A.1 cells. (Note: n=3, where n represents three different independent experimental replicates for all the experiments; ** denotes $p<0.005$, *** denotes $p<0.001$)

5.4.3 BLIMP-1 negatively regulate p53 expression: In order to decipher exact mechanism behind negative regulation of TAK1 with BLIMP-1 expression, we found TAK1 to activate p53 molecule (tumor suppressor gene). Despite our vast knowledge about multifunctional role of p53, its involvement in the host defence against pathogenic infections remains unknown. Modulation of the immune response, cell death and cell metabolism control are three possible strategies that have been put forwarded by which p53 interferes with bacterial infection are postulated (Siegl and Rudel, 2015). Recently, p53 has been found to serve major roles in inflammation and immunity in addition to its well characterized role in tumor

suppression and thus providing new dimensions to its role in human infection (Lowe et al., 2014). Interestingly, we found reduced expression of p53 level in the *Leishmania* infected cells as compared to uninfected cells by immunoblot (Figure 5.4). In agreement with this, flow cytometry analyses demonstrate that cells were stained more with p53 antibody in *blimp-1* deficient cells infected with promastigotes as compared to infection control (Figure 5.5). We can hypothesize that the parasites actually inhibit pyroptosis (non-canonical cell death pathway) by downregulation of p53 which is a transcriptional activator of human caspase 1 (Gupta et al., 2001).

5.4.4: BLIMP-1 dependent reduced cytochrome c expression during infection: The involvement of p53 expression in mediating non-canonical forms of cell death has been explored and mentioned earlier (Gupta et al., 2001). The reduced intracellular ROS level during infection as per our previous results (Saha et al., 2019) can also be explained in a way that p53 induces the level of BAX protein (pro-apoptotic) followed by increase in cytochrome c (Cyt c) level and thereby formation of intracellular ROS by the mitochondria which helps in either pathogen clearance or death of infected cells (Ranjan and Iwakuma, 2016). In constrast, there is reduced level of p53 in the infected cells leading to decreased Cyt c formation in the infected cells (Figure 5.4). Moreover, the level of p53 (Figure 5.4 & 5.5) and Cyt c (Figure 5.4) has been shown to be resumed in *blimp-1* deficient cells infected with promastigotes suggesting an antagonistic role of BLIMP-1 on p53 dependent activation of cell death (Yan et al., 2007).

