# IMPACT OF ALTERED EXTRACELLULAR MATRIX PROTEIN DISTRIBUTION ON IMMUNE CELL FUNCTION DURING INFLAMMATION

**Ph.D. THESIS**

***by***

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**OCTOBER 2019**

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**A THESIS**

***Submitted in partial fulfillment of the requirements for the award of the degree***

***of***

**DOCTOR OF PHILOSOPHY**

***in***

**DEPARTMENT OF BIOTECHNOLOGY**

***by***

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# INDIAN INSTITUTE OF TECHNOLOGY ROORKEE

## STUDENT'S DECLARATION

I hereby certify that the work presented in the thesis entitled **"IMPACT OF ALTERED EXTRACELLULAR MATRIX PROTEIN DISTRIBUTION ON IMMUNE CELL FUNCTION DURING INFLAMMATION** "is my own work carried out during a period from **July, 2014 to October, 2019** under the supervision of **Dr. Pranita P. Sarangi,** Assistant Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

The matter presented in the thesis has not been submitted for the award of any other degree of this or any other Institute.

Chandra Bhan

**Dated:** _____ **CHANDRA BHAN**

## SUPERVISOR'S DECLARATION

This is to certify that the above mentioned work is carried out under my supervision.

Pranita P Sarangi

**Dated:** _____ **PRANITA P. SARANGI**

# ABSTRACT

Extracellular matrix proteins (ECM) form the structural support for the migration of immune cells and provides multiple signals to assist in the functions of immune cells during physiological and pathological conditions. Secretion of inflammatory mediators such as cytokine and MMP (expand) s could influence the expression and distribution of ECM in the inflamed tissues, which in turn could modify cellular functions. Previous reports have shown changes in the expression of ECM proteins during local inflammatory responses such as asthma, fibrosis, chronic obstructive pulmonary disease (COPD), etc. However, limited knowledge is available on the changes occurring in the expression and distribution of important ECM proteins in multiple tissues during systemic inflammation.

One part of the doctoral work was focused on investigating, the expression profile of important ECM proteins in systemic inflammation using two systemic inflammation models, i.e, Lipopolysaccharide induced endotoxemia and Cecal Ligation and Puncture (CLP) induced polymicrobial sepsis were used having a distinct pattern of events which drives the systemic inflammations. Following LPS injection and CLP surgery, lung, liver, kidney & mesentery were isolated at 6 hr, 12 hr, 24 hr, and 36 hr time point. Using real-time PCR and western blot technique, expression of important ECM protein both at transcript and protein levels were measured. RT-PCR and Western blot analysis showed changes in the expression of various ECM proteins such as collagen 4, fibrinogen and vimentin. A unique expression pattern of these prominent ECM proteins was observed in both at the site of inflammation (mesentery) as well as in the visceral organs (Lung, liver & kidney) in both models, possibly due to the difference in inflammation induction pattern. Moreover, to confirm the importance of upregulated ECM protein on immune cell function and various inflammatory and functional signaling pathways, bioinformatics analysis with STRING software was performed to predict the important signaling pathways that get activated upon cell-matrix interaction such as activation, apoptosis, cytoskeleton modulation, integrin expression and migration during the inflammatory scenario both in murine and humans.

Secondly, the work also focused on assessing the effect of Fibulin7 (Fbln7), one of the newly identified adhesion matrix protein on neutrophils. Fibulin7 was recently identified as members of the fibulin family of secreted glycoproteins and was found to be expressed in developing tooth, bone, cartilage as well as immune-privileged locations such as eye and

placenta. Previous studies from our laboratory have shown that the Fbln7 full-length and its C terminal fragment (Fbln7C) have regulatory effects on human monocytes and macrophages but its effect on neutrophils is not known. Thus, experiments were designed to further understand the effects of Fbln7, specially, its C-terminal fragment-Fbln7C on the immunological functions of neutrophils which are key cell type of innate immune system and also play vital role in driving the pathological events such as multi organ failure in infection induced systemic inflammations. *In vitro* cell adhesion and inhibition assay showed that the neutrophils could bind to adhesion protein fbln7 via integrin β1and could compete with other ECM proteins such as fibronectin for binding to neutrophils. Significant reduction in ROS and inflammatory cytokine production (i.e. IL-6, IL-1β) was observed including reduction in Erk1⁄2 phosphorylation in neutrophils stimulated with LPS and fMLP in presence of Fbln7C compared to untreated controls. Furthermore, treatment of Fbln7-C in mice decreased the number of cells in both blood and peritoneum, as well as reduced the PMA induced ROS production from neutrophils isolated from peritoneum and lungs of endotoxemic group treated with Fbln7C compared to controls animals.

In summary, data from the ECM expression during systemic inflammation and immunomodulatory role ofFbln7-C will help in better understanding of ECM-Cell dynamics in the inflammatory microenvironment and may contribute to the development of cell adhesion based therapeutics.

# ACKNOWLEDGEMENTS

I take this opportunity to express my profound gratitude and deep regards to my supervisor Dr. **Pranita P. Sarangi** for her guidance and cooperation and for providing all assistance required for the completion of this work. Her painstaking efforts in going through the manuscript and suggestions for its improvement are gratefully acknowledged. I wish to express my heartiest thanks to Prof. A.K. Sharma, Head of the Department of Biotechnology, for providing the necessary facilities during the project period.

I express my sincere thanks to Student Research Committee (SRC) members, Dr. Shailly Tomar, Chairman SRC (Associate Professor, Biotechnology Department, IIT Roorkee), Dr. P. Gopinath, Internal expert (Associate Professor & Head, Biotechnology Department, IIT Roorkee, Dr. M. Sankar (Associate Professor, Department of Chemistry, IIT Roorkee) for their useful suggestions and support during the entire period of my research work.

I also express my gratitude to UGC- JRF Programme for proving scholarship for my research work at IIT Roorkee and giving me this opportunity of pursuing my PhD. The funding made it possible to join IIT Roorkee as a Research scholar to devote myself fully to the research

It is a pleasure to acknowledge the support extended by my friends and colleagues namely Miss Papiya Charkarboty, Mr. Pankaj Kumar, Mr. Shiba Dash, Mr. Puneet Kumar, and Mr. Kuldeep and Miss Sudeepa for their cooperation in various phases of my PhD. I would like to express my gratitude to all those who have contributed directly or indirectly in the course of my research work.

Last but not the least, I would like to Thank god for providing me with a wonderful family who have supported me during the entire life and doctoral program. My special thanks to my wife Dr. Kusum Lata who has provided much needed emotional support and participated in many and scientific discussions throughout my PhD program.

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# LIST OF ABBREVIATIONS

**ECM: Extracellular Matrix**

**CARS: Counter Anti-inflammatory responses**

**IL: Interleukins**

**TNFα: Tumor Necrosis Factor α**

**PMN: Polymorphonuclear Cells**

**LRR: Lucin Rich repeats**

**MMPs: Metalloproteinase**

**TIMP: Tissue Inhibitor of Metalloproteinases**

**TLR: Toll-Like Receptors**

**NFκB: Nuclear factor-κB**

**MAPK**: Mitogen-Activated Protein Kinase

**ERK: Extracellular signal regulated kinase**

**ROS: Reactive Oxygen Species**

**fMLP: *N*-Formylmethionyl-leucyl-phenylalanine**

**RT-PCR: Real Time- Polymerase Chain Reaction**

**PMA: Phorbol 12-myristate 13-acetate**

**Fbln7: Fibulin7**

**Fbln7-C: Fibulin7-C**

**SEM: Standard Error of The Mean**

**CASP: Caspase**

**ITG: Integrin**

**DAPI: 4′,6-diamidino-2-phenylindole**

**DCFDA: 2',7' –dichlorofluorescin diacetate**

# LIST OF PUBLICATIONS

## Peer Reviewed Journal

- *__Bhan, C__., *Dipankar, P., *Chakraborty, P., & Sarangi, P. P. (2016). Role of cellular events in the pathophysiology of sepsis. *Inflammation Research*, *65*(11), 853-868. (*Equal contribution)
- *__Bhan, C__., *Dash, S. P., Dipankar, P., Kumar, P., Chakraborty, P., & Sarangi, P. P. (2019). Investigation of Extracellular Matrix Protein Expression Dynamics Using Murine Models of Systemic Inflammation. *Inflammation*, Dec:42(6)2020-2031. (*Equal contribution)
- *Chakraborty P., *__Bhan C__., *Dalpati N., Dash S. P., Kumar, P., Sarangi P. P. (2019). C-terminal fragment of adhesion protein Fibulin7 improves survival in endotoxemic mice by regulating neutrophil functions (Submitted). (*Equal contribution)

## Book Chapter

- **Bhan C**., Dipankar P., Dash S. P., Chakraborty P., Dalpati N., Sarangi P. P. 2019. Recent advances in imaging and analysis of cellular dynamics in real time (Submitted)

## Conference Proceedings

- **Bhan C**, Chakraborty P,Dalpati N, Dash SP, Sarangi PP. Adhesion protein Fibulin7 modulates innate immune cell functions and improves survival in endotoxemia mice. IMMUNOCON 2018, 45$^{th}$ Annual conference of the Indian Immunological society at Institute of science, THSTI, Delhi, India.
- **Bhan C**, Dash SP, Chakraborty P, Dipankar P, Sarangi PP. Study in a murine model of sepsis reveals systemic inflammation induced dynamics of extracellular matrix protein expression in visceral tissues. IMMUNOCON 2017, 44$^{th}$ Annual conference of the Indian Immunological society at Institute of Science, Nirma University, Ahmedabad, India

# CHAPTER: 1

# Introduction-Literature Review and Project Rationale

In our immune system, Inflammation is a complex biological response against different infectious and non-infectious harmful insults such as pathogen associated molecular pattern (PAMP), and Damage associated molecular patterns (DAMP) (Chen et al. 2018). It is a protective mechanism that involves cellular and molecular mediators such as immune cells, cytokines, chemokines, complement factors, lipid mediators, etc.(Turner et al. 2014). The main purpose of inflammation is to clear the infection, remove necrotic cells and damaged tissue components from the site of injury and to initiate wound healing. However, chronic injury or tissue damaging events could provoke a systemic inflammatory response (Chen et al. 2018). This process is similar to local tissue inflammation, but is not confined to a particular location and involves the changes in endothelium and interstitial tissues of multiple organ systems such as in case of sepsis (Hotchkiss et al. 2016).

## 1.1. Sepsis:

Sepsis is defined as a systemic inflammatory response with a confirmed source of infection (Singer et al. 2016). In other words, sepsis develops due to a dysregulated host immune response to an infection. Depending on the severity, it is classified as SIRS, sepsis, severe sepsis, and septic shock. It is one of the frequently occurring fatal conditions in adult intensive care units and a leading cause of death in adults (Martin 2012). A study by Angus et.al showed that, approximately 750,000 cases of sepsis occur each year in North America, with a mortality rate of 30% to 50% depending on the severity of cases(Angus et al. 2001). In the United States, it is responsible for as many deaths annually as those from myocardial infarctions. Sepsis could result from the host response against various kinds of infections. According to the international sepsis registry published in 2009, among the 12881 enrolled patients, Gram-negative (41.4%) and gram positive (32.4%) organisms were the main cause of infection followed by fungal (8.7%) and viral (1.3%)(8). According to the aforementioned study, the lung was the primary origin of infection followed by abdominal, genitourinary and blood. To date, no effective treatment strategy available for sepsis but, according to the severity of sepsis different treatment strategies are used to clear infection(Vincent 2016). For example, when a patient is under high risk of infection they

are treated with prophylactic antibiotics and immune stimulators. However, when the infection has occurred, it is treated with antibiotics and antitoxin. If the infection is not controlled, it leads to SIRS and during this condition patient is given anticoagulant and antioxidant. If inflammation further progresses it leads to septic shock causing a severe condition where the patient is treated with anti-inflammatory cytokine or inflammatory cytokine antagonist (Pop-Began et al. 2014). The major caveat of available treatment strategies is that, they could produce an excess amount of PAMP and DAMP, which can further activate immune cells and increase the intensity of hyper inflammatory responses (Pop-Began et al. 2014). Therefore, further investigation is needed to find potential therapeutic targets for treating sepsis.

Depending upon distinct cellular and molecular patterns during the progression of sepsis, the entire set of events is classified into pro-inflammatory (cytokine storm) and anti-inflammatory stages (Rittirsch, Flierl, and Ward 2008). The pro-inflammatoryy stage is responsible for the recruitment of innate immune cells such as macrophages and neutrophils that release a massive amount of pro-inflammatory mediators systemically in order to control ongoing infection. Besides this, they are also responsible for the transmission of signals to the adaptive cells for boosting the immune responses for pathogen clearance. In sepsis, hyper-inflammatory responses are often dysregulated due to the abnormal innate immune response to an ongoing infection. Activated macrophages/monocytes are responsible for producing pro-inflammatory cytokines, chemokines, and nitric oxide through the signaling via toll-like receptors (TLRs), which causes the activation of the critical pro-inflammatory transcription factor, nuclear factor κB (NF-κB) leading to massive cytokine production (Bhan et al. 2016). TNF-α plays a critical role in the early phases of shock state (i.e hypotension, fever) and may have a role in septic shock related organ dysfunction. In addition to TNF-α, there is also the production of acute phase proteins, such as C-reactive protein, which leads to the activation of humoral defense mechanisms (complement system), resulting in the production of pro-inflammatory mediators, as well as C5a which further enhances the cytokine and chemokine production (Ward 2010). Moreover, it causes the activation of the coagulation system through various mechanisms, which lead to disseminated intravascular coagulopathy. Many of these mediators activate the Phagocytic cells such as (neutrophils and macrophages) which respond to this stimuli by producing granular enzymes and reactive oxygen species (ROS) such as H2O2, which have bactericidal activity and also capable of causing tissue damage, resulting into

increased vascular permeability and organ injury (Schieber and Chandel 2014). The second anti-inflammatory stage is also known as compensatory anti-inflammatory response syndrome was proposed initially to follow SIRS and is usually defined as the inability of the body to respond to a defined antigenic and/or infectious challenge. This condition is characterized by a decrease in delayed-type hypersensitivity (DTH) response and inefficiency to handle secondary infections, leading to the development of nosocomial infections (Hotchkiss and Karl 2003). Leukocytes isolated from the septic patients at the immunosuppressive stage show an altered immunological status. For example, they produce more IL-10, T cells show anergy with a shift to Th2 phenotype, reduced MHC-II expression on antigen presenting cells, etc. This stage is similar to the immunosuppressive stage found in most cases of severe injuries like surgical procedures, blunt trauma, severe burn injuries, or hemorrhage. In such cases, frequent complications occur due to multi organ failure and sepsis. In spite of these evidence from septic patients and animal studies, much more remain to be understood about how sepsis develops to septic shock and which aspects of immunological changes could be explored for the therapeutic and early diagnostic purpose.

## 1.2. Animal Models of Sepsis

Sepsis occurs as a life-threatening complication, when the reactions of the body to infection causes self-damage to its own tissues and organs. This complication is followed by systemic inflammation involving excessively produced pro-inflammatory cytokine such as interleukin-1β (IL-1β) and tumor necrosis factors-α (TNF-α). In sepsis research, multiple animal models have been created to mimic human sepsis and they are used for studying the pathogenesis of sepsis as well as for testing prospective therapeutic agents. Among different animal models of sepsis, LPS induced endotoxemia and cecal ligation and puncture (CLP) model, are two extensively used animal models for studying systemic inflammation.

### Overview of LPS-Induced Sepsis Mice Models

Lipopolysaccharides (LPS), the outer membrane element of Gram-negative bacteria, can cause a powerful immune response in livestock and are thus commonly used in sepsis studies as a pathogen factor. LPS administration induces systemic inflammation that imitates many of the early clinical characteristics of sepsis such as the excess generation of the pro-inflammatory

cytokine, which results in multi-organ failure and high Mortality rate. TLR-4 is the main receptor for LPS. Binding of LPS with TLR-4 can activate the inflammatory signaling pathway such as mitogen-activated protein kinase MAPK and NF-κB pathways in immune cells, which plays a very important role in the regulation of inflammatory response in diseases condition(Chen et al. 2018). LPs induced endotoxemia is considered a very acute model of inflammation and the majority of the events occur immediately after LPS injection. Depending on the dose of LPS, various levels of mortality could be achieved using this model

.

**The Induction of Sepsis in mice**

The animals are given LPS intraperitoneally and are provided a moist and soft diet. The animals are observed for the development of systemic inflammation. Blood and different tissues are collected for analysis at different time points as per the study requirement

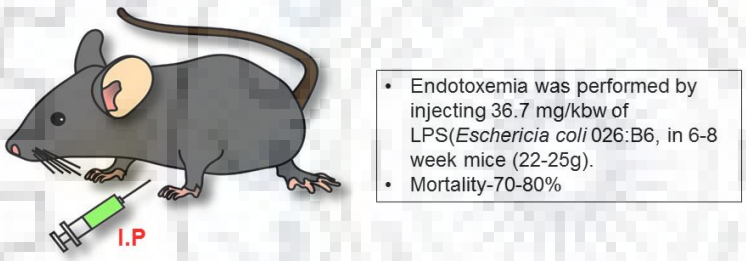



**Figure. 1 LPS-induced Endotoxemia model of sepsis**. In this model, LPS is injected intraperitoneally to induce systemic inflammation

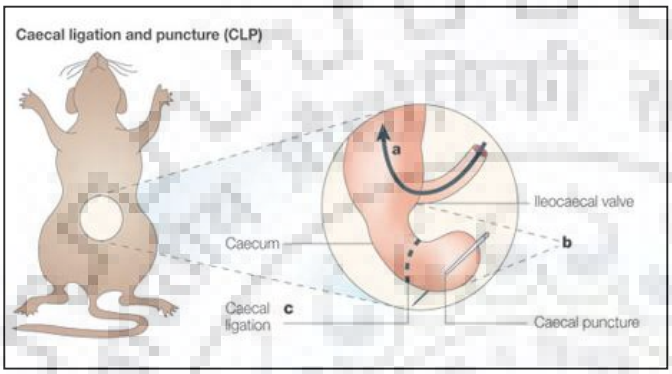
**Advantages and Disadvantages**

LPS administration is an easy and sterile way of inducing sepsis and has the same clinical feature with the human initial sepsis stages. However, in preclinical research, the LPS-induced sepsis model demonstrated weakness. First, sepsis-induced by Gram-negative bacteria shows a very fast and transient rise in the concentration of pro-inflammatory cytokines, whereas human sepsis is characterized by continuous elevation with a smaller magnitude. However, this model is very useful to study the initial stages of systemic inflammation and immune responses (Lewis, Seymour, and Rosengart 2016).

