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# Role of Bovine Ileal Sub-epithelial Myofibroblasts and Epithelial Cells in Innate Immunity

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# ROLE OF BOVINE ILEAL SUB-EPITHELIAL MYOFIBROBLASTS AND EPITHELIAL CELLS IN INNATE IMMUNITY

BY

TIRTH UPRETY

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ROLE OF BOVINE ILEAL SUB-EPITHELIAL MYOFIBROBLASTS AND EPITHELIAL CELLS IN INNATE IMMUNITY

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This thesis is approved as a credit and independent investigation by a candidate for the Master of Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of the thesis does not imply that the conclusion reached by the candidates are necessarily the conclusion of the major department.

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# LIST OF ABBREVIATIONS

ALR: Absent in melanoma 2 (AIM2) like receptor

AP1: Activator protein 1 (AP1)

ASC: Apoptosis related spec like protein

BIEC-c4: Bovine intestinal epithelial cell

Bmp: Bone morphogenesis protein

CARD: Caspase recruitment domain

CBP: cAMP-responsive-element-binding protein (CREB)-binding protein

CD: Cluster of differentiation

cDNA: Complementary DNA

DAMPs: Danger Associated Molecular Patterns

DMEM: Dulbecco's modified eagle media

DNA: Deoxyribonucleic acid

ERK: Extracellular signal regulated kinases

FLA: Flagellin

GALT: Gut associated lymphoid tissue

GlcNAc: N-acetlyglucosamine

HBSS: Hanks Balanced Salt Solution

iE-DAP: γ-D-glutamyl-meso-diaminopimelic acid

IFN: Interferon

Ig: Immunoglobulin

IL: Interleukin

IL-1R: Interleukin-1 receptor

IRAK: IL-1R associated Kinase

IRF3: Interferon regulatory factor 3

ISEMF: Intestinal sub-epithelial myofibroblast

ITAM: Immunoreceptor tyrosine-based activation motif

JNK: Janus Kinase

LGP2: Laboratory of genetics and physiology 2

LPS: Lipopolysaccharide

MAMPs: Microbe associated molecular patterns

MAPK: Mitogen associated protein kinase

MDA5: Melanoma differentiating gene 5

MDP: Muramyl dipeptide

Mincle: Macrophage-inducible C-type lectin

miRNA: micro RNA

mRNA: messenger RNA

MurNAC; N-acetylmuramic acid

MyD88: Myeloid differentiation primary-response protein 88

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NLR: Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs)

Nlrp3: NLR pyrin domain

OLR: Oligoadenylate synthase (OAS)-like receptor

PAMPs: Pathogen Associated Molecular Patterns

PBS: Phosphate buffered saline

PGN: Peptidoglycan

pIgR: Polymeric immunoglobulin receptor

Poly (I:C): Polyinosonic:polycytidylic acid

Poly (I:C)/lyovec: Poly I:C complexed with lyovec

PRR: Pattern Recognition Receptor

REGIIIγ: Regenerating islet-derived protein-IIIγ

RHIM: Receptor interacting protein (RIP) homotyping interaction motif

RICK: Receptor-interacting serine/ threonine kinase

RLR: Retinoic acid-inducible gene (RIG) like receptor

RNA: Ribonucleic acid

RT-qPCR: Real Time-Quantitative Polymerase Chain Reaction

SIGIRR: Single immunoglobulin IL-1R-related molecule (SIGIRR)