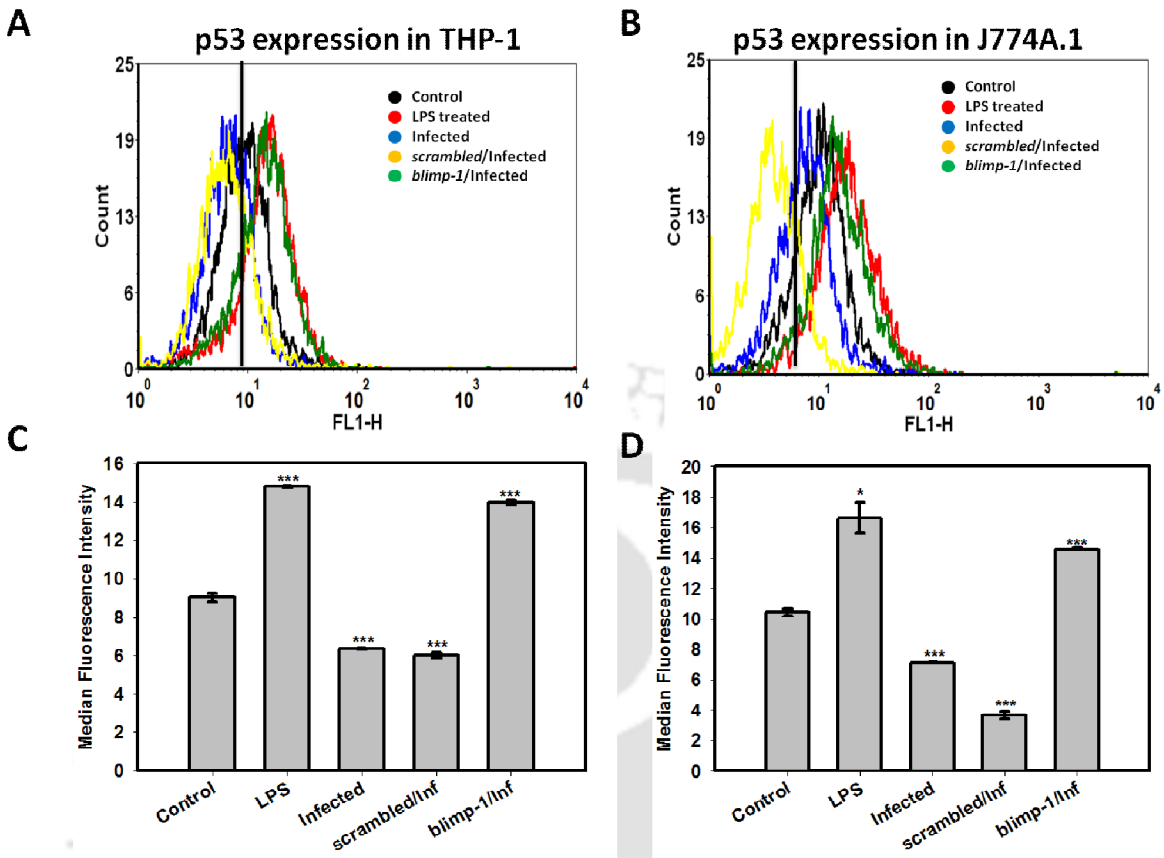

Figure 5.5: Flow cytometry analysis of p53 expression in *blimp-1* deficient cells infected with promastigotes. BD FACS Calibur was used to run the samples and green fluorescence of FITC was measured in FL1-H channel. FCS Express 5 software was used to analyze the data. Overlaid histogram plot was shown for (A) THP-1 cells and (B) J774A.1 cells. Median Fluorescence Intensity (MFI) has also been plotted for (C) THP-1 and (D) J774A.1 stained cells. (Note: n=3, where n represents three different independent experimental replicates for all the experiments; * denotes $p<0.05$ & *** denotes $p<0.001$)

5.5 Discussions & Conclusion

Among various kinds of PTMs, phosphorylation and ubiquitanations are the most characterised ones in the regulation of inflammasome signaling, catalyzed by hundreds of enzymes. The highly diverse and versatile PTMs plays an important role in shaping the dynamics of inflammatory response during pathogenic infections and thus remains an exciting area of research for the foreseeable future. In this context, the downregulation of NEK7 and TAK1 expression haults NLRP3 inflammasome activation by their inability to phosphorylate and activate NLRP3 during *L. donovani* infection. This might be an explanation of how parasite evade the inflammatory response of NFκβ – NLRP3 signaling pathway. Moreover, reduced TAK1 expression has been linked with higher BLIMP-1

expression during infection. We have also found resumption of TAK1 expression in *blimp-1* deficient cells infected with promastigote. Recently, there has been a report highlighting TAK1 controlling cell death by activation of NFκβ (Mihaly et al., 2014) and also p53 (Stramucci et al., 2018). The tumour suppressor gene p53 is called "guardian of the genome"for its predominant role in regulating genomic stability and the DNA damage response in order to maintain cell integrity. In last few decade, the role of p53 signaling pathways have constituted promising strategies for cancer therapies, and developing new insights into metabolic transformation in cancer cells. Recent reports suggest that pathogens, especially those with an intracellular life cycle, modulate the human host cell to ensure their own survival (Eisenreich et al., 2019, Thakur et al., 2019). However, they also need to counteract the severe damage that infections often cause to the host in order to avoid the immediate loss of their replicative niche. For example, infection with *H. pylori* triggers DNA repair involving the ATM-dependent recruitment of 53BP1 as well as phosphorylation of H2AX22 (Toller et al., 2011). In same way, production of ROS during infections with the *Chlamydia trachomatis* was found to contribute to double stranded breaks (DSBs) by phosphorylation of H2AX and methylation of histone H3. It also interferes with DNA repair in the host cell by inhibiting the recruitment of phosphorylated ATM (pATM) and 53BP1 similar to *L. monocytogenes* (Chumduri et al., 2013). DNA damage and ROS being strong inducers of p53-mediated apoptosis, bacterial pathogens along with few virus and protozoan parasites have adopted various mechanisms to escape and bypass p53 signaling to ensure host cell survival, highlighting the importance of this pathway for intracellular pathogens (Zaika et al., 2015, Miciak and Bunz, 2016). In this context, human double minute 2 (HDM2) get activated by the malaria parasite, *Plasmodium yoelii*, which substantially decreases p53 protein levels during its liver-stage infection and thus evade cellular death of infected cells (Kaushansky et al., 2013). Here we report for the first time reduced level of p53 expression during infection leading to decrease in Cyt c release wherein the expression of these mediators (p53 and Cyt c) is resumed in *blimp-1* deficient cells infected with promastigotes.