**Cecum Ligation and Puncture (CLP)-Induced Sepsis Model**

Sepsis is a severe medical condition with dysregulated systemic immune responses accompanied by immunosuppression, which still a big challenge to researchers and clinicians. In order to

encourage the creation of new and more efficient therapeutics agents, a thorough inquiry into the pathogenic mechanism of these diseases is urgently needed. Different animal models have been created to study the pathophysiology of sepsis, among which the most commonly used model is CLP, as it strongly mimics the development and features of human sepsis such as hypothermia, tachycardia, and tachypnea.it is usually recognized that CLP more correctly represents clinical truth than prior methods.




**Jon A. et.al. . Nature Reviews Drug Discovery (2005)**

**Figure 2 The caecal ligation and puncture (CLP) model of sepsis.** In this model sepsis is induced by laparotomy and exposure of the junction between the large and small intestines and perforating the caecum through and through to induce polymicrobial sepsis.

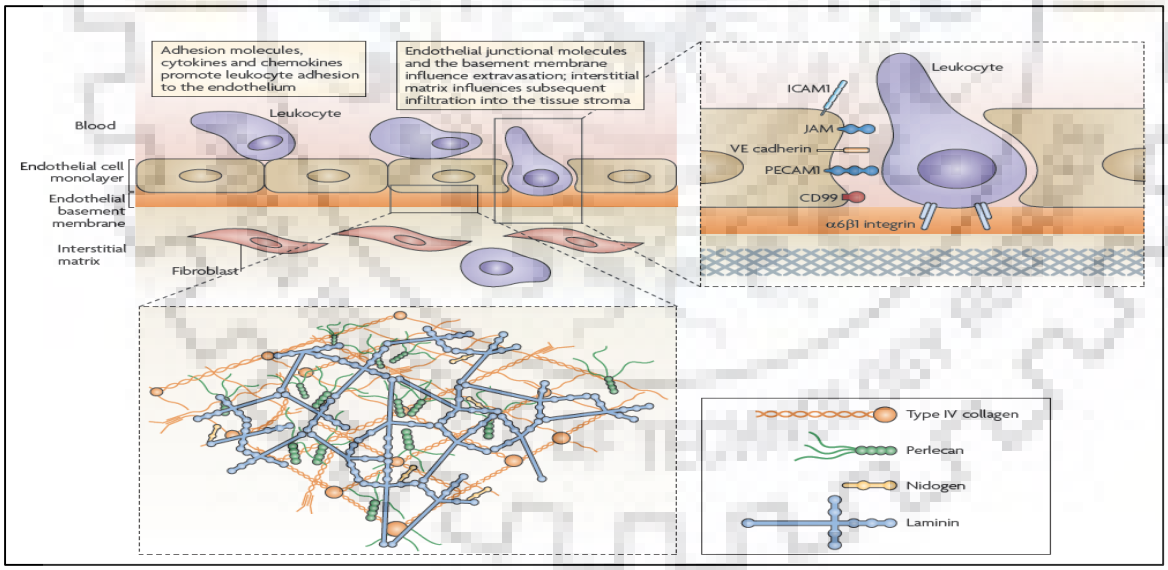
**Advantages of Cecum Ligation and Puncture (CLP) Model**

For its benefits over the other sepsis model, the CLP technique is a widely used and easily acceptable technique for inducing sepsis in vivo. First, CLP offers a better depiction of the complexity of human sepsis relative to other models. CLP has a ligation underneath the ileocaecal valve after laparotomy of the midline, followed by the puncture of the cecum with the help of a needle shown in Fig 2. Since the cecum is full of bacteria, its puncture leads to polymicrobial peritonitis, bacteremia, septic shock and ultimately death. Secondly, CLP pathogens are endogenous and thus imitates traumatic injury that leads to human peritonitis (Zhang et al. 2006).

## 1.3. Extracellular Matrix (ECM) Proteins:

Extracellular matrix (ECM), the material present surrounding the cells and tissues, is a complex structure formed by various secreted macromolecules that forms an organised, three-dimensional

scaffold to hold cells in place (Frantz, Stewart, and Weaver 2010). It comprises of regulated, tissue-specific composition, mainly different cell-secreted proteins, polysaccharides, sulphated proteoglycans and glycosaminoglycans (GAGs), forming a highly insoluble structure that exists in various biochemical and structural forms, namely the basement membrane and the interstitial matrix. The basement membrane forms a tightly knit substratum that gives support to epithelial cells and provides cell polarity. Basement membranes are composed mainly of few laminin isoforms, Type IV collagen, nidogen, perlecan and some minor glycoproteins (Sorokin 2010). The interstitial matrix, on the other hand, is made of fibrillar type I collagen (major component) and type III and V collagens, non-collagenous glycoproteins like tenascins, vitronectin and fibronectin and sulphated GAGs such as chondroitin sulphate and keratan sulphate(Sorokin 2010). The interstitial matrix is relatively loose and imparts tensile strength and elasticity to tissue. Apart from organizational and structural roles, the ECM also has a protective function by its buffering action, which maintains homeostasis and helps retain water (Frantz, Stewart, and Weaver 2010).




**Sorokin L, 712, Nature Reviews (2010)**

**Figure. 3: Description of ECM composition and various receptors associated with signaling processes during cell migration.**

## ECM composition and Functions:

The ECM protein made up of multiple matrix proteins that intertwined with each other to form the principal component of ECM, which provides vital support to cells, tissues, and organs in the body. Depending on their functions, they are divided into structural (e.g., elastin, collagen) and non-structural proteins (e.g., laminin, fibronectin). Growth factors, MMPs, and integrins are other essential ECM components that present in our body (Frantz, Stewart, and Weaver 2010).

**Collagens:**

Collagen is the most bountiful protein in our body and found in very high concentrations in the connective tissues like skin and tendons (Tanzer 2006, Kim, Turnbull, and Guimond 2011). They are produced by endothelium, fibroblasts, and epithelium. Generally, it contains three polypeptide chains with a triple helical structure. It can form a variety of supramolecular structures and divided into subgroups, based on their capability to form beaded filaments, fibrils, and networks, etc. To date, there are 30 types of collagen that have been identified. Among those few collagens such as Type I collagen is found in almost all the tissues. Whereas, other collagens like type II collagen found in the cornea & cartilages, Type III collagen in the blood vessel wall and collagen type IV found in basement membranes. It is one of the highly expressed structural protein present in ECM, that not only provide tensile strength but also involved in cell and ECM mediated processes such as cell migration and adhesion. Moreover, proteolytic cleavage of these proteins could result in the generation of matrikines, that could influence the cellular behaviors(Patel and Snelgrove 2018). Besides these, change in three-dimensional organization induces as a result of its interaction with other ECM components can expose the hidden cryptic sites on collagen proteins, that could modulate the immune cell functions (Mortimer and Minchin 2016).

**Elastin and Elastic Fibres:**

Elastin is a type of protein present in the tissues and is closely linked with collagen and it has the ability to stretch (Eckes, Nischt, and Krieg 2010). They are present in the body tissues like blood vessels, skin, bladder, etc. Elastic fibres are composed of the outer sheath of 10-12nm mainly of fibrillins which is surrounded by crossed linked elastin having 90% of fibres. Elastin is secreted as a soluble precursor (tropoelastin) mainly by chondrocytes, fibroblasts, and smooth muscle

cells. Mutation in the elastin gene can lead to Williams Beuren syndrome, which causes vascular defects like stenosis or dilation. However, if the condition persists, it can lead to cutis laxa(Tassabehji et al. 1998).

**Proteoglycans:**

They are the highly glycosylated proteins, generally made up of repeating units of disaccharides (glycosaminoglycans) with sulphate and carbonyl groups(Iozzo and Schaefer 2015). Usually, they are unified with collagen in ECM and confer additional properties besides structural stability (Yue 2014). It's highly negative charged nature results in the formation of space filling structures that helps in retaining divalent cations (calcium) and water. It also helps in the lubrication process. According to Hynes & Naba et.al Approximately 36 ECM proteoglycans are encoded by mammalian genomes (Hynes and Naba 2012). In ECM, different types of proteoglycans play varieties of functions. For example, perlecan is a principle component of the basement membrane, biglycan&decorin are LRR repeat proteins that are involved in matrix assembly and hyalectan family members (e.g., aggrecan, versican) bind to tenascins and other glycoproteins and modulate ECM-protein complexes.

**Glycoproteins:**

These are proteins, which consist of carbohydrate groups covalently attached to the polypeptide chain (Yue 2014). In mammals Mammalians approximately 200 glycoproteins could be found having a wide variety of functions such as ECM matrix assembly, adhesion, signaling, and reservoir for a growth factor (Yue 2014, Jones and Jones 2000). Matrix glycoproteins are distributed in almost all parts of the body, e.g. laminins in the basement membrane, fibrillins&fibulins in elastic fibres, fibrinogen in the vascular system, etc. Laminins are the most well documented glycoproteins having trimeric molecules (1α, 1β, 1γ chain), which is an essential component of the basement membrane. Other glycoproteins such as fibronectin exist as dimers whose two identical subunits are joined by a disulphide bond (Mao and Schwarzbauer 2005, Aumailley et al. 2005). It is usually produced by a single gene but alternate splicing results information of several isoforms of fibronectin. In vertebrates, fibronectin is present in two forms i.e soluble and insoluble form. Soluble fibronectin found in blood plasma, which is produced by hepatocytes in the liver whereas insoluble fibronectin, which is a major component of ECM, is

produced by fibroblasts, and is involved in various processes such as cell adhesion, and migration (To and Midwood 2011).

Fibulin is a family of secreted extracellular matrix glycoproteins that are linked to basements membranes, elastic fibres, and other matrices. Structurally it contains tandem repeats of epidermal growth factor (EGF)-like modules in the center, fibulin-type module at the C-terminus and a variable N-terminal domain. .During organogenesis and vasculogenesis, it can regulate cell-to-cell and cell-to-matrix communication and stabilize the extracellular matrix (ECM. Furthermore, it's also involved in cell morphology, developments, adhesion and motility acting as both tumor suppressors and oncogenic factors(Timpl et al. 2003). More recent studies have suggested their potential roles in inflammatory and autoimmune diseases. Fibulin-7 is a newly discovered member of the fibulin family, first identified in developing teeth. Unlike the other family members, at the N-terminus, fibulin7 has a sushi domain that involved in protein-protein interaction (de Vega et al. 2007). Recently, one of its C-terminal fragments (Fbln-7C) lacking the sushi domain has been shown to decreases the development of tubes and vessels in umbilical vein endothelial cells and prevent angiogenesis in an *in vivo* corneal mouse model (de Vega et al. 2014). Besides this, Fbln-7C has been shown to negative regulate differentiation and inflammatory function in monocytes and macrophages such as adhesion, migration and cytokine production (Sarangi et al. 2018).

## 1.4. Others Components Associated with ECM Proteins:

ECM proteins have the ability to bind with lots of different factors such as growth factors, MMPs, cytokines (Taipale and Keski-Oja 1997). That is why it's known to act as the reservoir for these proteins during an inflammatory and developmental process (Kim, Turnbull, and Guimond 2011). Growth factors are known to bind with ECM proteins through heparin sulphate(Wilgus 2012). During inflammation proteolytic degradation of ECM proteins results in releasing of these factors, that play a very decisive role in ECM remodeling and wound healing.

MMPs are zinc dependent enzymes (endopeptidases) consist of 23 proteins that play vital roles in ECM remodeling both at physiological and pathological conditions (Visse and Nagase 2003). According to their proteolytic activity they are classified into various subtypes such as collagenases (degrade fibrillar collagens, Col I, II, III), gelatinases (degrade Col IV & other basement proteins), membrane type MMPs, stromelysins (degrade non-collagen matrix proteins)

and matrilysins(Ricard-Blum 2011, Parks, Wilson, and López-Boado 2004). In tissues at the basal level, the MMP activities are very low but it could be modulated by several factors such as matrix interaction, cell-cell interaction and with the presence of various cytokines (Arend and Dayer 1995). Generally, MMPs are secreted in inactivated forms, which get activated by proteolytic removal of their prodomain. The mechanism of MMP activation is not fully understood but they can be regulated by oxidants, plasmin and other activated MMPs(Arend and Dayer 1995, Parks, Wilson, and López-Boado 2004). During inflammation activated MMPs play different roles such as degradation of ECM components, activation of other MMPs and releasing growth factors (Löffek, Schilling, and Franzke 2011). Its activity is regulated by TIMP, which bind to the active site of MMPs and block their functions that help in the maintenance of ECM homoeostasis.

## 1.5. ECM-Cell Interactions:

ECM interactions with cells are very dynamic and are constantly changing during inflammatory processes (Lu et al. 2011). Its intrinsic components form cell-matrix adhesions which play crucial roles in influencing immune cell activity and behaviors such as cell migration, activation, differentiation and proliferation (Lu et al. 2011). One way in which the cellular microenvironment is regulated for the above processes is by signalling via matrix-adhesion molecules such as integrin (Geiger and Yamada 2011). These receptors present on tissue-resident cells and transient leukocytes that extravasate during inflammatory responses interacts with matrix elements such as collagen, fibronectin, and laminins causing further changes in matrix composition and structure that in turn influences their actions (Weninger, Biro, and Jain 2014). However, cells could also use non-integrin based interactions to modulate cellular functions. Usually, ECM structures contain bioactive domains called “matricryptins”, which are usually hidden under physiological conditions. However, during inflammation, proteolytic degradation of ECMs results in the release of bioactive ECM fragments called "matrikines”. These matrikines interact with various receptor on the inflammatory cells such as of TLR2 and/or TLR4 and could trigger various inflammatory responses such as chemotaxis, activation, and survival of immune cells, thus helps in increasing the severity of inflammatory responses in certain disease conditions such as rheumatoid arthritis (RA), chronic lung diseases and skin inflammatory diseases (Gaggar and Weathington 2016).

Integrins are transmembrane, heterodimeric proteins that form receptors to adhere to ECM components on the extracellular surface, and intracellularly connect the cytoskeleton and activate a number of cell signalling pathways (Hynes 2002). 18 α and 8 β subunits dimerise to form 24 distinct combinations of integrin dimers. They are identified generally on their ligand-binding partners and are largely classified as β1, β2, and β3 or αv-containing integrins as these are the largest classes of integrins on the cell surface. Members of the β1 integrin family are expressed in most cell types whilst β2 integrins, also called leukocyte-specific receptors are expressed on most white blood cells in their active states (Hynes 2002). In addition to their extracellular ligands, each integrin is associated with the cytoskeletal network in the interior of a cell. Both internal and external surfaces combine to give integrins their property of bidirectional signalling that plays an important function in diverse ways such as in preimplantation development, inflammation, angiogenesis and homeostasis (Hynes 2002). Integrin dimers send signals across the cell membrane in both directions, which is facilitated by the connection with the cytoskeletal elements. Apart from their prominent role in cell-matrix and cell-cell interactions, they also transmit chemical signals as information into the cell on the location of the cell and nature of the extracellular environment, termed "Outside-In" signalling. Another process is “Inside-Out” signalling in which intracellular signals to the cytoplasmic ends cause integrins to undergo conformational changes in their extracellular domains, rendering them active (Harburger and Calderwood 2009). Activation caused by the binding of proteins such as talins and kindlins to the cytosolic tails causes dimers to cluster together, making them more adhesive for their counter-receptors or ligands. Systematic interactions and coordination between multitudes of cellular proteins and pathways are involved in integrin signalling that works along with external protein networks to relay the information across the cell membrane (Kim, Turnbull, and Guimond 2011).

## 1.6. ECM Remodeling:

Proper cellular and tissue functions require a tightly organized ECM homeostasis and an impaired regulation or irregular ECM remodeling could lead to life threatening conditions such as poor wound healing, defective organ development, cancer, connective tissue inflammation, and fibrosis (Cox and Erler 2011). One of the prominent features of ECM remodeling involves changes in 3D-organization that expose its hidden cryptic sites, which can interact with cell

receptors such as integrins and modulate carious activities like cell migration, adhesion, and activation. MMPs are the main proteolytic enzymes that are associated with altered ECM remodeling. As mentioned earlier, under physiological conditions, various MMPs show basal level expression but during the pathological condition, their expressions are elevated which results in dysregulation of ECM remodeling (Stamenkovic 2003). In brain injury such as ischemic conditions, it's known to be associated with aberrant ECM remodeling. It is also supported by Cuadrado et al study on brain injury where abnormal expression of MMP-10 was observed in ischemic brain injuries compared to healthy ones (Cuadrado et al. 2009). Besides this, MMPs are also known to be involved in tumor invasion and infiltration (Kessenbrock, Plaks, and Werb 2010).

## 1.7. Excess ECM production May Lead To Pathological Fibrosis

Uncontrolled ECM remodeling is shown to be associated with multiple pathological conditions. For example, in both acute and chronic injuries, loss of regulation in the ECM homeostasis could lead to altered ECM expression and distributions, which in turn could lead to disease conditions such as fibrosis (Bonnans, Chou, and Werb 2014). This abnormal healing process, if not checked on time could cause organ failure, like myelofibrosis in the bone marrow or cirrhosis of the liver. Such a phenomenon is also associated with escalated risk of cancers in patients; for e.g, cirrhotic liver heightens the chances of liver carcinoma (20–30%) and repeated mammography increased the risk of breast cancer (Davis et al. 2008, El-Serag 2011). In fibrotic conditions, TGFβ is the most well researched pathway which is responsible for the translocation of transcription factors (SMAD2–SMAD3 complex) into the nucleus and directly regulate the expression of about 60 ECM-matrix related genes including collagen I, TIMP1, etc (Verrecchia, Chu, and Mauviel 2001).