SILT: Solitary isolated lymphoid follicles

α-SMA: Alpha-Smooth muscle actin

SOCS1: Suppressor of cytokine signaling 1

STAT: Signal transducer and activator of transcription

SV40: Simian virus 40

TAK: Transforming growth factor -β (TGF-β)-activated kinase

TIR: Toll/Interleukin-1 receptor

TLR: Toll Like Receptor

TRAF6: Tumor Necrosis Factor (TNF)-receptor associated factor 6

TRIF: TIR-domain-containing-adapter-inducing interferon-β

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# ABSTRACT


## ROLE OF BOVINE ILEAL SUB-EPITHELIAL MYOFIBROBLASTS AND EPITHELIAL CELLS IN INNATE IMMUNITY

TIRTH UPRETY

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Gastro-intestinal (GI) tract harbors largest number of microbiota as well as the largest number of immune cells for a given tissue. The host needs to mount an effective immune response against invading pathogens and tolerance against commensals. Thus, regulatory mechanism and barrier function of the GI tract are of utmost importance for appropriate host microbe interaction and gut homeostasis. Intestinal epithelial cells (IECs) act as the first line of defense against invading pathogens. IECs recognize pathogens and commensals and mount an effective innate immune response. Such recognition of pathogens is mediated through germ line encoded pattern recognition receptors (PRRs). Intestinal sub-epithelial myofibroblasts (ISEMFs) reside just beneath the surface epithelium and are involved in maturation and differentiation of epithelium. ISEMFs protect from pathogens that breach surface epithelium by expressing PRRs. Lack of stable intestinal epithelial and sub-epithelial myofibroblast cell lines has slowed down scientific studies on these cells. In this study, we established and characterized ISEMF cells from the ileum of a 2-day old calf. We also had generated stable bovine ileal epithelial cell (BIEC-c4) cultures in our lab. On real time-quantitative polymerase chain reaction (RT-qPCR) analysis both these cell types expressed Toll-like receptors (TLRs) 1-9. To investigate their responses to various pathogen-associated molecular patterns

(PAMPs), we stimulated both cell types for 3 hours and 24 hours with various PAMPs. The RT-qPCR assay was used to investigate changes in TLR gene expression and in cytokine genes following stimulation. Lipopolysaccharide, peptidoglycan, and flagellin were used as bacterial ligands of surface PRRs. Similarly, γ-D-Glu-mDAP, muramyl dipeptide, polyinosonic:polycytidylic acid, poly I:C complexed with lyovec, and imiquimod were used as ligands of cytosolic and endosomal PRRs. Bovine ileal ISEMFs responded to bacterial PAMPs and to ligands of cytosolic and endosomal PRRs by significantly altering TLR gene expression. Unlike bovine ISEMFs, BIEC-c4 cells responded only to bacterial ligands. Thus, we conclude that bovine ileal ISEMF can be a good model to study innate immune responses and signaling pathways occurring at sub-epithelial compartment. However, BIEC-c4 cells may serve as a good in-vitro model to study enteric infectious disease pathogenesis and innate immune responses associated with them.

# Chapter 1. Introduction and Objectives

## 1.1 Introduction

Humans and animals being constantly exposed to myriad pathogens, within and outside of the body, need a robust system that can rapidly respond to invading pathogens. The immune system has evolved with evolving pathogens into an intricate system where it is difficult to compartmentalize it into sub-systems. In general, the immune system can be broadly classified into innate immunity and adaptive immunity.

Innate immunity comprises of anatomical barriers and germ line encoded receptors that recognize molecular patterns conserved across microorganisms and pathogens whereas adaptive or acquired immunity relies mainly on T-cell and B-cell responses against pathogens. Traditionally, innate immunity was characterized as non-specific immunity, but a recent understanding of innate immune system has shown the specificity in recognizing a virus or bacteria or intracellular and extracellular pathogens. Innate immunity's ability to recognize danger associated molecular patterns (DAMPs) that arise after necrotic cell death restricts the extensive cellular damage. Moreover, to mount a strong adaptive immune response requires stimulatory signaling from the innate immune system. Innate immunity's rapid response to invading pathogens precedes that of adaptive immunity and thus is important in limiting initial invasion by pathogens. Recent understanding of the significance of innate immunity has led to a renaissance of researches aimed at the better understanding the molecular mechanism of innate immune responses.