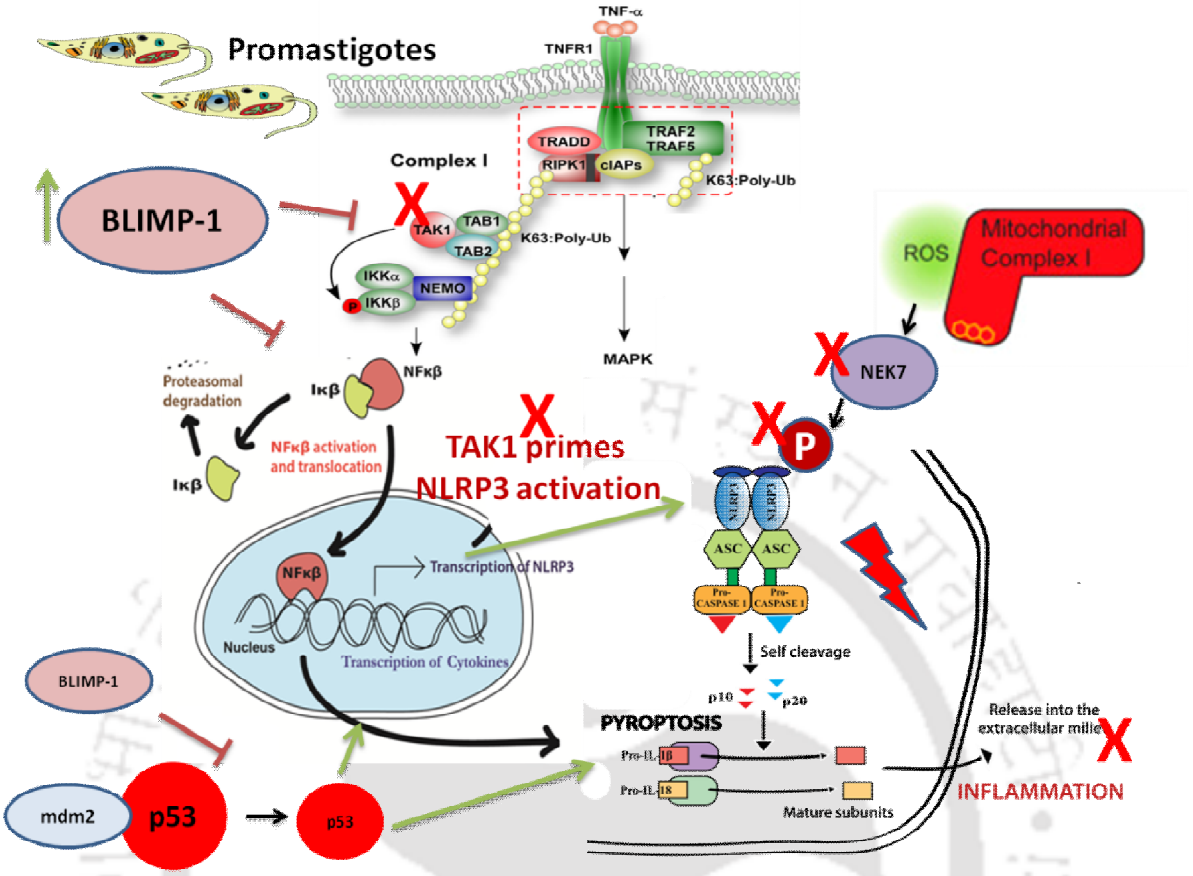

Figure 5.6: Schematic representation of *Leishmania* modulated immunosuppression by reducing TAK1 expression. This inhibition results in p53 destabilization without any Cyt c release resulting in reduced production of intracellular ROS tampering NLRP3 inflammasome activation by inhibiting NLRP3 phosphorylation caused by reduced expression of NEK7.