Some pro-fibrotic cytokines such as IL-33 also play a very significant role in fibrosis by regulating immune cells function. In the liver, IL-33 facilitates the increase of resident IL-13 secreting innate lymphoid cells and in turn, it activates the main ECM producing hepatic stellate cells in the liver(Mchedlidze et al. 2013, Bailey et al. 2012). Besides this, IL-33 also promotes fibrosis by downregulating MMPs, stimulating collagen accumulation, inducing fibroblasts differentiation into myofibroblasts, increasing TGFβ expression and recruiting innate immune cells (pro-fibrotic) (Bailey et al. 2012). It is also shown that in chronic lung diseases like chronic

obstructive pulmonary disease (COPD), which is characterized by blockage in airflow of lung tissue by ECM, is shown that, the elastin breakdown by neutrophil elastase and defective degradation of type VI collagen is responsible for increase in COPD related disease severity (Bihlet et al. 2017). Another study showed that in COPD, there is an increase in the accumulation of collagen type -I, -III, and -IV, fibronectin, and laminin $\beta_2$ in the lung tissues (Kranenburg et al. 2006). In the lung, structural remodeling of the airway can lead to an increase in ECM accumulation and abnormal scar tissue formation, which can lead to airway blockage, contraction of the lumen and loss of function. Additionally, fibronectin fragment (EDA), is known to increase in scleroderma and could induce collagen synthesis via TLR4 signaling, modulate myofibroblast differentiation and stiffness of ECM (Saetta et al. 2001, Bhattacharyya et al. 2017). All these studies emphasize there is the necessity of regulated ECM homeostasis in both physiological as well as in pathological conditions.

## 1.8. Rationale of the Study

To date, many studies have focused on the molecular mechanisms of altered trafficking of immune cells and disease specific local ECM remodeling. For example, previous studies in local inflammatory conditions such as arthritis, brain injury, lung injury, etc., have shown changes in the expression of multiple ECM proteins such as collagen-IV (Col-IV), fibrinogen, vimentin, fibronectin, and laminin (Table.1). Not much is known about the changes that occur during systemic inflammatory responses as sepsis. Thus, detailed knowledge on the pattern of ECM protein expression could help in understanding the cell migration patterns and designing cell adhesion based therapeutics.

**Table 1: ECM protein Expression under various inflammatory conditions.**

| **Disease Model** | **Change in ECM Proteins** |
|---|---|
| Lung injury | Collagen I, III, V, Laminin, Fibrinogen &Fibronectin |
| Liver injury | Collagen IV, Laminin, Nidogen and Gelatin |
| Kidney injury | Collagen 13 |
| Systemic Inflammation | ECM???? |

Similarly, the second rational of this study is based on testing the effect of a C terminal fragment of the newly identified adhesion protein Fibulin7 (Fbln7-C) that binds to immune cells via their integrin (Fbln7-C) in modulating the immune cell function, especially neutrophils. Fbln7 is a newly identified protein which has shown immunomodulatory effects on the human monocytes and macrophages, but not much is understood about its effect on the neutrophils. This study will decipher the effects of Fbln7-C on the functions of neutrophils.

# OBJECTIVES OF THE STUDY

1. To study the expression and distribution pattern of various ECM proteins in local inflammation sites during systemic inflammation.

2. To study the expression and distribution pattern of various ECM proteins in distant visceral organs during systemic inflammation.

3. To study the immuno-modulatory effect of adhesive fibulin7 protein on neutrophils function.

# CHAPTER: 2

## Investigation of Extracellular Matrix Protein Expression Dynamics Using Murine Models of Systemic Inflammation

### Introduction

Extracellular matrix (ECM), is a key modulator of inflammation. It not only provides the structural support for migration of immune cells but also acts as a repository of extracellular signaling molecules (Kim, Turnbull, and Guimond 2011). Following extravasation, the immune cells interact with a variety of matrix components such as collagen, fibronectin, laminin which could modify their functional properties (Bonnans, Chou, and Werb 2014). Similarly, secretion of inflammatory mediators such as cytokines, MMPs, protease from inflammatory cells influence the expression and distribution of ECM in the inflamed tissue contributing to different cellular phenotypes (Overstreet et al. 2013). Thus, ECM remodeling is a prominent pathological feature in many inflammatory diseases (Bonnans, Chou, and Werb 2014, Shimshoni et al. 2015).

Sepsis, is defined as a dysregulated systemic inflammatory response to an uncontrolled infection and is a leading cause of death in adult intensive care units around the world (Bhan et al. 2016). Previous histopathological analysis of visceral tissue from septic patients have demonstrated that the dysregulated microenvironment could lead to hyper activation, altered migration and activation of innate immune cells such as neutrophils and macrophages (Sarangi et al. 2012, Rittirsch et al. 2008). Furthermore, excessive production of pro-inflammatory mediators, matrix metalloproteinases (MMPs) and reactive oxygen species by innate immune cells could lead to further tissue damage leading to multi organ failure (Bhan et al. 2016). To date, many studies have focused on the molecular mechanisms of altered trafficking of immune cells and disease specific local ECM remodeling. For example, previous studies in local inflammatory conditions such as arthritis, brain injury, lung injury etc., have shown changes in the expression of multiple ECM proteins such as collagen-IV (Col-IV), fibrinogen, vimentin, fibronectin, and laminin. (Bonnans, Chou, and Werb 2014, Cox and Erler 2011, Kumawat et al. 2013).

The current study is based on the hypothesis that, systemic inflammation could differentially alter the expression and distribution of ECM proteins in both local and distant visceral tissues. Our results from two murine models of systemic inflammation which involve

different inflammatory events, showed tissue specific differences in the expression of Col-4, fibrinogen and vimentin at mRNA and protein level with few unique patterns in both models. The changes in the expression of these proteins were observed earlier at the site of inflammation compared to the distant visceral organs. These changes could be associated with sequence of inflammatory changes taking place in various tissues at a given time. Further, bioinformatics analysis showed multiple pathways which could be associated with specific ECM proteins under inflammatory conditions both in human and mouse. The results from this study will open a new avenue towards understanding the complex pathophysiology of sepsis and may lead to development of cell-adhesion based therapeutics.

## 2.2. Materials and Methods:

**2.2.1. Sepsis Mouse Model:** CLP and endotoxemia were conducted on C57BL/6j in accordance with protocol approved by the Animal Ethics Committee at Indian Institute of Technology Roorkee, India. For both procedures, protocols were followed as described earlier by Sarangi et. al .(Sarangi et al. 2017).6-8 weeks C57BL/6 male mice were purchased from National Animal Resource Facility for Bio-Medical Research, Hyderabad, India. Endotoxemia was performed by injecting36.7mg/kbw of Lipopolysaccharide (LPS) (*Eschericia coli* 026:B6, Sigma Aldrich, St. Louis, MO) in 6-8 week mice(22-25g) intraperitoneally as per the titration in the animals to achieve 70-80% mortality. CLP surgery was performed in mice anaesthetized with Ketamine and Xylazine. Following midline incision, cecum was taken out and ligated with silk suture. Following ligation, the caecum was punctured through and through using a 21-gauge needle. Following wound closure with silk suture, 1% lignocaine was applied to the surgery site. 1ml of Dextrose Normal Saline (DNS) was injected subcutaneously to resuscitate the animals following CLP surgery. For both the models, access to soft pellets and water was made available through-out the experimentation period.

**2.2.2. Real Time- PCR:** Total RNA was extracted from the tissues using TRIzol Reagent (Life technologies, Carlsbad, CA) and reverse transcription of the extracted RNA was performed using Accuscript High Fidelity 1$^{st}$ Strand cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. Real Time- PCR was performed using Power

SYBR Green PCR master mix and Power UP SYBR Green master mix (Applied Biosystems, Foster City, CA) with Primers listed in the table-2. Samples were normalized to the endogenous control HPRT and relative gene expression was measured by $2^{-\Delta\Delta CT}$method.

**Table. 1. List of Primers**

| S.No. | Primer (gene) name | Primer sequence |
|---|---|---|
| 1. | HPRT-F | GTTAAGCAGTACAGCCCCAAA |
| 2. | HPRT-R | AGGGCATATCCAACAACAAACTT |
| 3. | Collagen-Iα1-F | ACCTCAAGATGTGCCACTCT |
| 4. | Collagen-Iα1-R | TGACCTGTCTCCATGTTGCA |
| 7. | Collagen-IVα1- F | GGGGCGAGAACTCCATAAGA |
| 8. | Collagen-IVα1- R | GGTTGGACAGCACTCACATC |
| 9. | Fibronectin –F | CCGTTCCAGGAGAGTTCTGA |
| 10. | Fibronectin –R | TCGCACTGGTAGAAGTTCCA |
| 11. | Fibrinogen α –F | CAGGGTCAAAGGCAGAAAAC |
| 12. | Fibrinogen α –R | CAGGGTTCCGATTTCCATCA |
| 13. | Laminin-IVα- F | CCCCTGCCTTTTGATGTTCA |
| 14. | Laminin-IVα- R | GCGTGGTCTCTTCTCTCTCT |

**2.2.3. Protein isolation and Western blotting:** Total protein was isolated from the animal tissues using T-PER$^{TM}$ Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Walthem, MA). An equal amount of protein was loaded for SDS- PAGE. The separated proteins were transferred onto BioTrace NT nitrocellulose membrane (PALL Corporation, New York) and blocked with 5% BSA or 5% dried, fat-free milk in PBS-0.05% Tween-20 (PBST) for 1–2 h at room temperature, then incubated with primary antibody in blocking buffer for overnight at 4°C. The primary antibodies used in the assays were anti-□-actin (Cell Signaling Technology, Danvers, MA), anti-fibrinogen (Santa Cruz Biotechnology, Dallas, TX), anti-vimentin (Santa

Cruz Biotechnology, Dallas, TX), anti-collagen 1 (Gel Electrostatic Surface Potential of Macrophages Correlates with Their Functional Phenotype l Signaling Technology, Danvers, MA), and anti-collagen IV (Southern Biotech., Birmingham, AL). Membranes were then washed 4 times with PBST and incubated with HRP-conjugated secondary antibody at in 5% blocking buffer for 1 hr at room temperature. Membranes were again washed 4 times with PBST. The bands developed by immune-reactive complex were visualized by means of a Chemidoc (Bio-Rad, Hercules, CA). All densitometry analysis of protein bands was performed using ImageJ software (National Institute of Health, Bethesda, MD).

**2.2.4. Bioinformatics Analysis:** Analytical determination of the investigated proteins (vimentin, Col-IV, and fibrinogen) for both physical (direct) and functional (indirect) aspects were analysed through Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, version 11.0) database (https://string-db.org) (Szklarczyk et al. 2018). Medium confidence (0.400) parameter was chosen for the minimum required interaction score and inflation parameter was set to 3 in MCL clustering. Also the structure preview inside network bubbles was disabled to simplify the display output. All the proteins were individually searched in STRING database for *Homo sapiens* and *Mus musculus* separately to analyse their interacting partners and their respective functional relevance.

**2.2.5. Statistical analysis:** All values are presented as mean ± Standard error mean (SEM). Data analysis was carried out using GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA) using student t- test or nonparametric Mann-Whitney test, Kruskal-Wallis with Dunn's multiple comparisons test and One-way ANOVA with Dunnett's multiple comparisons test. However, according to suggestions by Wasserstein et. al., the phrase 'statistically significant has not been used in this study (Wang et al. 2019).

## 2.3. Results:

### 2.3.1. Systemic inflammation induces modulations in the expression of ECM proteins at the transcript level in both local inflammation site and visceral tissues

Changes in the distribution and modulation of ECM expression have been previously reported in different models of local inflammation but not much is known about the detailed distribution pattern in systemic inflammations (Fu et al. 2019). Therefore, we first investigated the expression dynamics of these ECM proteins at the transcript level using LPS induced

endotoxemia and CLP surgery murine models of systemic inflammation. Following LPS injection and CLP surgery, mesentery, lungs, kidney and liver samples were isolated at 6hr, 12hr, 24hr and 36 hr time points and the mRNA expression was analyzed using real time PCR. Induction of local and systemic inflammation was confirmed by monitoring IL-6 mRNA expression in mesentery and lungs for chosen time points (Fig. 1).

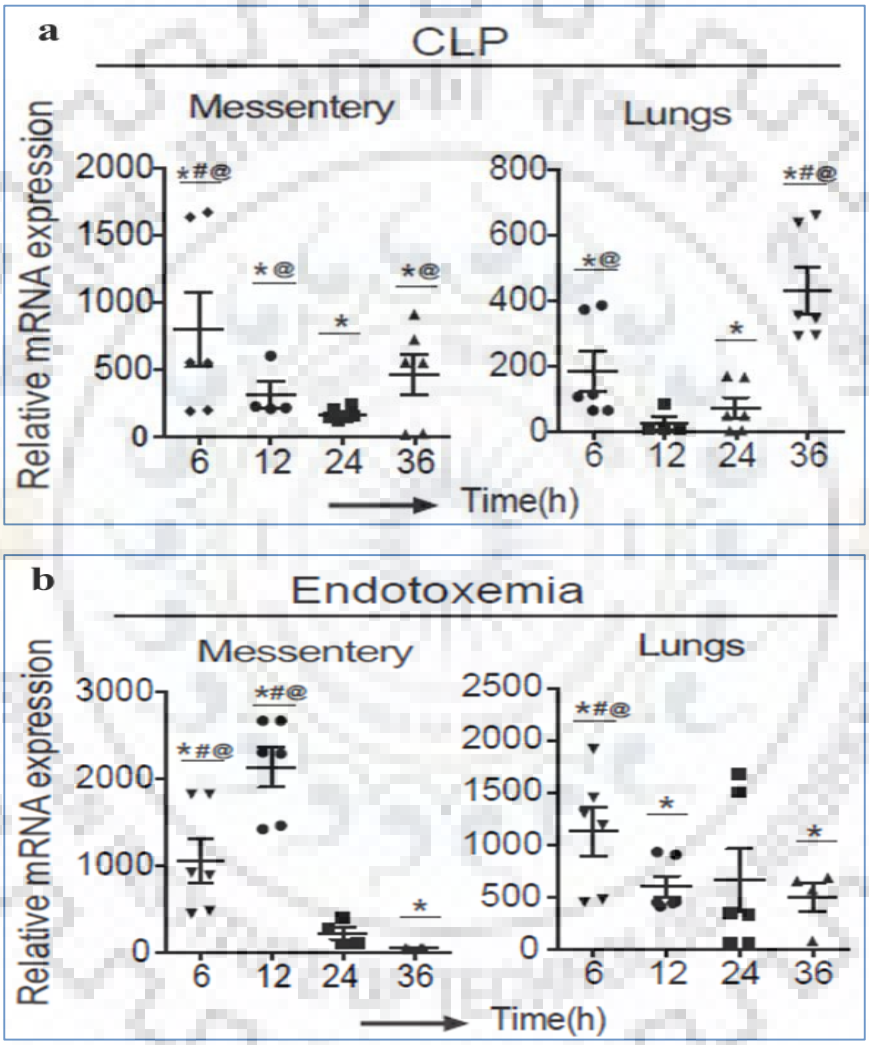



**Figure. 1. Expression of IL-6 mRNA in mesentery and lungs following induction of systemic inflammation.** Relative expression kinetics of IL-6 mRNA in mesentery and lungs harvested from CLP and LPS animals was measured using quantitative real-time PCR. The dot-plots represent the relative IL-6 mRNA level in mesentery and lungs of CLP (a) and endotoxemia (b) animals compared to the same tissue harvested from naïve animal. The data are presented as mean ± SEM. (N=4-6/time point) ($p \leq 0.05$- *-Student t- test, #- One-way ANOVA

with Dunnett's multiple comparisons test and @- Kruskal-Wallis test with Dunn's multiple comparisons test

After confirming the inflammatory responses in both the models of sepsis, the levels of various extracellular matrix proteins were measured by Real-time PCR technique as described in the methodology. All the primers were used as shown in the table 2. Interestingly, as is shown in Fig. 4 and 5, different expression pattern of ECM protein expression was observed for both LPS induced endotoxemia and the CLP induced polymicrobial sepsis models, possibly due to difference in inflammation induction pattern in both models (Sarangi et al. 2012, Wasserstein, Schirm, and Lazar 2019). As shown in Fig. 2 and 4, up to 30 fold increase in the mRNA of Col-IV with a 4 and 6 fold increase in the transcripts of fibrinogen and Col-1 respectively were observed in the mesentery tissue within 6h of surgery. A four-fold increase in the transcripts of laminin-IV was also observed by 12 h post CLP induction.