One of the various methods of pathogen recognition by the innate immune system is through recognition of pathogen associated molecular pattern (PAMPs) and the recognizing receptors are pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are transmembrane proteins that are part of PRR family and recognize conserved regions in bacteria, viruses, fungi or protozoa through leucine-rich repeat in their extracellular domain. Researchers are aiming at harnessing the potential therapeutic application of TLR signaling, especially in vaccine synthesis where TLR agonist are used as adjuvants for eliciting a strong immune response. Such a novel approach includes vaccination strategies for influenza vaccine (1), *Mycobacterium tuberculosis* vaccine (2), cancer vaccine like human papilloma virus (HPV) (3). A better understanding of cellular responses to various TLR agonists will help in designing approaches to manipulate immune response for therapeutic purposes. These PRRs constantly interact with microorganisms and mount a pro-inflammatory or anti-inflammatory response, whichever deemed essential.

Of various sites of host-microbe interaction, the gut epithelium is of utmost importance as it harbors trillions of commensals and pathobionts forming an ecological community called as gut microbiota. Intestinal epithelial cells have two major functions, segregation of gut microbiota and host intestine and mediate signals between microbiota and immune cells. Intestinal epithelial cells are constantly producing antimicrobial peptides, mucins, cytokines like IL-12, IL-27, IL-17 and chemokines to ward off pathobionts and pathogens (4). Constant production of pro-inflammatory cytokines by intestinal epithelial cells can lead to disease like Inflammatory Bowel Disease (IBD). Thus, intestinal epithelial cells need to balance the pro- and anti- inflammatory immune response. Just beneath the intestinal mucosa lies mesenchymal cells notably fibroblasts and myofibroblasts. These cells provide structural support as well as play vital role in maturation and differentiation of epithelial cells (5) and in immune regulation at mucosal and sub mucosal levels (6). Many studies have focused to understand their role in innate immunity when the first line of defense is compromised.

In vivo efforts to better understand the mechanisms by which intestinal epithelial cells maintain the harmony between pro and anti-inflammatory responses in response to constant interaction with gut microbiota is challenging. In vitro studies on intestinal epithelial cells enable to investigate mechanisms at cellular and molecular level. Researchers routinely use cell lines to study biological processes, however, unavailability of genotypically and phenotypically characterized bovine intestinal epithelial cell lines and intestinal sub-epithelial myofibroblast cell lines has made it difficult to study innate immune responses at intestinal mucosal and sub-mucosal level in bovine species. The development and characterization of a stable bovine intestinal sub-epithelial myofibroblast cell line will help us to investigate immune responses occurring beneath the mucosal level. The study proposed here will serve to understand the responses of intestinal epithelial cells and intestinal sub-epithelial myofibroblasts to various bacterial and viral ligands in terms of expression of TLRs,cytokines and chemokines. The research findings will help researchers in designing approaches to use TLR agonists for further immune system modulation and therapeutic strategies.

**1.2 Objectives**

The objectives of the study:

1. To establish and characterize a stable bovine intestinal sub-epithelial myofibroblast (ISEMF) cell line from the ileum of the 2-day old calf.

2. To analyze and study the expression of Toll like receptors (TLRs) by ISEMFs and investigate their responses to pathogen associated molecular patterns (PAMPs).
3. To analyze and study the expression of Toll like receptors (TLRs) by bovine intestinal epithelial cells line (BIEC-c4) and investigate its responses to pathogen associated molecular patterns (PAMPs).

# Chapter 2. Review of literature

## 2.1 An overview of the immune system

Edward Jenner by vaccinating against small pox laid the foundation for research into the field of Immunology. Shibasaburo Kitasato and Emil von Behring led the foundation for passive immunization by using antitoxins against tetanus. This led Paul Ehrlich to propose side chain theory which later was considered as a mechanism of antibody production from B-cell. Elie Metchnikoff observed cells that could engulf bacteria and coined them as phagocytes. In doing so, he laid the foundation for studies on innate immunity (7). During the early phase, scientists debated on what protected the body from pathogens. Some argued for cells like phagocytes while other supported humoral components like the then antitoxins (antibodies). Early research in immunology was dominated by humoral immunology as it could explain many immunopathologies. Later dichotomies like delayed type hypersensitivity and allograft rejection led to an appreciation of cell-mediated immunity.