Summarizing the cross talks among all the host mediators in NLRP3 inflammasome driven cell death pathway, we can conclude that TAK1 induces the activation of NFκβ followed by NLRP3 inflammaome activation. This triggering of cellular environment results in p53 stabilization which induces Cyt c release resulting in increased production of intracellular ROS favoring NLRP3 inflammasome activation by facilitating NLRP3 phosphorylation by NEK7. The downstream effect of NLRP3 inflammasome activation cause cells to undergo caspase 1 dependent pyroptosis, releasing IL-1β and IL-18 (Saha et al., 2019). The above cross-regulation of pathways get halted during *L. donovani* infection *in-vitro* by over-expression of BLIMP-1 protein. In a nutshell, we conjecture that the parasites control pyroptosis in the infected cells by BLIMP-1 mediated suppression of TAK1 and p53 which impair the NFκβ – NLRP3 inflammasome signaling affecting the pro-inflammatory response due to decreased IL-1β processing as discussed in our early report (Saha et al., 2019). Few

reports and studies have also been claimed about the relationship between p53 and NLRP3 inflammasome driven human disease but with conflicting and ambiguous results at present (Licandro et al., 2013, Haasken and Sutterwala, 2013). Thus interesting facts have been observed on how *L. donovani* parasites modulate various host cell signaling pathways and exploit mediator molecules to cross talk between different pathways for their long term survival and progression of infection.

CHAPTER 6

SIGNIFICANCE OF THE THESIS WORK

The research work conducted during PhD was mainly focussed on the host pathogen interactions involving *Leishmania donovani* as the model pathogen. During all pathogenic encounter, the host cell's immune system plays a vital role as a defense barrier to our body. Unfortunately, a successful progression of infection continues due to the suppression of host immune system by various ways. These pathogens including bacteria, virus, fungi, parasite, etc actually evade the immune surveillances posed by array of host immune cells like neutrophils, fibroblasts, dendritic cells, monocytes and macrophages. In case of visceral leishmaniasis, commonly called as "*kala-azar*" in India, the protagonist parasite, *L. donovani* modulate various host signaling pathways essential for recruiting immune cells to the site of infection or release of pro-inflammatory cytokine leading to Th1 immune response as per previous reports.

In our current research work, we have deciphered novel immunosuppressive mechanisms adopted by these parasites to evade the inflammatory immune reactions. Overall, our study suggests that the parasite suppresses caspase 1 dependent, IL-1β and IL-18 mediated pyroptotic death of macrophage. We have also found on how BLIMP-1, a transcriptional reprresor of IFN-β is responsible for cross regulating a tightly regulated NFκβ – NLRP3 pathway crucial for host defense system during pathogenic encounters. We speculate that these parasites modulate NFκβ – NLRP3 signaling pathway by prompting BLIMP-1 over-expression to disrupt the Th1/Th2 balance of the host cells and eventually evading pyroptosis of infected cells. This work also unravels the importance of BLIMP-1 in reversing the pro-inflammatory reactions and arresting pyroptosis pathway mediated by NFκβ – NLRP3 signaling pathway. In addition, the downregulation of NEK7 and TAK1, enzymes involved in the phosphorylation of NLRP3 has been shown during infection along with how BLIMP1

upregulation actually induces the repression of TAK1 and its downstream molecule p53. Apart from p53 role in tumor suppression, it also regulate pyrotopsis and work in coordination with NFκβ activation in human macrophages. The antagonistic role of BLIMP-1 on p53 and Cyt c expression, in the infected cells have also been shown and discussed .

Comprehensive explorations into the mechanistic insight need attention to decipher the exact nature of cross regulations during pathogenic assaults and come up with better therapeutic strategies.Thus, better understanding of these mechanisms behind immunosuppression adopted by the parasites needs to be validated in *in-vivo* models which will be instrumental in designing modern immunotherapeutics against *kala-azar*.

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LIST OF PUBLICATIONS

PhD thesis related works

Journals

1. **Gundappa Saha**, Bakulesh M. Khamar, Prerna Kumari, Manish Kumar and Vikash Kumar Dubey (2019) BLIMP-1 plays important role in the regulation of macrophage pyroptosis for the growth and multiplication of Leishmania donovani. ***ACS Infectious Diseases***. 5(12): 2087-2095 **[IF: 4.919]**

2. **Gundappa Saha**, Bakulesh M Khamar, Om Prakash Singh, Shyam Sundar and Vikash Kumar Dubey (2018) Leishmania donovani evades Caspase 1 dependent host defense mechanism during infection. ***International Journal of Biological Macromolecules***. 126: 392-401 (**Media Coverage** as "Potential therapy for Kala-azar" in Nature India – January 2019 by **Nature Publishing Group**) **[IF: 4.784]**