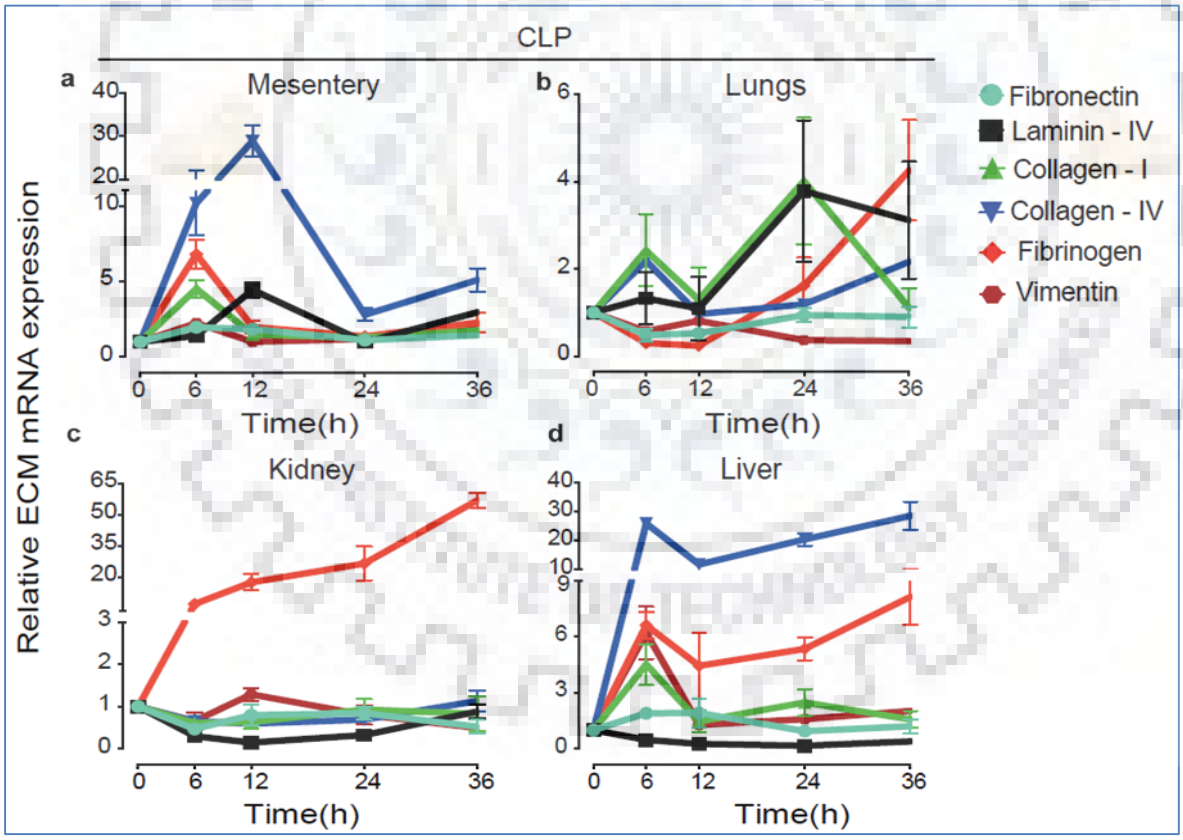



**Figure. 2. Expression of ECM proteins at the transcript level in different tissues following induction of systemic inflammation by CLP surgery.** Relative expression kinetics of ECM

proteins at mRNA level in various tissues isolated from CLP animals was measured using real-time PCR as described in the methods. The line diagrams show the expression of various ECM proteins at mRNA level in CLP model. Results are expressed as fold change in comparison to the expression level of respective ECM protein in the same tissue harvested from naïve animals. The data are presented as mean ± SEM. (N=4-6/time point)

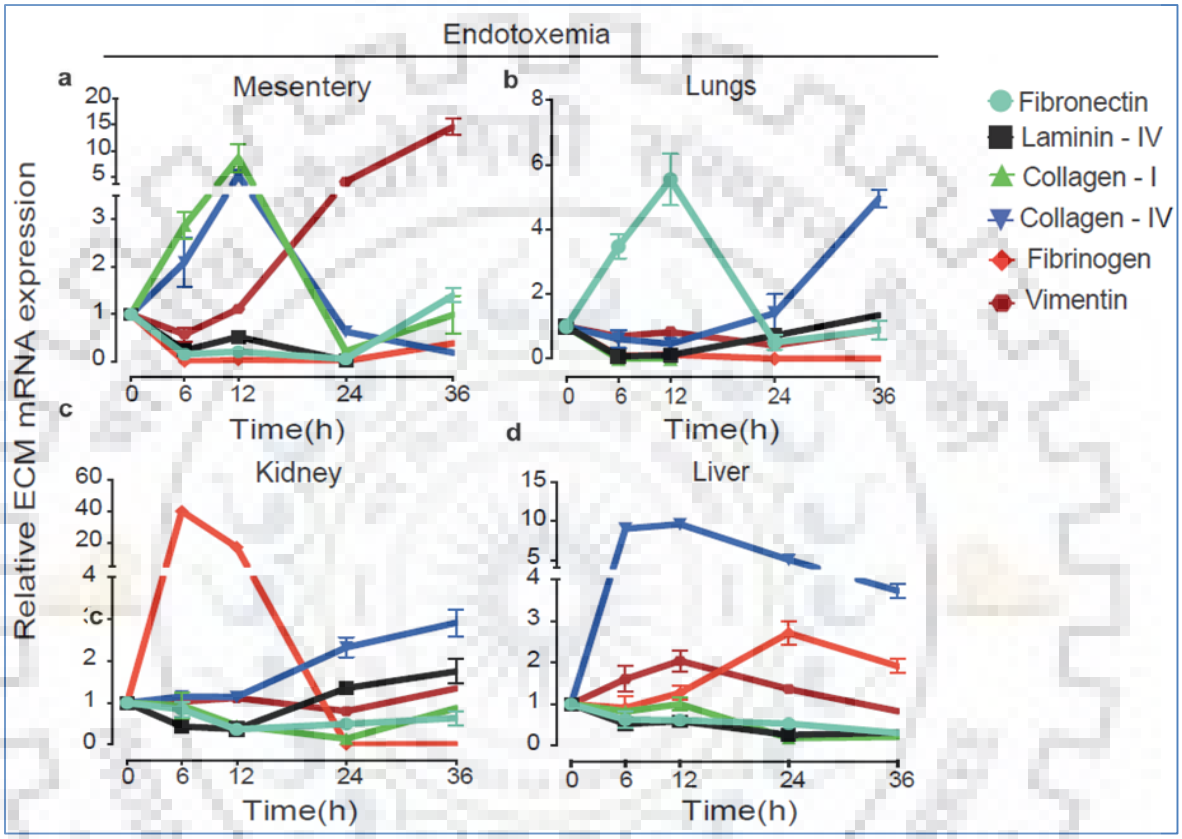



**Figure 3. Expression of ECM proteins at the transcript level in different tissues following induction of systemic inflammation by LPS administration.** Relative expression kinetics of ECM proteins at mRNA level in various tissues isolated from endotoxemia animals was measured using real-time PCR as described in the methods. The line diagrams show the expression of various ECM proteins at mRNA level in endotoxemia animals. Results are expressed as fold change in comparison to the expression level of respective ECM protein in the same tissue harvested from naïve animals. The data are presented as mean ± SEM. (N=4-6/time point)

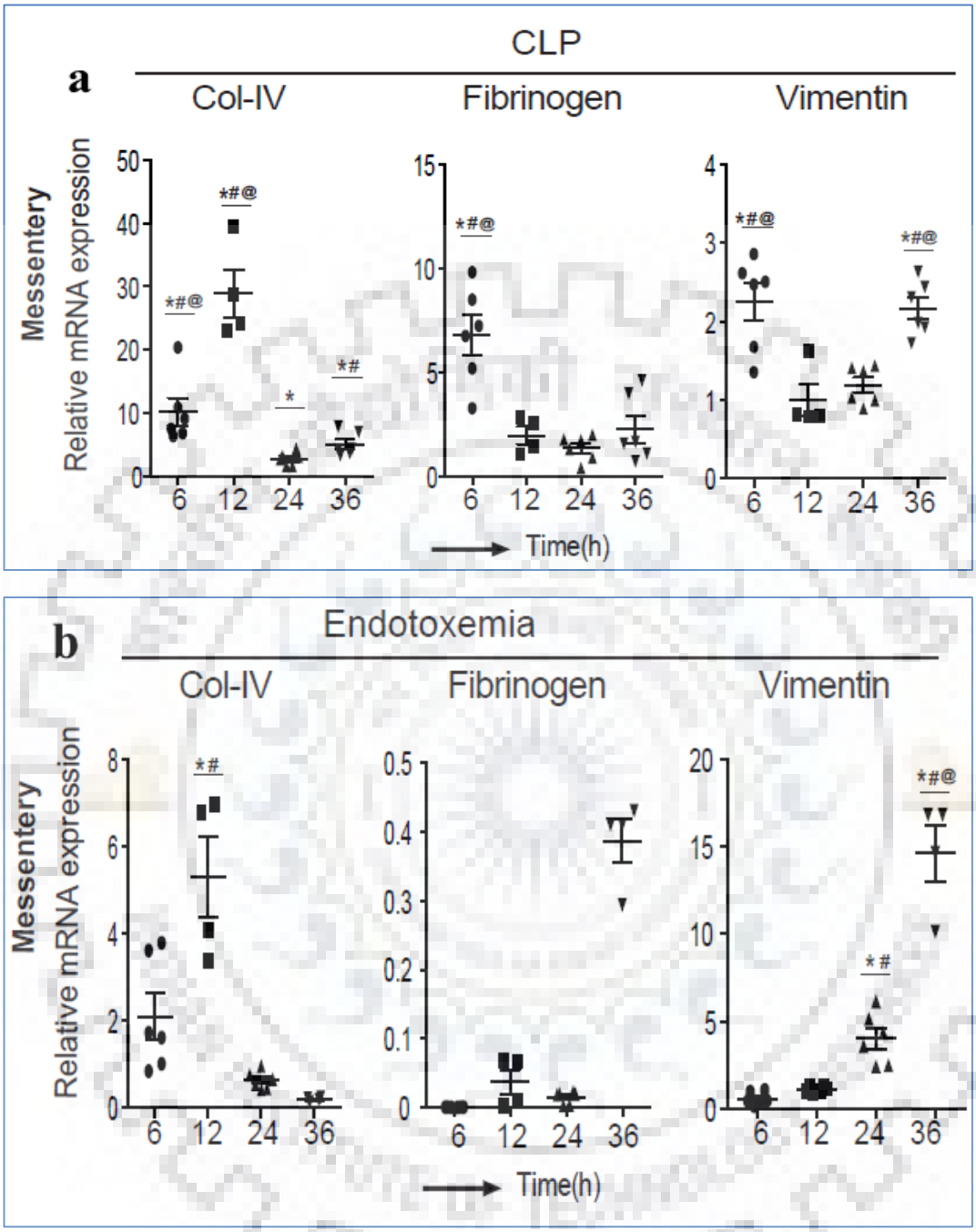
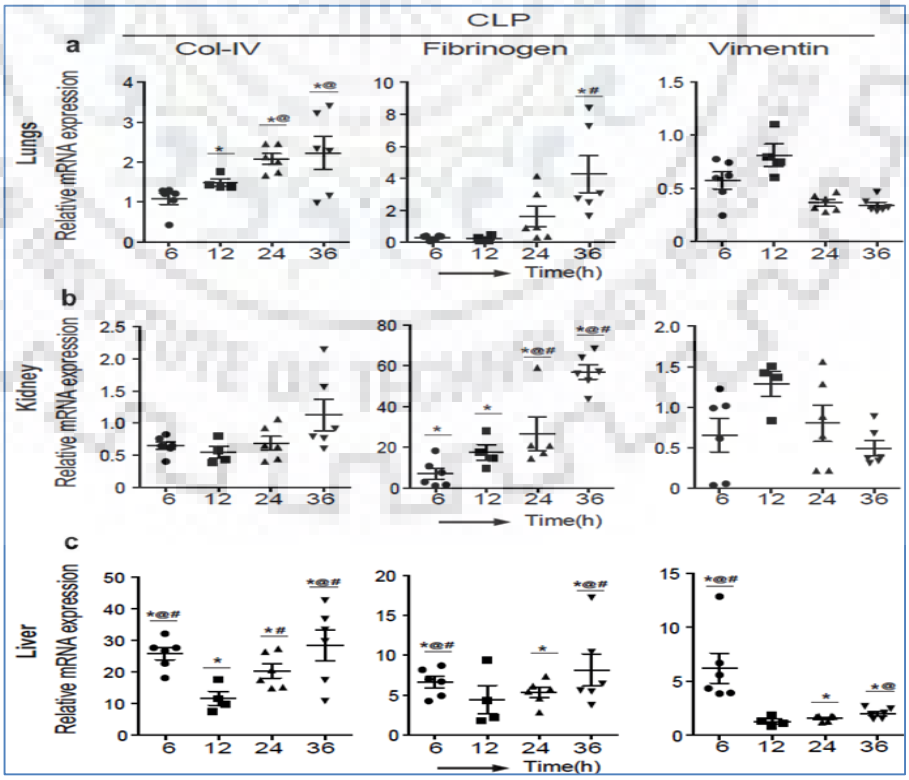



**Figure. 4. Expression of ECM proteins at the transcript level in inflammatory site following induction of systemic inflammation.** Relative expression kinetics of ECM proteins at mRNA level in mesentery isolated from CLP and endotoxemia animals was measured using quantitative real-time PCR as described in the methods. The dot-plots show the expression of various ECM proteins at mRNA level in mesentery of CLP (a) and endotoxemia (b) animals. Results are expressed as fold change in comparison to the expression level of respective ECM protein in the same tissue harvested from naïve animals. The data are presented as mean ± SEM. (N=4-6/time point) ($p \leq 0.05$- *-Student t- test, #- One-way ANOVA with Dunnett's multiple comparisons test and @- Kruskal-Wallis test with Dunn's multiple comparisons test)

Upon analysis of tissues from visceral organs, a similar trend for Col-IV was also observed in the lungs (Up to 3 fold) and liver (Up to 30 fold) with an earlier upregulation of the same in the liver tissues (Figure 5a, and c & Figure 2 b and d). In contrast, as shown in Fig.5b and 2 c, fibrinogen transcripts showed up to 40-60 fold increase in CLP surgery group compared to naïve animals. Unlike other three tissues, levels of fibrinogen mRNA was dramatically higher compared to Col-IV. A smaller change of up to 5-6 fold increase in the transcripts of vimentin was also observed in the lungs, liver and kidney tissues (Figure 5a, b, and c & Figure 2 b, c, and d).

In LPS model, up to 4 fold and 10 fold increase in the level of Col-IV was observed in mesentery and liver respectively within 6hr to 12hr of LPS administration (Figure 4b and 5f & 3a and d). However, in lungs and kidney the upregulation in Col-IV transcript was observed during later time points i.e. 24-36hr after injection (Figure 5d and e & 3b and c). Elevated level of fibrinogen transcripts was also observed in liver and kidney, whereas the upregulation in fibronectin transcript level was limited to lungs only. As shown in Fig.4b considerable changes (up to 15 fold, $p > 0.003$) in the expression of vimentin mRNA was also observed within 36h in mesentery. So, in both the model of systemic inflammation, Col-IV, fibrinogen and vimentin were the prominent ECM proteins showing remarkable changes in the expression at transcript level.



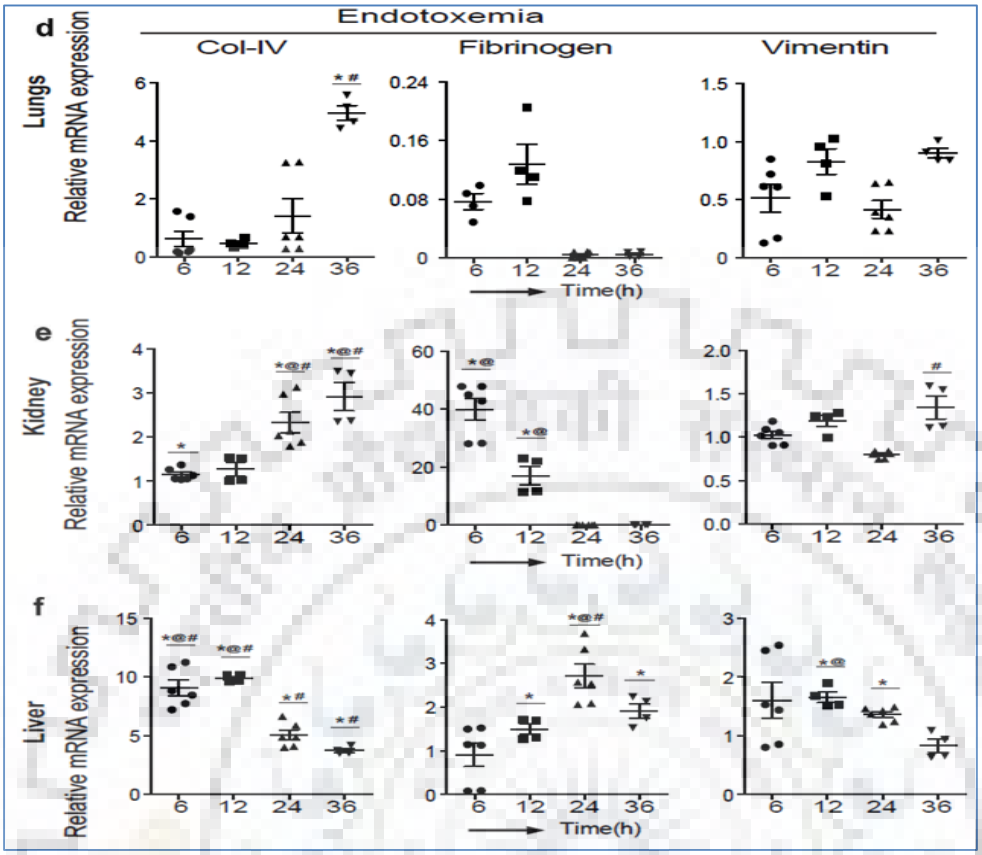



**Figure. 5. Expression of ECM proteins at the transcript level in visceral tissues following induction of systemic inflammation.** Relative expression kinetics of ECM proteins at mRNA level in lungs, kidney and liver isolated from CLP and endotoxemia animals was measured using quantitative real-time PCR as described in the methods. The dot-plots show the expression of various ECM proteins at mRNA level in visceral organs of CLP (a, b and c) and endotoxemia (d, e and f) animals. Results are expressed as fold change in comparison to the expression level of respective ECM protein in the same tissue harvested from naïve animals. The data are presented as mean ± SEM. (N=4-6/time point) ($p \leq 0.05$- *-Student t- test, #- One-way ANOVA with Dunnett's multiple comparisons test and @- Kruskal-Wallis test with Dunn's multiple comparisons test)

### 2.3.2. Protein analysis shows collagen-IVα, fibrinogen, and vimentin are modulated at the site of inflammation

To check if the modulations observed in the ECM expression at mRNA level reflect at protein level, total protein was extracted from the inflamed mesentery tissues from both CLP and LPS induced animals. Expression of various proteins that showed modulation at the transcript levels were analysed using western blot technique. As shown in Figure 6a, in the mesentery tissues of CLP animals, up to two fold change in the expression of ECM proteins such as Col-IV, fibrinogen and vimentin were observed as early as 6hr post induction of systemic inflammation

and was sustained till 24hr post-surgery. Interestingly, as perceived from the band density, level of fibrinogen protein expression was found to be much more compared to others.