The immune system has evolved considerably over the course of evolution. From toxic peptides and gene inactivating process to forestall pathogens employed by simplest eukaryotes to development of an arsenal of cells capable of detecting pathogens and mounting a specific response in higher vertebrates, it has developed into a complex system with no single definition to address this complexity (8).

The immune system is an intricate network of immune and non-immune cells, tissues, and organs that protect the body from pathogens and harmful substances. Broadly the immune system can be classified into innate immunity and adaptive immunity and the four major components of the immune system are barrier functions, immune tissues, immune cells, and protein/peptide defense (9).

**2.2 Components of innate immunity**

Innate immunity relies on germ line encoded receptors to mount an immune response against invading pathogens. Initially, it was considered as non-specific immunity. Research in the field of innate immunity has led to discoveries that show innate immunity to be specific. More than 90% of animal species rely solely on innate immunity for protection against pathogens (8).

Anatomical and physiological barriers provide initial defense against pathogens. These barriers include skin, cilia, low pH of the intestine, and antimicrobial peptides. Innate immunity along with the barriers serves as the first line of defense. Traditionally innate immunity was described only as host component. Efflux of information from microbiome studies has shown that innate immunity is a result of complex interplay between host and microbes.

Innate immunity relies on physical barriers, germ line encoded receptors, complement proteins, phagocytic cells, innate effector cells, and regulatory molecules like chemokines and cytokines (10). The absence of immunological memory separates innate immunity from adaptive immunity. Recent studies have shown a paradigm shift as innate immunity is shown to have some degree of immunological memory (11, 12). Innate immune cells like macrophages and natural killer cells (NK cells) upon reintroduction of similar infection show enhanced immunity. This enhanced immunity is independent of either B-cell or T-cell and is termed as ‘trained immunity’. This trained immunity could result from metabolic reprogramming of innate immune cells (13, 14).

Such immunological memory is also observed in copepods that lack an adaptive immune system and could possibly be a function of innate lectins (11). Trained immunity is also shown to exist in disease models of human neonates (15).

### 2.2.1 Pattern Recognition Receptors (PRRs)

In 1989 Charles Janeway Jr proposed the Pattern Recognition Receptor (PRR) theory. The central theme of this theory was that immune cells have receptors that recognize the microbial pattern and mount an effective immune response and provide necessary co-stimulation to an adaptive immune system for the further response (16). Such microbial patterns are conserved across the microbial groups and called as pathogen associated molecular patterns (PAMPs) or microbe associated molecular patterns (MAMPs). There are 6 families of these PRRs (17):

i. Toll-like receptors (TLRs)
ii. C-type Lectins
iii. Nod-like receptors (NLRs)
iv. RIG-I like receptors (RLRs)
v. AIM-2 like receptors (ALRs)
vi. OAS-like receptors (OLRs)

#### 2.2.1.1 Toll like receptors (TLRs)

Toll-proteins were initially identified in *Drosophila* as a transmembrane protein involved in the organization of dorso-ventral polarity of embryos (18). Later in 1996, researchers identified that Toll protein was involved in protection against fungal infection (19). A detailed history of discoveries in TLR study is enlisted in Fig 1 which is adapted from (20).