3. **Gundappa Saha**, Adarsh Kumar Chiranjivi, Bakulesh M. Khamar, Manish Kumar and Vikash Kumar Dubey. BLIMP-1 dependent down-regulation of TAK1 and p53 molecules is responsible for resisting pathogen clearance during Leishmania infection **(Under Revision)**

4. **Gundappa Saha** and Vikash Kumar Dubey. Balancing between Life & Death: Insight into the role of innate immunity and inflammation during parasitic infection. (Review Article) **(Manuscript drafted for communication)**

PhD thesis unrelated works

Book Chapter

5. **Gundappa Saha**, Prakash Saudagar and Vikash Kumar Dubey. Virus like particles (VLPs): Nano-carriers in targeted therapeutics, In: Encapsulation of active molecules and their delivery system **(Submitted)**

Journals

6. Shweta Raj, **Gundappa Saha**, Santanu Sasidharan, Vikash Kumar Dubey, Prakash Saudagar (2019). Biochemical characterization and chemical validation of Leishmania MAP Kinase-3 as a potential drug target. ***Scientific Reports*** 9: 16209 **[IF: 4.525]**

7. Adarsh Kumar Chiranjivi, **Gundappa Saha**, Pranjal Chandra and Vikash Kumar Dubey. The role of amino acid C15, C38, A48, D49G, and A54 in unexplored variant of dihydrolipoamide dehydrogenase from *L. donovani* **(Communicated)**

8. Kamalesh Verma, **Gundappa Saha,** Lal Mohan Kundu and Vikash Kumar Dubey (2018) Biochemical characterization of a stable azoreductase enzyme from *Chromobacterium violaceum*: Application in industrial effluent dye degradation. ***International Journal of Biological Macromolecules*.** 121:1011-1018. **[IF: 4.784]**

9. Jay Prakash, Sunita Yadav, **Gundappa Saha**, Adarsh Kumar Chiranjivi and Vikash Kumar Dubey (2018) Episomal expression of human glutathione reductase (HuGR) in *Leishmania* sheds light on evolutionary pressure for unique redox metabolism pathway. ***International Journal of Biological Macromolecules.*** 121:498-507. **[IF: 4.784]**

10. Jiban Barman#, Ritesh Kumar#, **Gundappa Saha**, Kartikeya Tiwari and Vikash Kumar Dubey (2018) Apoptosis and its interplay with other cell death processes. ***Current Pharmaceutical Biotechnology*** 19(8): 644 - 663 2018. **(Review Article) [IF: 1.819]**

11. Mousumi Das, **Gundappa Saha**, Anil Kumar Saikia, and Vikash Kumar Dubey (2015) Novel agents against miltefosine-unresponsive *Leishmania donovani*. ***Antimicrobial Agents & Chemotherapy*** 59:7826 –7829 **[IF: 4.715]**

LIST OF CONFERENCES ATTENDED

1. **Late – Breaker Poster Presentation** in the "17th International Congress of Immunology (IUIS – 2019)" organized by Chinese Society of Immunology from October 19 – 23, 2019 in Beijing, China.
2. **Best Poster Award** in the "National Conference on Emerging Trends in Disease Model Systems" organized by NCCS Pune and NASI from March 25-26, 2019.
3. **Best Young Investigator's Talk Award** in the "Recent Advancements in Biochemical Engineering & Biotechnology (RABEB 2019)" organized by IIT (BHU), Varanasi from March 15-16, 2019.
4. Poster presentation in the 86th Conference of Society of Biological Chemists, India titled "Emerging Discoveries in Health and Agricultural Sciences" organized by School of Life Sciences, Jawaharlal Nehru University, New Delhi from November 16-19, 2017.
5. **Best Poster Award (Institute Level & Departmental Level)** sponsored by Springer in "Research Conclave 2017" organized by Student Academic Board, Indian Institute of Technology Guwahati held from March 16 – 19, 2017.
6. Poster Presentation in the "Meeting on Cell Biology of Infections" held on October 13-14, 2016 at National Centre for Biological Sciences – TIFR, Bangalore
7. Oral Presentation of a research proposal entitled "Development of Novel Vaccine Candidates to combat Leishmaniasis" in Assam Biotech Conclave – 2015, organized by Guwahati Biotech Park during 20th – 21st November, 2015.

LIST OF AWARDS/RECOGNITIONS

1. Recipient of **Federation of Immunological Societies for Asia-Oceania (FIMSA) Travel Award** for 17th International Congress of Immunology (IUIS – 2019).

2. Recipient of **Prime Minister's Fellowship Award for Doctoral Research** - May 2016.

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