In contrast to CLP model, endotoxemia animals showed a delayed upregulation of both Col-IV and fibrinogen protein in the mesentery tissues that peaked around 24 hr following LPS injection. (Figure 7a). However, in line with the CLP model, a two-fold increase in the expression levels of vimentin was observed within 6hr of LPS injection. (Figure 7a)

### 2.3.3. Modulation of ECM expression extends to visceral organs during systemic inflammation

When a local inflammation is not contained, it spreads throughout the body resulting in systemic inflammation in which visceral organs are also seriously affected. So, to study the modulations in ECM expression in visceral organs, total protein was isolated from lungs, liver and kidney harvested from LPS and CLP induced animals, and western blot was performed. Lungs are one of the vital organ that gets affected relatively quicker following the spread of any infection into the circulation. When analysed at different time points, a moderate upregulation of Col-IV, fibrinogen and vimentin were upregulated in the lung tissues within 6-12 hr post-surgery. Although detected in liver and kidney of inflamed animals, no significant modulations were observed in such tissues in CLP animals.

In contrast to CLP animals, as shown in Figure 7b, up to three-fold increase in Col-IV and upto two-fold upregulation in the expression of fibrinogen and vimentin were observed in the lung tissues. Possibly as intense inflammatory response following LPS injection resulted in the modulation of ECM structure of the lung tissues in such animals. Interestingly, as shown in the Figure 7c, in the kidneys of endotoxemia animals, marked upregulation of fibrinogen ($p > 0.04$) was observed as compared to naïve tissues suggesting an important role of this protein in the modulation of inflammatory events. As shown in Fig. 7d, the change in the ECM protein expression was not prominent compared to other tissues

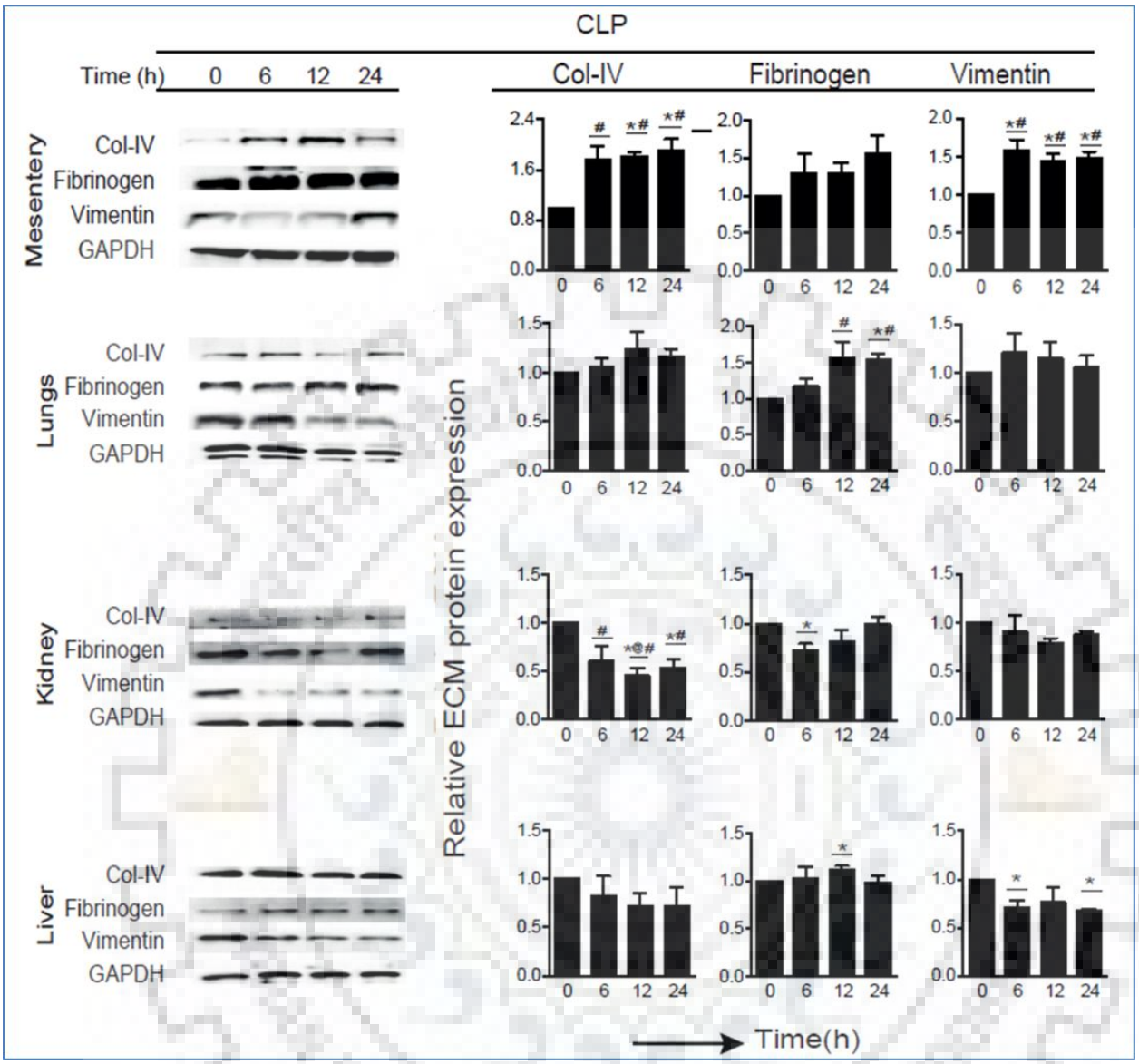



**Figure. 6. Expression of ECM proteins at the local inflammation site and distant visceral organs in CLP induced systemic inflammation.** Relative expression of ECM proteins in different tissues at different time points following CLP surgery was measured using western blot as described in the method section. GAPDH was used as endogenous control for all analysis. The left panels in Fig. a, b, c and d shows the western blot pictures of respective proteins at different time points in the tissue as labelled in respective figures. The bar diagrams on right panels show the fold change in the expression of specific protein compared to that of corresponding organs of naïve animals as measured by densitometry analysis of individual bands using ImageJ software. The data are presented as mean ± SEM. ($p \leq 0.05$- *-Student t- test, #- One-way ANOVA with Dunnett's multiple comparisons test and @- Kruskal-Wallis test with Dunn's multiple comparisons test)

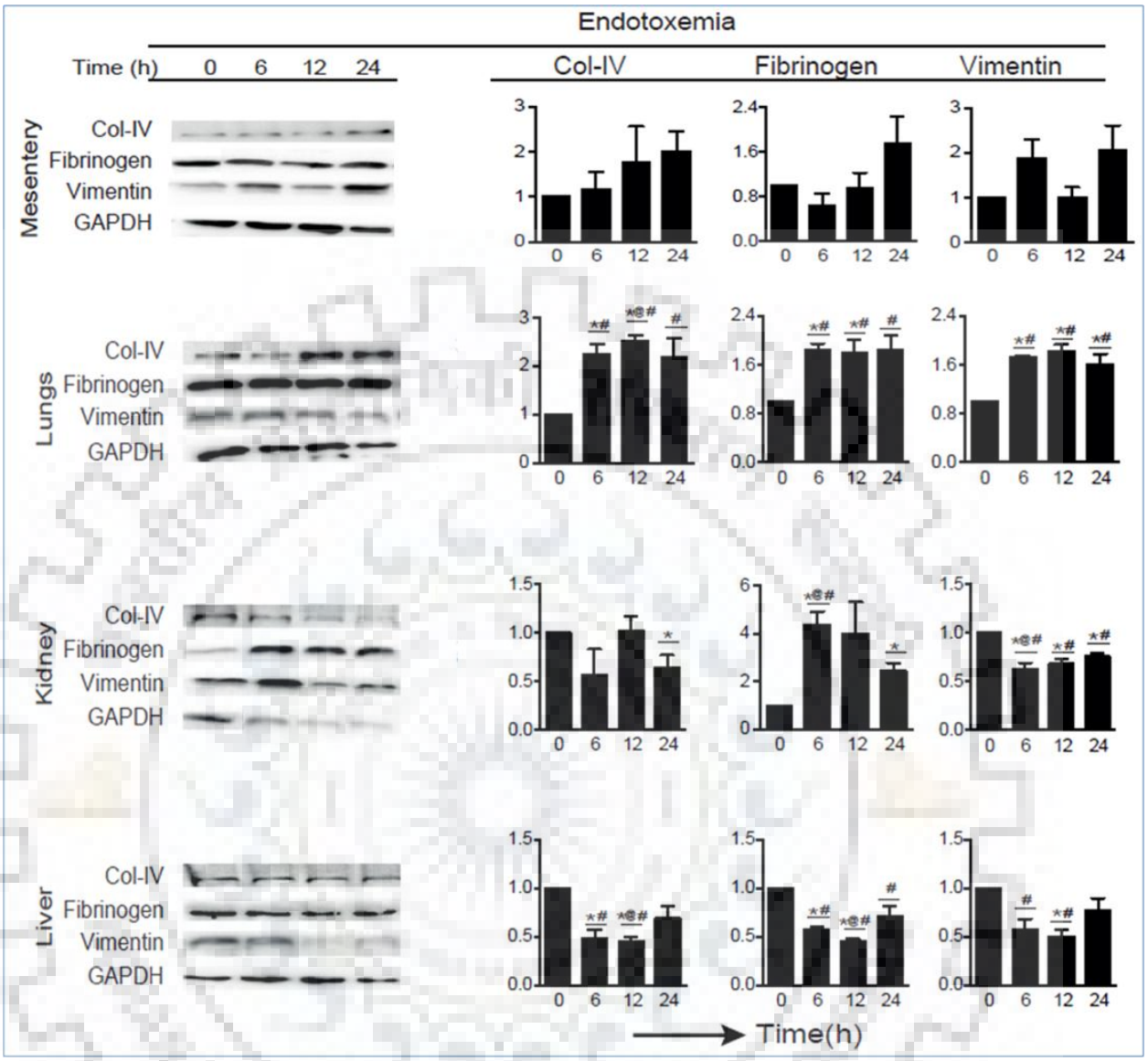



**Figure. 7. Expression of ECM proteins at the local inflammation site and distant visceral organs in LPS induced endotoxemia.** Relative expression of ECM proteins in different tissues at different time points following LPS injection was measured using western blot as described in the method section. GAPDH was used as endogenous control for all analysis. The left panels in Fig. a, b, c and d shows the western blot pictures of respective proteins at different time points in the tissue as labelled in respective figures. The bar diagrams on right panels show the fold change in the expression of specific protein compared to that of corresponding organs of naïve animals as measured by densitometry analysis of individual bands using ImageJ software. The data are presented as mean ± SEM. ($p \leq 0.05$- *-Student t- test, #- One-way ANOVA with Dunnett's multiple comparisons test and @- Kruskal-Wallis test with Dunn's multiple comparisons test)

### 2.3.4. Bioinformatics study of possible signalling pathways associated with systemic inflammation

In order to further analyze the possible effects of the modulation in the levels of specified vimentin, Col-IV and fibrinogen, bioinformatics analysis of their respective interacting molecules and the signalling pathways was performed using STRING. The proteins and their interacting molecules were compared both in mouse and human to assess the relevance of the experimental data with respect to inflammatory diseases. The network edges shown in Fig. 8 represent the confidence of the data supported from different sources like text-mining, co-expression, experiments etc. where greater thickness of line correlate with better strength of the data support. As depicted in the Fig. 8 (left panel), in human, the vimentin (VIM) showed association with CASP3, CASP7, CASP8, AKT1 protein that were involved in TNF signalling pathway (hsa04668, KEGG pathway) which is very well pronounced in systemic inflammation (Lu et al. 2011, Lerman et al. 2014). Proteins like COL1A1, COL1A2, COL4A2, COL6A1, ITGA1 were found common in the Col-IV (COL4A1) mediated ECM-receptor (hsa04512, KEGG pathway) and integrin cell surface interactions (HSA-216083, Reactome pathway) pathways suggesting its primary association with molecular signals originating from cytoskeletal structures which guide immune cell extravasation and tissue migration.

The fibrinogen (FGA) showed interactions with FGB, FGG, F2, F13B, SERPINC1 which mediate the complement and coagulation cascades (hsa04610, KEGG pathway), where notably the communication between FGA and F2 governs the inflammation and possibly are expressed in the tissues for would healing purpose (Kuzmich et al. 2017, Gordon et al. 2004, Solovjov, Pluskota, and Plow 2005). As demonstrated in Fig. 8 (left panel) and Table-2, human proteins also showed some unique interactions of CDC5L, AKT1 and STAT3 with vimentin, ITGA1 and COL16A1 with Col-IV, and ALB, HRG with fibrinogen that were absent in mice model.

Similarly, when compared to the murine model, multiple similarities were found in the interacting partners of vimentin, Col-IV and fibrinogen. For example Casp3, Casp7 and Casp8 associated with TNF signalling pathway (mmu04668, KEGG pathway), Col1a1, Col1a2, Col4a2, Col6a1, Itgb1 associated with ECM-receptor (mmu04512, KEGG pathway) and Fgb, Fgg, F2, F13b, Serpinc1, Plg associated with complement and coagulation cascades (mmu04610, KEGG pathway) were found to be some common pathways in both human and mouse. As shown in human protein, murine proteins also showed multiple unique binding partners. In Fig. 8 (right

panel) and Table-2, Tmp4, Tnnt3 and Neb specifically interacted with mouse vimentin, Itgb1 and Plod2 with mouse Col-IV and Plg, Apoa2 with mouse fibrinogen proteins.

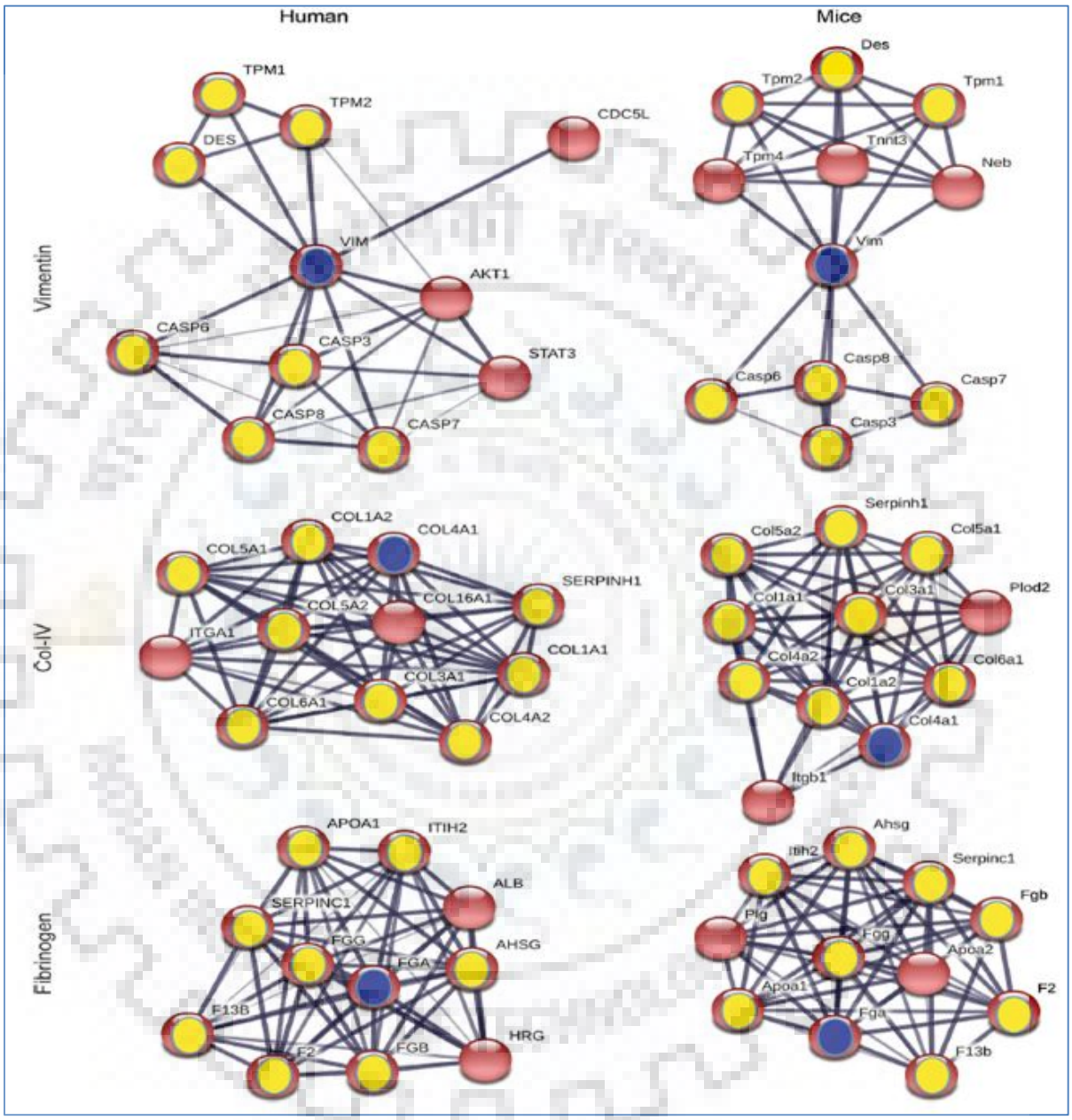



**Figure 8. Protein-Protein Interaction (PPI) network of investigated ECM proteins.** Vimentin, Col-IV, and fibrinogen interacting PPI network for human (left panel) and mice (right panel) acquired from STRING v11.0. The network edges show the confidence where darker line thickness reflects better strength of the data support.