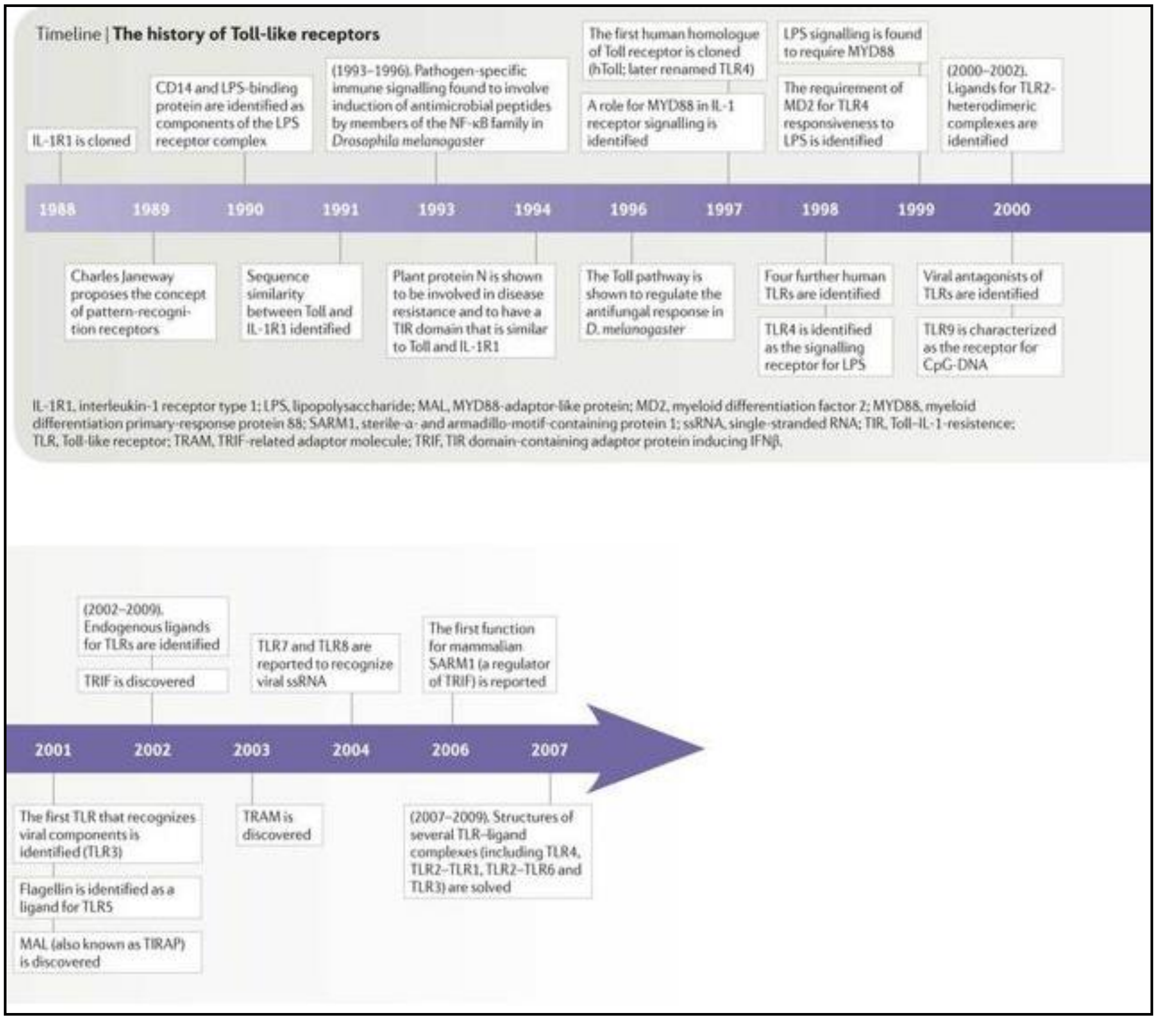


Fig 1: The history of Toll like receptors. Figure adapted from (20)

TLRs are a type-I transmembrane glycoproteins with N-terminal ligand recognition domain, single transmembrane helix, and C-terminal cytoplasmic signaling domain. The cytoplasmic region has considerable homology to other receptors of Interleukin-1 receptors family (IL-1R) and thus signaling domains of TLR is called as Toll/Interleukin-1 receptor (TIR) domain. The transmembrane domain contains a stretch of nearly 20 hydrophobic residues. TLRs that sense nucleic acid PAMPs use the transmembrane domain to interact with nucleic acid PAMPs and directs these TLRs to endocytic compartments (21, 22). The ectodomain region of TLR is different from that of

IL-1R. TLR ectodomain contains leucine rich repeats which are stretch of 22-29 hydrophobic residues in distinct interval involved in sensing of pathogens and the cytoplasmic region is involved in signal transduction (23, 24). Synthesis of TLR occurs in Endoplasmic Reticulum which is then trafficked to Golgi and ultimately recruited to the cell surface or to endosomes (25). Most mammals have ten TLRs (TLR 1-10) while a mouse has 13 TLRs (TLR 10 being a pseudogene) (26, 27). Sensing of PAMPs occurs through TLR ectodomain. PAMPs associated with various PRRs are listed in Table 1.