**Table 2: Predicted downstream protein network upon cell-matrix interaction using STRING tool**

| Sl. No. | ECM Proteins | Associated Signaling Pathway | Background Gene Count | Interacting Gene number and names |
|---|---|---|---|---|
| **HUMAN** | | | | |
| 1 | Collage-IV | ECM-Receptor interaction | 81 | 6 genes - COL1A1,COL1A2,COL4A1,COL4A2,COL6A1,ITGB1 |
| 2 | Collage-IV | PI3K-Signaling Pathway | 349 | 6 genes- COL1A1,COL1A2,COL4A1,COL4A2,COL6A1,ITGB1 |
| 3 | Fibrinogen | Platelet Activation and Aggregation | 256 | 8 genes- AHSG,ALB,APOA1,F2,FGA,FGB,FGG,HRG |
| 4 | Fibrinogen | Surface Integrin Interaction | 283 | 3 genes- FGA,FGB,FGG |
| 5 | Vimentin | Cancer Signaling | 515 | 5 genes- AKT1,CASP3,CASP7,STAT3 |
| 6 | Vimentin | Apoptosis | 135 | 5 genes- AKT1,CASP3,CASP6,CASP7CASP8 |
| 7 | Vimentin | JAK-STAT Signaling | 160 | 2 genes- AKT1,AKT3 |
| 8 | Vimentin | Chemokine Signaling | 181 | 2 genes- AKT1,STAT3 |
| **MOUSE** | | | | |
| 1 | Collage-IV | ECM-Receptor interaction | 81 | 6 genes- Col1a1,Col1a2,Col4a1,Col4a2,Col6a1,Itgb1 |
| 2 | Collage-IV | PI3K-Signaling Pathway | 349 | 6- Col1a1,Col1a2,Col4a1,Col4a2,Col6a1,Itgb1 |
| 3 | Fibrinogen | Platelet Activation and Aggregation | 242 | 7 genes- Ahsg, Apoa1, F2, Fga, Fgb, Fgg, Plg |
| 4 | Fibrinogen | Surface Integrin Interaction | 68 | 3 genes- Fga, Fgb, Fgg |
| 5 | Fibrinogen | Immune system | 879 | 5 genes- Ahsg, F2, Fga, Fgb, Fgg |
| 6 | Vimentin | Apoptosis | 135 | 4 genes- Casp3, Casp6, Casp7, Casp8 |
| 7 | Vimentin | TNF-Signaling | 108 | 3 genes- Casp3, Casp7, Casp8 |

**Background Gene Count:** Total number of genes involved in associated signaling pathway.
**Interacting Gene number and names:** Total number of genes involved in correlation with respective ECM proteins.

## 2.4. Discussion

Sepsis induced multiple organ failure is a prominent pathophysiological condition where excessive and altered migration of inflammatory leukocytes to the tissue is detrimental (Kopec et al. 2017). In order to migrate through the tissue, leukocytes interact with the ECM via their integrin receptors expressed on cell surfaces (Mariscal and Joanne 2011). The current study shows a parallel comparison between the expression pattern of different ECM proteins such as Col-IV, fibrinogen and vimentin at different time points in different tissues using two different models of systemic inflammation having separate mechanisms and district pattern for onset of inflammatory events (Sarangi et al. 2012, Rittirsch, Flierl, and Ward 2008, Lerman et al. 2014). Our data shows more prominent modulations at the site of inflammation and the lung tissues compared to liver and kidney with different thresholds in both the models.

Previous studies have shown that inflammation induced ECM remodeling could modulate the severity of inflammatory diseases by altering immune cell migration and functions. (Overstreet et al. 2013, Bekaert et al. 2017). Studies also indicate that tissue specific expression of ECM proteins could modulate the abnormal influx of inflammatory cells into the local site as well as distant visceral tissues. Data presented in the current study shows that both CLP and endotoxemia models demonstrated a unique time and tissue specific changes in the expression of ECM proteins. Interestingly, in endotoxemia although the modulation in ECM protein expression was less intense at the inflammation site, has more intense changes in the lungs when compared to the same proteins in CLP suggesting an inflammatory stimulation dependent modulation of inflammatory cells. This contrasting observation between CLP and endotoxemia may be the result of rapid dissemination of LPS throughout the body causing a more complex immune response compared to CLP, where the diffusion of polymicrobial infection is comparatively slow. Previous studies have also demonstrated that, instead of having comparable mortality levels, it could be possible that the inflammatory components such as cellular infiltrates, cytokines, chemokine, proteases, reactive oxygen and nitrogen species could be differentially expressed at various time points in CLP and endotoxemic animals, which could contribute to the different expression pattern of ECM proteins in both the models (Sarangi et al. 2012, Lerman et

al. 2014). Alternately, These changes in matrix proteins may also influence the differences seen in the inflammatory cell influx and secreted mediators from such cells in the peritoneum and other visceral tissues in both the models (Sarangi et al. 2012).

Fibrinogen, a 340-kDa multimeric glycoprotein has an important role in vascular homeostasis. Although fibrinogen is normally present at a basal level in blood, during inflammation, its concentration increases and serves as an important biomarker for inflammation (Davalos and Akassoglou 2012). Previous study in acute phase response showed the role of fibrinogen as an adhesive protein for the intra-alveolar aggregation and adherence of *P. carinii* and subsequent immune response causing severe tissue damage (Simpson-Haidaris et al. 1998). Fibrinogen is also responsible for chemotaxis of pro-inflammatory neutrophils and macrophage via binding with integrin $\alpha_M\beta_2$ in inflammatory conditions (Solovjov, Pluskota, and Plow 2005). It also participates in repair mechanism where it binds to macrophage causing secretion of MMP-12 that mediates wound healing (Kopec et al. 2017). Our study showed upregulation of fibrinogen at both mRNA and protein level at the site of inflammation as well as visceral organs. As a matter of fact, fibrinogen was one of the highly expressed proteins as compared to others when assessed from the density of the bands with equal protein loading. Further bioinformatics analysis on interaction of fibrinogen with other molecules revealed the possibility of FGA-F2 interaction which is reported to be involved in the healing process. Possibly, the upregulation in fibrinogen promotes chemotaxis of inflammatory immune cells, thus exaggerated immune response, tissue damage and multi organ failure. On the other hand, the possibility of role of fibrinogen in the healing mechanism cannot be ruled out.

Vimentin is a cytoskeletal protein that helps in maintaining the cell structure and integrity. It is also associated with regulation of cell migration and invasion (Nieminen et al. 2006). Additionally, it plays a crucial role in inflammation, where it is responsible for NLRP-3 activation and transcellular migration of immune cells to damage site (Dos Santos et al. 2015). The direct interaction of vimentin with integrin $\beta_1$ is also known to facilitate the binding of $\alpha_5\beta_1$ to fibronectin (Kim et al. 2016). Our data shows upregulated expression of vimentin in lungs and mesentery during sepsis. Bioinformatics analysis showed the interaction of vimentin with different signalling molecules such as, CASP3, CASP7, CASP8 and AKT1 which are involved in the TNF signalling pathway responsible for regulation of cell survival. In accordance to

previous reports, our data suggests the possibility of role of vimentin in increasing the severity of the disease by mediating the infiltration of inflammatory immune cells to organs.

Col-IV is a vital component of the basement membrane responsible for its integrity and stability. During inflammation, destruction of basement membrane by MMPs secreted by infiltrated immune cells is responsible for generation of matrikines that have chemotactic effect for variety of immune cells (Pipoly and Crouch 1987, Gaggar and Weathington 2016). Moreover, it is also involved in transmigration of neutrophils through basement membrane by binding with L-selectin expressed on immune cells (Iwabuchi et al. 1996). Further bioinformatics analysis by STRING revealed the possible interaction of Col-IV and integrin suggesting its possible association with transmigration and interstitial migration inside tissue. We observed high Col-IV expression mainly in lungs and mesentery of septic animals. This upregulation might be involved in the infiltration of inflammatory neutrophils, which causes further damage to the affected organs.

Previously, the changes in Col-IV, fibrinogen, vimentin, laminin etc. were reported in brain injury, lung injury, arthritis, cancer etc. (Bonnans, Chou, and Werb 2014, Cox and Erler 2011). These investigations on altered ECM distribution and expression were only limited to local inflammatory condition. The current report using two well-characterized mouse models shows the dynamic changes of ECM expression during systemic inflammation. The dynamic changes might be responsible for infiltration/chemotaxis and downstream function of immune cells that can play crucial role in driving the disease severity. The inflammatory events in human might differ from mice up to some extent. However, our bioinformatics analysis showed interactions of the reported proteins with molecules involved in signaling pathways associated with systemic inflammation, which were common to human and mice. Moreover, extensive study on the functional relevance of the bioinformatics data is needed for validation. Further research and development based on the current findings will help more to understand the complex pathophysiology of sepsis and may lead to development of a new therapeutic approach.

## 2.5. Conclusion:

In summary, the first part of the present study examines the extracellular matrix protein expressions dynamics in two well established murine model of systemic inflammation i.e Lipopolysaccharide-induced endotoxemia and Cecal Ligation and Puncture (CLP) induced

polymicrobial sepsis. Our RT-PCR and western blot study on harvested tissue at different time point from sepsis-induced mice, revealed a distinctive expression of collagen 4, fibrinogen and vimentin at the site of inflammation(mesentery) as well as in the visceral organs (Lung, liver & kidney) in both models, potentially owing to difference in inflammation induction pattern. Additionally, effect of upregulated ECM protein on immune cell function and inflammatory signaling pathways such as activation, apoptosis, cytoskeleton modulation, integrin expression, and migration was analyzed using bioinformatics STRING software. Results obtained from our study show the tissues and time-specific expression of important ECM proteins in systemic inflammation. Besides this, it also anticipated significant signaling pathway both in murine and humans that get activated upon cell-matrix interaction during the inflammatory scenario. However, it is imperative to perform a comprehensive study on the functional relevance of bioinformatics data is needed for validation.

# CHAPTER: 3

## A C-terminal fragment of adhesion protein Fibulin7 regulates neutrophil functions *in vitro* and *in vivo*

### 3.1. Introduction:

Sepsis is a serious, life-threatening, immune system malfunction resulting from systemic spread of infection (Hotchkiss et al. 2016). According to the latest world health organization report, it affects more than 30 million individuals worldwide, resulting in 6 million deaths per year (Fleischmann et al. 2016). Despite numerous efforts in improving diagnosis and therapies, currently no specific drugs are available to cure or slow down the progression of sepsis to severe sepsis or septic shock (Cohen et al. 2015). Neutrophils are versatile cells of the host innate immune system and the presence of potent cytotoxic components such as proteases, ROS and pro-inflammatory cytokines in their granules make them an essential part of the anti-microbial defense (Soehnlein et al. 2017). During the initial phase of sepsis, circulating neutrophils migrate to the site of inflammation and perform phagocytosis of the invading pathogen and contribute to the inflammatory processes (Bhan et al. 2016). However, with the progress of sepsis, prolonged exposure to excessive inflammatory substances results in an altered migration pattern and generation of hyperactive neutrophil subsets that are associated with tissue injury and multi-organ failure (Bhan et al. 2016, Lerman et al. 2014). Of note, reports from multiple groups have demonstrated that inhibition of neutrophil trafficking using reagents against adhesion receptors such as integrin could improve the survival of septic animals (Lerman et al. 2014, Sarangi et al. 2012).

Fibulins are secreted glycoproteins which play an important role in cell adhesion, protein-protein interactions and could regulate different cellular functions (De Vega, Iwamoto, and Yamada 2009). To date, seven members of fibulin family of proteins have been identified which contain similar structural components such as series of epidermal growth factor (EGF)-like modules and a fibulin-type module at the C-terminus. The latest member of the family, fibulin7 (Fbln7), which in addition to fibulin type domains, also contains a sushi domain on the N terminus. Fbln7 was first identified in the developing tooth and subsequently was shown to be expressed in several other tissues such as cartilage, bone, placenta and cornea (de Vega et al. 2007). Recently, one of its C-terminal fragments which lacks the sushi domain was shown to

diminish tube formation and vessel sprouting in Umbilical Vein Endothelial Cells (HUVEC) and inhibit angiogenesis in an *in vivo* mouse corneal model (de Vega et al. 2014, Ikeuchi et al. 2018). Fbln7-C was also demonstrated to negatively regulate differentiation and inflammatory functions such as adhesion, ROS and cytokine production in human monocytes and macrophages (Sarangi et al. 2018). In the current study, we have evaluated the effect of Fbln7-C on the neutrophil adhesion, migration and inflammatory functions using *in vitro* and *in vivo* inflammatory conditions. Our data show that Fbln7-C could modulate neutrophil functions and its administration could improve the survival in a LPS induced murine endotoxemia. The *in silico* studies suggests that only the confirmation of Fbln7-C and not the full length or wild-type (WT) facilitates interactions with integrin β1 and possible could interfere with binding to other ligands such as fibronectin.

## 3.2. Material and Methods

### 3.2.1. Isolation of human peripheral blood neutrophils

Blood was collected from healthy donors according to the Institute Human Ethics Committee, Indian Institute of Technology Roorkee approved protocol, in heparin containing vacutainers. Granulocytes and erythrocytes were separated from whole blood by centrifugation through 1-step Polymorph Prep (Axis Shield, Dundee, UK) density gradient. Remaining erythrocytes were removed by hypotonic lysis, yielding a neutrophil purity of > 95%, which was confirmed via flow cytometry in a FACSVerse flow cytometer (BD Biosciences, San Diego, CA).

### 3.2.2. *In vivo* model of murine endotoxemia and Fbln7-C protein administration

LPS-induced endotoxemia was performed in 8-12 week old C57BL/6 male mice procured from the Indian Institute of Microbial Technology, Chandigarh, India, according to the protocol approved by Institute Animal Ethics Committee, Indian Institute of Technology, Roorkee. Endotoxemia assay was performed as described before (Sarangi et al. 2018). Briefly, Lipopolysaccharide (LPS) (*E. coli* 026:B6, Sigma Aldrich, St. Louis, MO) was injected in 8-12 week mice intraperitoneally (36.8 mg/Kg body weight as per titration of the received lot of LPS). Recombinant Fbln7-C (amino acid residues 135-440, 33 kDa, 10 μg/dose) was administered in 2 doses, 2hr and 10hr post LPS injection intraperitoneally and intravenously (IV) in retro-orbital venous plexus respectively. Animals were sacrificed at 12 hr and the blood, bone marrow, and peritoneal lavage cells were collected and processed for flow cytometric analysis. Lungs were

digested using collagenase IV (Sigma-Aldrich, St. Louis, MO) to prepare a single cell suspension for flow cytometric analysis

### 3.2.3. Flow cytometry

Cells isolated from blood, peritoneum and lungs at 12 hr time point and surface staining was performed using purified anti-mouse Fc-receptor (CD16/CD32), F4/80-Alexa488, Ly6G-PE, MHCII-PerCP, CD11b-APC, CCR-2-FITC, and CXCR-4-APC antibodies (Biolegend, San Diego, CA). BD Horizon™ Fixable Viability Stain 450 was used to gate on live cells (FVS450; BD Biosciences, San Jose, CA). All samples were fixed with 1% paraformaldehyde and collected on a FACSVerse flow cytometer (BD Biosciences, SanDiego, CA). The data was analyzed using FlowJo software.

### 3.2.4. Cell adhesion assay

Cell adhesion assay was performed in 96-well, flat-bottom plates (Genetix, New Delhi, India). The wells were coated overnight at 4°C with 50 µl of fibronectin and various amounts of Fbln7-C and then blocked with 2% bovine serum albumin (BSA) for 1 h at 37°C. The human neutrophils (1 x $10^5$ cells in 100 µL per well) were plated and incubated for 2h at 37°C. After washing, the attached cells were measured using the CCK8 cell counting kit (Dojindo, Rockville, MD). For the cell adhesion inhibition experiments, cells were incubated with blocking antibodies (10 µg/ml) against integrin $\alpha_5\beta_1$, $\alpha_2\beta_1$, and $\alpha_v\beta_3$ (Millipore, Billerica, MA) for 30 min before plating.

### 3.3.5. ROS detection Assay

A cell permeable, oxidation sensitive dye, 2′,7′-Dichlorodihydrofluorescein diacetate (DCFDA) (Sigma-Aldrich, St. Louis, MO) was used to detect the intracellular ROS production. For *in vitro* studies, neutrophils were isolated as described above and were incubated for 30 min in presence/absence of Fbln7-C (10µg/ml). Next, the cells were stimulated with 100ng/mL of LPS, 10ng/mL of IL-1β or 1µM of fMLP for 15 mins. The cells were washed with PBS and were further incubated with 10uM DCFDA for 15 min at 37°C. Following DCFDA treatment, the cells were washed with FACS buffer and were acquired using FACSVerse flow cytometer. The data was analyzed using BD FACSuite™ software (BD Biosciences, San Diego, CA). For *ex vivo* ROS assays, cells were isolated from bone marrow, blood, peritoneal lavage, and lungs of animals treated with Fbln7-C or PBS at 12 hr following LPS injection and were immediately processed for ROS assay as described above.

### 3.2.6. Quantitative RT-PCR:

The total RNA was isolated from the neutrophils at different time points using TRIzol Reagent (Life Technologies). The RNA was reverse transcribed using iScriptTM reverse transcription supermix for RT-qPCR (Bio-Rad, Hercules, CA), and. Real-time- PCR was performed using Power SYBR Green PCR master mix and Power Up SYBR Green master mix (Applied Biosystems, Foster City, CA). Samples were normalized to the endogenous control GAPDH and relative gene expression was measured by 2-ΔΔCTmethod. The primers used were human GAPDH forward (TCCTCTGACTTCAACAGCGACAC) and reverse (TCTCTCTTCCTCTTGTGCTCTTGC), human-IL-6 forward(GAAAGCAGCAAAGAGGCACT) and reverse (TTTCACCAGGCAAGTCTCCT), human IL-1β forward (AGGGACAGGATATGGAGCAA) and reverse (ACGCAGGACAGGTACAGATT)

### 3.2.7. Protein isolation and Western blotting:

For the detection of total and phosphorylated Erk1/2 (extracellular-signal-regulated kinase), total proteins were isolated from neutrophils (1 x $10^6$/ml of RPMI 1640-10% FBS) stimulated with LPS in the presence of absence of Fbln7-C using CelLytic Reagent (Sigma Aldrich, St. Louis, MO). Protein samples were stored at -80 $^0$C with protease inhibitor Cocktail until further use. Protein of the equal amount were loaded for SDS-PAGE. The separated proteins were transferred onto BioTrace NT nitrocellulose membrane and blocked with 5% dried, fat-free milk (HiMedia, West Chester, PA) in PBS-0.05% Tween-20 (PBST) for 1 hr at room temperature. Next the membranes were incubated with primary antibody (Cell Signaling Technology, Danvers, MA) in blocking buffer overnight at 4°C. Membranes were then washed 4 times with PBST and incubated with HRP-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA) at 1:1000 in 5% blocking buffer for 1 h at room temperature. Membranes were washed 4 times with PBST and were developed by immune-reactive complex were visualized in Chemidoc (Bio-Rad, Hercules, CA). For all the samples ®-actin was used as endogenous control, (Cell Signaling Technology, Danvers, MA).

### 3.2.8. Immunofluorescence staining of actin

Glass chamber slides (Millipore, Darmstadt, Germany) were coated with recombinant proteins, fibronectin (5 μg/ml) or Fbln7-C (20 μg/ml), overnight at 4°C. The wells were washed and blocked with 2% BSA. After washing, neutrophils (1 x $10^4$ in 200 μl per well) were plated in the

presence of LPS (100 ng/mL) and allowed to attach for 30 mins. Once the cells were attached, they were washed with pre-warmed PBS, fixed with pre-warmed 4% PFA for 10 min, and permeabilized with 0.1 % Triton-X-100 for 5 min. Actin staining was performed using phalloidin-rhodamine (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions followed by DAPI (Life Technologies, Carlsbad, CA) staining and mounting. Images were acquired and analyzed using a fluorescence microscope (Scope.A1; Zeiss, Germany) at 63X magnification.

#### 3.3.9. Statistical Analysis

All values are presented as mean Standard error mean (SEM). Data analysis was carried out using GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA) using non-parametric Mann-Whitney

## 3.3. Results:

### 3.3.1. Fbln7C binds to integrin β1 in dose dependent manner and inhibits neutrophil spreading

To study the modulatory effects of Fbln7-C on human neutrophils, peripheral blood neutrophils were isolated from healthy donors using polymorph prep density gradient. Purity was confirmed with flow cytometry (Fig. 1A) and binding assay was performed using plates coated with different concentrations of Fbln7-C. As shown in Fig. 1B, a dose dependent binding of neutrophils to Fbln7-C was observed with significantly higher binding of neutrophils at 5, 10 and 20 ug/mL Fbln7-C concentration compared to uncoated and fibronectin (5 ug/ml) coated wells. And as shown in Fig. 1C, presence of blocking antibody against integrin β1, reduced the binding of human neutrophils to Fbln7-C compared to isotype control treated wells confirming integrin β1 as one of the receptors for Fbln7-C on neutrophil surface as demonstrated previously on HUVEC cells and human monocytes. Further, to study the effect of Fbln7-C on neutrophil spreading, fluorescence staining with actin-phalloidin was performed in LPS treated and untreated neutrophils adhered on Fbln7-C (10 ug/mL) coated plates. Fibronectin (5 ug/mL) was used as positive control for the experiments. As shown in Fig. 1D, (upper panel), binding of neutrophils to fibronectin coated plates, promoted adhesion and spreading which was further enhanced with LPS (50 ng/mL) activation. However, spreading and actin fiber formation was significantly reduced in Fbln7C coated plates both in unstimulated and LPS stimulated

neutrophils (Fig. 1D, Lower Panel). Furthermore, reduction in spreading on Fbln7-C was confirmed from the reduced cell area (Fig. 1E) and perimeter (Fig. 1F) measurements of neutrophils adhered on Fbln7-C coated plates compared to fibronectin.

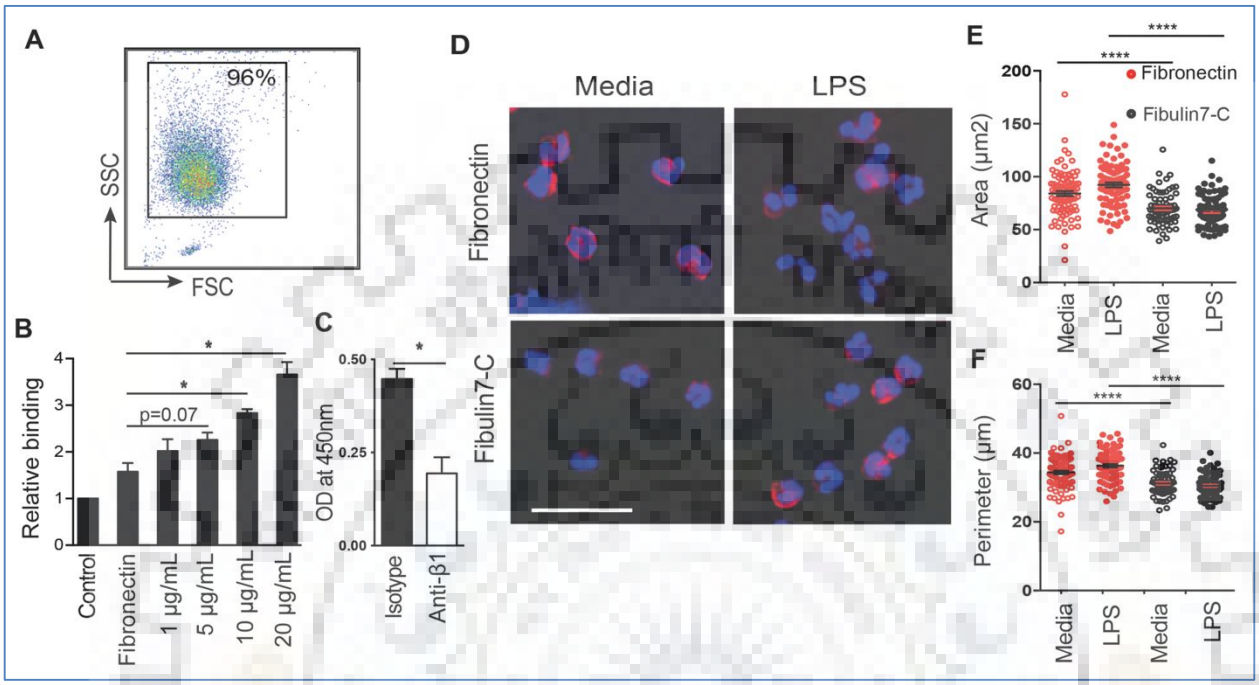



**Figure. 1. Fbln7C binds to Human neutrophils via integrins and inhibits their spreading. (a).** Representative dot plot showing purity of neutrophils isolated from healthy donors. (**b**). The bar graph shows the relative binding of human neutrophils on different doses of Fbln7-C and fibronectin (5 µg/mL) compared to uncoated control. **(c)** The bar graph shows the binding of human neutrophils to Fbln7-C (10 µg/mL) in presence of isotype or anti-β1 blocking antibody. The bar graphs in (a) and (b), represent the data from three independent experiments performed in duplicate **(d)**. LPS (100 ng/mL) stimulated neutrophils were allowed to adhere on either fibronectin (5 µg /mL) or Fbln7-C (10 ug/mL) coated plates for 30 min and were processed for actin phalloidin staining. Images were taken at 63X objective. Scale bar, 50 µm. **(e) and (f)**. In a separate experiment, quantification of the cell area and perimeter of the neutrophils adhered on the fibronectin (5 µg /mL) or Fbln7-C (10 ug/mL) coated plates was performed. Bar graphs represents the results from 70-90 cells/group counted from three separate fields of immunofluorescence images taken at 20X objective. The results are expressed as the mean ± SEM. * $p<0.05$

### 3.3.2. Fbln7-C compete with fibronectin and inhibits spreading and migration of neutrophils

Next, we proceeded to study whether Fbln7-C could compete with other ECM proteins for binding to neutrophils and modulate neutrophil functions *in vitro.* For this study, human neutrophils were allowed to adhere to fibronectin-coated plates following pretreatment with

Fbln7-C (10 ug/mL), blocking antibody against integrin ®1(10ug/mL) or isotype control. Adhered cells were fixed and actin-phalloidin staining was performed to compare the spreading of neutrophils under above conditions. As shown in Fig. 2A, neutrophils adhered and demonstrated spreading on fibronectin in both control and LPS stimulated wells with relatively larger cell areas and perimeters (Fig. 2A left panel, 2B and 2C) in LPS stimulated wells compared to unstimulated controls. In contrast, the presence of Fbln7-C or blocking antibody against integrin ®1, significantly reduced the cell spreading as evident from smaller cells with nearly spherical morphologies (Fig. 2A right panel), with smaller cell area (Fig. 2B) and perimeter (Fig. 2C) measurements compared to untreated and isotype control treated neutrophils both in LPS stimulated and unstimulated conditions

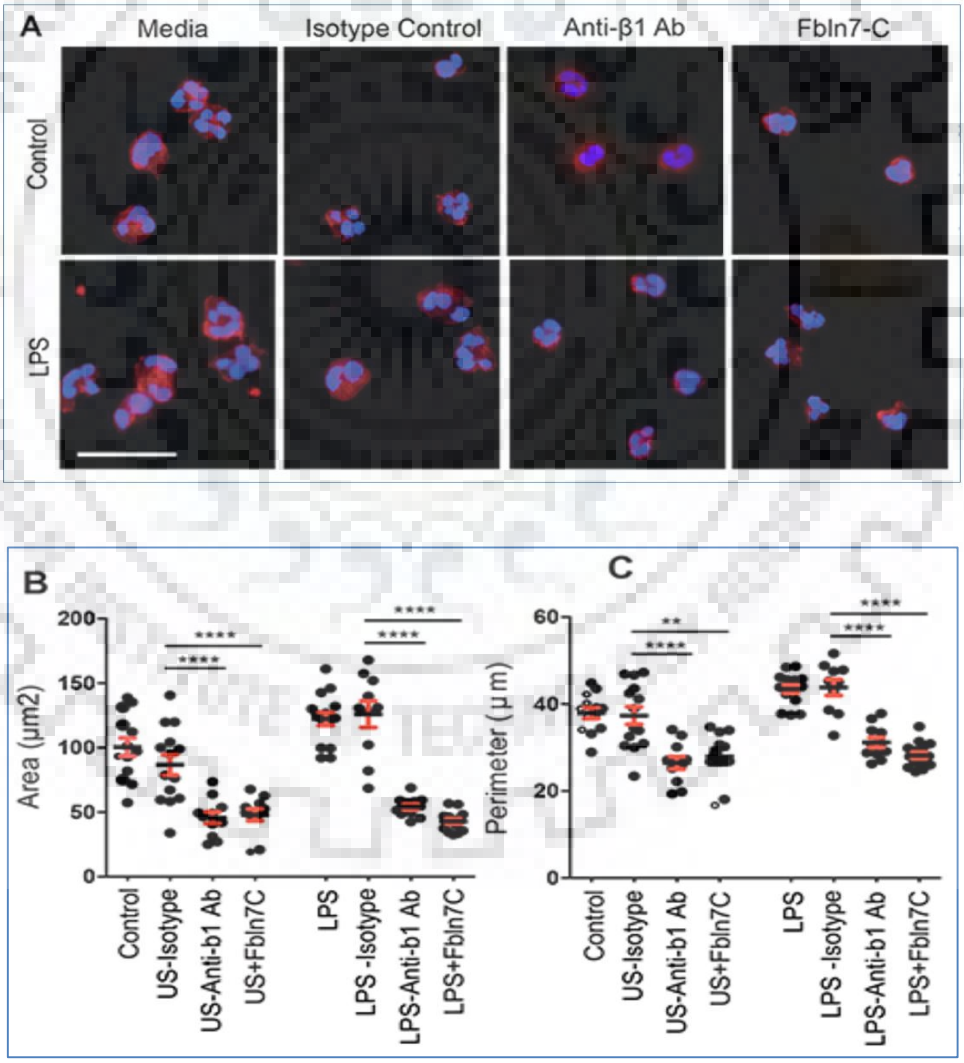


**Figure 2. Fbln7-C competes with fibronectin to bind with human peripheral blood neutrophils. (a).** Images showing actin phalloidin immunofluorescence staining in LPS stimulated or unstimulated neutrophils adhered on fibronectin (5 µg/mL) coated plates in the presence of absence of Fbln7-C (10 µg/mL). **(b) and (c)** Quantification of the cell area and perimeter of the attached cells (10-15 cells) counted from the representative snapshots (63x) of immunofluorescence images of neutrophils adhered on fibronectin and Fbln7-C. Scale bar, 50 µm.

### 3.3.3. Fbln7-C reduces the inflammatory mediators and Erk1/2 phosphorylation in human peripheral blood neutrophils

After confirming an inhibitory effect of Fbln7-C on human neutrophil spreading and migration, we further analyzed the effects of Fbln7-C protein on the immunological functions of neutrophils such as inflammatory cytokine production, both at transcript and protein level. For measuring the levels of pro-inflammatory cytokines at the mRNA level, freshly isolated neutrophils were stimulated with either LPS or fMLP for 1- and 2 hr and the expression kinetics of IL-6 and IL-1β mRNA were measured using real time PCR. As shown in Fig 3. 1A and B, stimulation of neutrophils with either LPS or fMLP increased the expression of both IL-6 and IL-1β mRNA. However, presence of Fbln7-C resulted reduced expression levels compared to the control wells

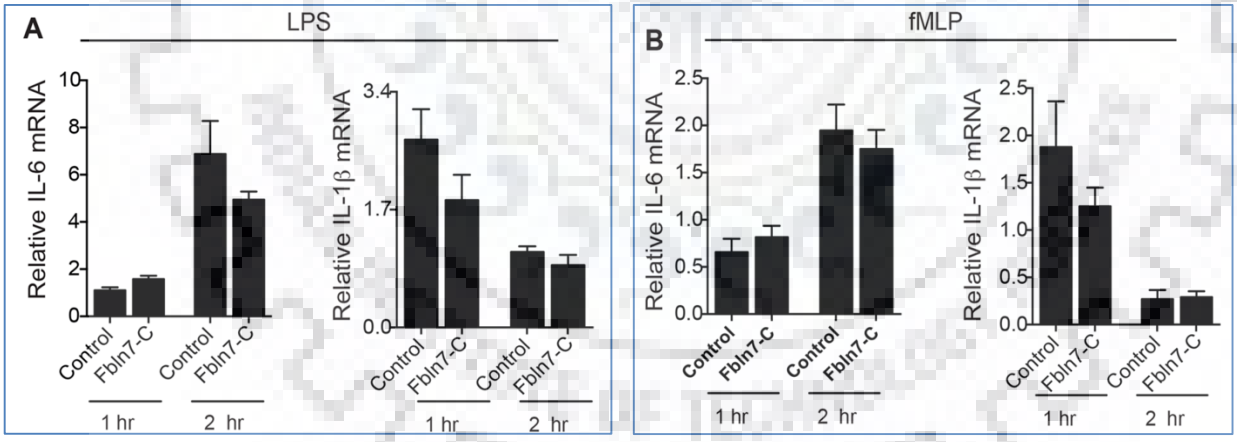



**Figure. 3. Effects of Fbln7-C on the expression of pro-inflammatory cytokine mRNA in activated neutrophils. (a) and (b)**. Time course of IL-6 and IL-1β mRNA expression in neutrophils stimulated with either LPS or fMLP in the presence or absence of Fbln7-C was measured at different time points. The bar graphs show the fold changes compared with the unstimulated neutrophils before stimulation.

Similar results were also obtained with the measurement of IL-6 and IL-10 cytokines in the supernatants collected from neutrophils cultured for 12 hr in presence or absence of Fbln7-C.

As shown in Fig. 4A and B, at 12 hr following stimulation with LPS or fMLP, higher levels of cytokines were observed in the supernatant compared to unstimulated conditions and presence of Fbln7C reduced the release of IL-6 and IL-10 with more prominent effect on IL-6 production by neutrophils. In addition to pro-inflammatory cytokines, neutrophils also generate large amounts of ROS to perform anti-microbial and inflammatory function. Therefore, ROS measurement was performed in LPS and fMLP stimulated neutrophils. As shown in Fig. 4C, in line with the cytokine expression data, there was a significant reduction in ROS production in Fbln7-C treated neutrophils compared to controls in all the stimulation conditions indicating an inhibitory effect of Fbln7-C protein on the inflammatory functions of human neutrophils.

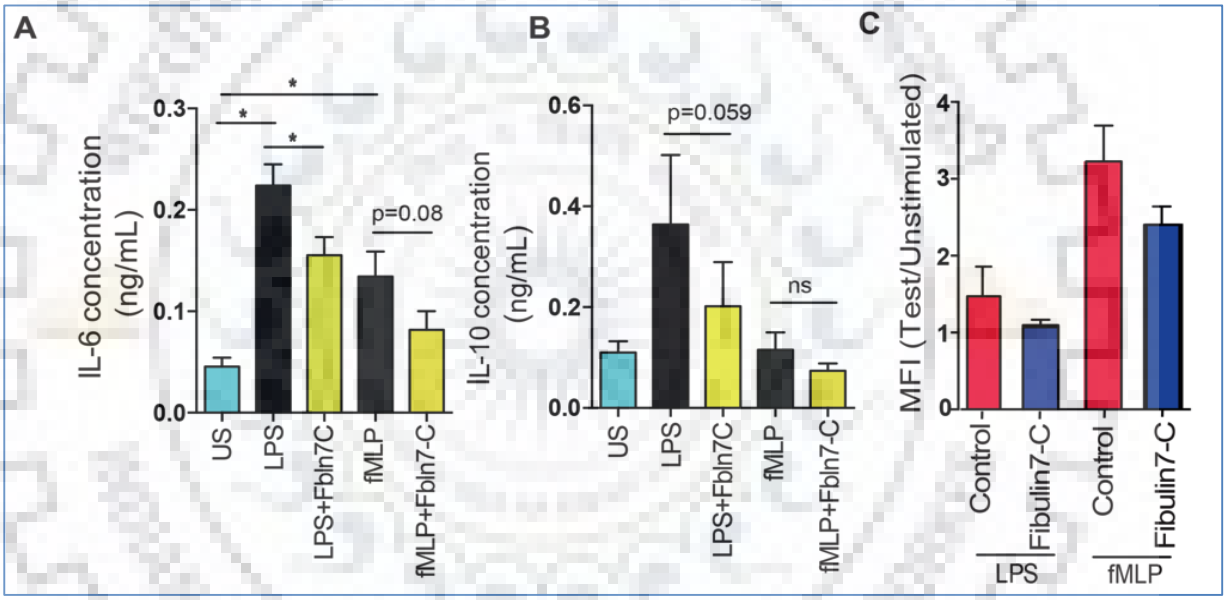



**Figure. 4. Effects of Fbln7-C on the expression of pro-inflammatory mediators in activated neutrophils. (a) and (b).** Expression of the IL-6 and IL-10 protein in the supernatant produced by neutrophils stimulated with either LPS (100 ng/mL) or fMLP (1 uM) for 12 hr in the presence or absence of Fbln7C. The proteins were quantitated using the sandwich ELISA. (**c)** Neutrophils isolated from healthy donors were stimulated with LPS for 15 min in the presence or absence of Fbln7-Cfollowed by a 15 min of incubation with DCF-DA. ROS production by neutrophils under different conditions was compared by measuring oxidized DCF-DA by a flow cytometry. The bar graphs represent the average MFI of DCF-DA$^{hi}$ neutrophils.

Next, we analyzed the possible signaling pathways involved in Fbln7-C mediated immunomodulation of neutrophil functions. Previous studies have shown that pro-inflammatory mediators such as LPS could activate the Erk1/2 and MAPK signaling pathway and aggravates

inflammatory responses in neutrophils and could enhance neutrophil survival by delaying their apoptosis (El Kebir and Filep 2013). Therefore, we studied the effect of Fbln7C on the kinetics of Erk1/2 in LPS stimulated neutrophils. As shown in Fig. 5A, phosphorylation of Erk1/2 was reduced at the indicated time points suggesting that Fbln7-C could modulate inflammatory properties of neutrophils by inhibiting signaling through Erk1/2 signaling pathway.

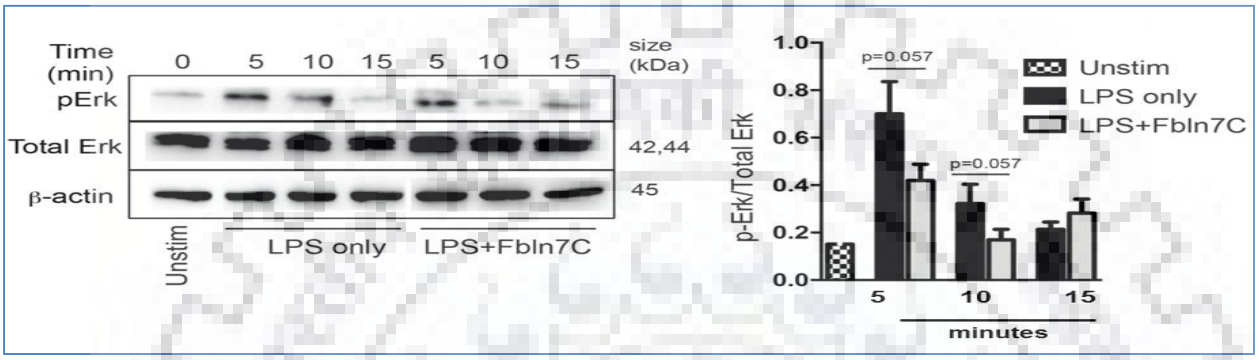



**Figure. 5. Effects of Fbln7-C on the expression of Erk1/2 signaling molecules in activated monocytes (d)**. Expression kinetics of total and phosphorylated Erk1/2 was measured using western blot technique. β-actin was used as endogenous control for the experiments. Densitometric analysis of each band was performed using ImageJ software. The bar graph on the right shows the average band density of three separate experiments. The IL-6 mRNA, protein and DCF-DA expression data represents the average of five independent experiments performed in duplicates. The results are expressed as the mean ± SEM. * $p<0.05$.

### 3.3.4. Fbln7-C inhibits the migration of neutrophils into inflamed tissues in endotoxemic animals

Functions of neutrophils could be considered as double-edged sword. Multiple reports have shown that neutrophils play vital role in the pathophysiology of LPS induced endotoxemia and that inhibiting neutrophil recruitment and functions could improve LPS induced lethality in animals (Bhan et al. 2016). Therefore, next we evaluated the efficacy of Fbln7-C in modulating systemic inflammation mediated lethality and neutrophil infiltration using in an *in vivo* model of murine endotoxemia. To this end, Fbln7-C or PBS was administered intraperitoneally at 2 hr and intravenously via retro-orbital venous plexus at 10 hr and 34 hr post LPS injection and survival was monitored for 120 hrs. Our data showed that, administration of Fbln7-C significantly improved the survival of endotoxemic animals compared to PBS controls. In a separate experiment, peritoneal lavage was isolated at 12 hr following LPS injection and detailed analysis of neutrophil infiltration and distribution was performed. As shown in Fig. 6A and B, both

percentage and total number of Ly6G$^{+}$ neutrophils were reduced in the peritoneum of Fbln7-C treated mice as compared to control animals. Interestingly, as shown in Fig. 6C, infiltrated neutrophils in the peritoneum of Fbln7C treated animals expressed lower levels of CD11b suggesting lesser activation levels of neutrophils in such animals. Additionally, there was a moderate increase in the percentage of viable cells (Fig. 6D). Furthermore, experiments were performed to investigate the inflammatory status of infiltrating neutrophils and ROS activity of cells isolated from peritoneal lavage was measured using DCFDA reagent following stimulation with PMA. Our data demonstrated that, ROS activity of peritoneal neutrophils was diminished in Fbln7-C treated mice compared to PBS treated group suggesting a generalized inhibitory activity of Fbln7C on neutrophil activity.

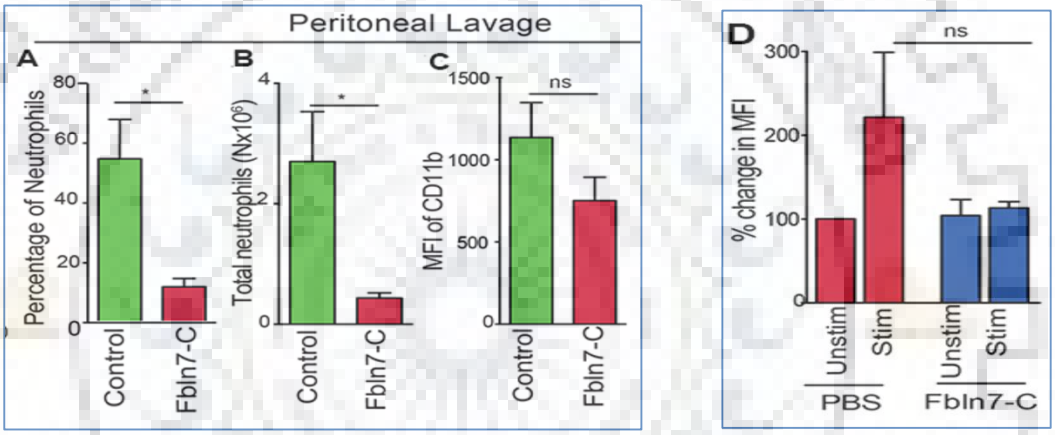



**Figure. 6. Fbln7-C Fbln7-C reduces neutrophil infiltration and inflammatory function in endotoxemic animals.** Endotoxemia assay was performed, Cells were isolated from the peritoneal lavage of endotoxemic mice (12h after LPS injection) administered with PBS, or Fbln7-C (10 µg/dose) in two doses at 1h and 4h (1$^{st}$ dose IP and 2$^{nd}$ dose IV). FVS450$^{-}$ cells were gated (live cells) for analysis. **(a) &(b)** The bar graph shows the frequencies and total numbers of Ly6G$^{hi}$ cells in the peritoneal lavage. **(c)** The bar graph shows the MFI of CD11b expression when gated on (CD11b$^{hi}$Ly6G$^{hi}$) cells in peritoneal lavage. N= 4/group**. (d).** Cells were isolated from peritoneal lavage of Fbln7C and PBS treated animals at 12 hr post LPS injection. ROS generation in the inflammatory cells was measured using DCF-DA assay as described in the method section. The bar graph shows the MFI of DCF-DA$^{hi}$ granulocytes. The results are expressed as the mean ± SEM.

## 3.4. Discussion

About a decade ago, Fbln7 was first identified in the developing tooth and due to its structural resemblance, it was added as a new member of the fibulin family of secreted glycoproteins (de Vega et al. 2007). Fbln7 is also expressed in other tissues such as bone, cartilage, and immune

privileged sites such as eye and placenta (de Vega et al. 2007). Since its discovery, reports from various labs have improved our understanding about the roles and effects of Fbln7 and Fbln7 derived fragments and peptides under physiological and pathological conditions (de Vega et al. 2014, Ikeuchi et al. 2018, Sarangi et al. 2018, de Vega et al. 2016, de Vega et al. 2019, Tsunezumi et al. 2018). In a recent report, we showed that Fbln7-C and up to a moderate extent Fbln7 full-length protein could regulate macrophage differentiation and functions (Sarangi et al. 2018). However, effect of Fbln7-C on other immune cell types such as neutrophils under inflammatory and pathological conditions required further investigation. In this study, we show that Fbln7-C could bind to human neutrophils via integrin β1 and modulate expression of inflammatory mediators and Erk1/2 signaling pathway in such cells. We also show that administration of Fbln7-C could inhibit cell infiltration in an endotoxemia model of murine sepsis.

The pathophysiology of sepsis is often associated with increased number of immature neutrophils in the blood (Shen et al. 2017, Bekaert et al. 2017). It is also showed that activated neutrophils could accumulate and obstruct microvasculature in visceral organs and cause collateral tissue damage leading to organ dysfunction and multi organ system failure (Shen et al. 2017). Many reports have demonstrated that reagents which could reduce the infiltration and functions of such hyperactive neutrophils could significantly alleviate organ dysfunction and improve survival in sepsis (Sônego, Alves-Filho, and Cunha 2014). In this line, the recombinant human activated protein C (rhAPC), the only FDA approved drug for treating severe sepsis have shown promising results in improving survival in sepsis by targeting neutrophil chemotaxis via binding to their β1 and β3 and mutant APC with higher affinity of neutrophil expressing $\alpha 3\beta 1^{hi}$ was able to significantly improve the survival in endotoxemic animals (Elphick et al. 2009). Secondly, rhAPC binding to its receptor was demonstrated to inhibit proinflammatory cytokine production by blocking NF B transcription factor and was able to reduce the expression of adhesion receptors on immune cells (Sarangi, Lee, and Kim 2010). Previous study by Lerman *et al* have also demonstrated that integrin depletion of $\langle \alpha 3\beta 1^{hi}$ hyperactive neutrophils by its antagonistic peptide could reduce the cytokine production and improve survival in sepsis (Lerman et al. 2014). In the current report, our *in vitro* and *in vivo* studies showed that Fbln7-C via binding to neutrophil surface integrin could inhibit neutrophil recruitment in to the site of inflammation. Fbln7-C was able to inhibit the cytokine and ROS production from fMLP and LPS

stimulated neutrophils *in vitro*. Thus, our data shows exposure of circulating neutrophils to Fbln7-C could modulate their inflammatory phenotype and inhibit their migration. It is also possible that binding of Fbln7-C to surface integrin may interfere with its cross talk to other receptors such as TLRs, which was shown to induce inflammatory cytokine production from activated neutrophils (Lerman et al. 2014).

Previous reports have shown that under physiological conditions, neutrophils express very low level of β1 integrin. However, exposure to inflammatory stimulations results in the upregulation of 1integrins on neutrophil surface and is significantly increased on the extravasated neutrophils (Lerman et al. 2014, Sarangi et al. 2012). Therefore, it is conceivable that the effect of Fbln7-C could be more pronounced on the neutrophils, which have higher expression of surface integrin receptors. In our studies, *ex vivo* stimulation of peritoneal cells isolated from Fbln7-C treated endotoxemic mice with PMA resulted in lesser levels of ROS (gated on granulocytes) compared to cells isolated from PBS injected animals. This observation of lower inflammatory state of neutrophils in Fbln7-C treated animals was also confirmed from their lower CD11b expression compared to PBS injected mice. However, such an effect was not observed with cells isolated from blood. In contrast, there was a moderate increase in the ROS production from granulocytes in the circulation (Data not shown). One possible explanation for such observation could be higher β1 expression in the cells in the peritoneum resulting in better binding to Fbln7C and better suppression of inflammatory neutrophils (Sarangi et al. 2012). Alternately, exposure to Fbln7-C in the circulation may interfere with the extravasation process of activated neutrophils resulting in the recruitment of neutrophils with lower expression of β1 integrins into peritoneum. In other words, binding of Fbln7C to surface integrins may restrict the interaction between neutrophil integrin with endothelium and basement membrane, which mediate neutrophil extravasation and interstitial migration. ROS released by hyperactive neutrophils could cause vascular leakage and septic shock in terminal patients thus is considered as a poor prognosis in sepsis (Meegan et al. 2017, Berton et al. 1992). Recent studies have shown the involvement of both β1 and β2 integrin in ROS production from neutrophils via Syk tyrosine kinase signaling pathways (Berton et al. 1992, Nguyen, Green, and Mecsas 2017). In this study, experiments we show that Fbln7C binds to integrin β1 receptor expressed on neutrophils and possibly this ligation blocked integrin dependent ROS production from neutrophils.

Fbln7-C, was initially shown to have anti-angiogenic effects *in vitro,* where Susana et al showed that Fbln7C could inhibit tube formation in HUVEC cells by inhibiting the formation of mature focal adhesions (de Vega et al. 2014). Later Ikeuchi et al demonstrated an anti-angiogenic effect of Fbln7 full-length and Fbln7C in controlling angiogenesis in a rat corneal model where Fbln7C could inhibit Erk1/2 and VEGF receptor-2 phosphorylation (Ikeuchi et al. 2018). It is well documented that inflammatory mediators such as LPS could increase the lifespan of neutrophils and increase the inflammatory functions by turning on the Erk1/2 phosphorylation in the activated neutrophils (Shen et al. 2017). Our study shows that presence of Fbln7-C inhibited the phosphorylation of Erk1/2 in LPS treated neutrophils.

## 3.5. Conclusion:

The second part of the work presented data provides evidence to show that treatment with Fbln7-C could reduce migration and functions of neutrophils both *in vivo* and *in vitro* and also could trim down the inflammatory state of neutrophils present in various compartments in addition to local site during an ongoing inflammation as evident from lower ROS production from neutrophils isolated from bone marrow, blood, peritoneum and lungs of Fbln7C treated endotoxemic mice compared to control. Therefore, Fbln7-C may be used as a potential therapy for sepsis to improve the overwhelming immune response observed. Currently we are investigating the effect of Fbln7-C on immune cells under additional pathological conditions such as cancer. A detailed computational analysis of the structural components and binding partners would improve our understanding of Fbln7C and its bioactive molecules as a potential immune-modulatory therapeutic for various diseases.

# CHAPTER: 4.

## Conclusion and Future Prospect

ECM intertwined with networks of proteins play a vital role in the activation and migration of immune cells. During inflammation, the extravasated leukocytes communicate with different components of ECM such as collagen, laminin, etc. by their cell surface receptor. This Cell-Matrix interaction can result in outside-in and inside-out signaling that can regulate activation, migration, differentiation and cellular functions of immune cells. During inflammatory responses secrete inflammatory mediators such as MMPs and proteases could degrade the ECM proteins and release the bioactive matrikines. These matrikines could further interact with immune cells and regulate their activity. There is a need for further research to understand the aberrant behavior of ECM proteins in pathophysiological conditions and their profound effects on ECM-Cell dynamics.

First part of this work has explored the dynamics of extracellular matrix protein expression in two well established systemic inflammatory murine models i.e. Lipopolysaccharide-induced endotoxemia and Cecal Ligation and Puncture (CLP) mediated polymicrobial sepsis and have showed a unique expression pattern of collagen 4, fibrinogen and vimentin in both models at the site of inflammation (mesentery) as well as in the visceral organs (Lung, liver & kidney), possibly due to difference in the mode of inflammation induction. The data from our research indicate tissues and time-specific expression of vital ECM proteins in systemic inflammation. In addition, a major signaling pathway is also anticipated in both Human and Mice that are triggered during inflammatory conditions.

In the second part of the study, using human neutrophils and LPS-induced systemic inflammation mouse model, showed that the C terminal fragment of the adhesion protein, Fbln7-C is a negative regulator of inflammation and neutrophils functions, which are important cell types of the innate immune system and also play a crucial role in driving pathological occurrences such as infection-induced multi-organ failure in systemic inflammation. Our study thus shows that treatment of Fbln7-C can modulate the inflammatory phenotype and prevent infiltration of circulating neutrophils to the site of inflammation. Further studies are needed to understand the receptor and conformational dynamics of Fbln7-C for further understanding its mechanisms of action.

## SUMMARY OF KEY FINDINGS AND FUTURE PROSPECTS:

- Our study in both CLP and endotoxemia models demonstrates a unique time and tissue-specific dynamics in the expression of ECM proteins such as Col-IV, Fibrinogen, and Vimentin both at the site of inflammation and visceral organs.
- This study will help in a better understanding of possible signaling events associated apoptosis, activation, and cytoskeleton modulation, etc. from ECM protein in the inflammatory microenvironment.
- To study the correlation between changes in the expression pattern of ECM protein and its effect on immune cell function such as activation, integrin expression, and migration, etc.
- This study will help in a better understanding of the complex pathophysiology of sepsis and may lead to the development of a new cell adhesion based therapeutic approach.
- Fibulin7-C has anti-inflammatory and immunoregulatory effects on neutrophil function both *in vitro* and *in vivo*
- The results will help in determining the implication of the various drugs targeting important molecules in the cell-matrix interactions in inflammatory conditions.

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