Table 1: TLR specific ligands. Adapted from (23).

| Receptor | Ligand | Origin of ligand |
|---|---|---|
| TLR1 | Triacyl lipopeptides<br>Soluble factors | Bacteria and mycobacteria<br>*Neisseria meningitidis* |
| TLR2 | Lipoprotein/lipopeptides<br>Peptidoglycan<br>Lipoteichoic acid<br>Lipoarabinomannan<br>Phenol-soluble modulin<br>Glycoinositolphospholipids<br>Glycolipids<br>Porins<br>Atypical lipopolysaccharide<br>Atypical lipopolysaccharide<br>Zymosan<br>Heat-shock protein 70* | Various pathogens<br>Gram-positive bacteria<br>Gram-positive bacteria<br>Mycobacteria<br>*Staphylococcus epidermidis*<br>*Trypanosoma cruzi*<br>*Treponema maltophilum*<br>*Neisseria*<br>*Leptospira interrogans*<br>*Porphyromonas gingivalis*<br>Fungi<br>Host |
| TLR3 | Double-stranded RNA | Viruses |
| TLR4 | Lipopolysaccharide<br>Taxol<br>Fusion protein<br>Envelope protein<br>Heat-shock protein 60*<br>Heat-shock protein 70*<br>Type III repeat extra domain A of fibronectin*<br>Oligosaccharides of hyaluronic acid*<br>Polysaccharide fragments of heparan sulphate*<br>Fibrinogen* | Gram-negative bacteria<br>Plants<br>Respiratory syncytial virus<br>Mouse mammary-tumour virus<br>*Chlamydia pneumoniae*<br>Host<br>Host<br>Host<br>Host<br>Host |
| TLR5 | Flagellin | Bacteria |
| TLR6 | Diacyl lipopeptides<br>Lipoteichoic acid<br>Zymosan | *Mycoplasma*<br>Gram-positive bacteria<br>Fungi |
| TLR7 | Imidazoquinoline<br>Loxoribine<br>Bropirimine<br>Single-stranded RNA | Synthetic compounds<br>Synthetic compounds<br>Synthetic compounds<br>Viruses |
| TLR8 | Imidazoquinoline<br>Single-stranded RNA | Synthetic compounds<br>Viruses |
| TLR9 | CpG-containing DNA | Bacteria and viruses |
| TLR10 | N.D. | N.D. |

#### 2.2.1.1.1 TLR signaling

Sensing of PAMPs by TLRs leads to homo-dimerization or hetero-dimerization of TLR ectodomain. Dimerization of TLR ectodomains brings cytoplasmic domains near for dimerization and initiate downstream signaling (28-31). Dimerization of the cytoplasmic domain is essential for the recruitment of signaling molecules. Downstream signaling molecules include adaptor protein called myeloid differentiation primary-response protein 88 (MyD88), IL-1R associated Kinases (IRAKs), transforming growth factor -β (TGF-β)-activated kinase (TAK1), TAB2, and tumor necrosis factor (TNF)-receptor associated factor 6 (TRAF6) (32, 33).

**2.2.1.1.1.1 MyD88 dependent signaling**

The MyD88 protein has a death domain (DD) at N-terminal and a cytoplasmic TIR domain. MyD-88 recruits IRAK to IL-1R complex by the interaction of DDs. It forms a homodimer of DD-DD and TIR domain-TIR domain when recruited to the receptor complex. It acts as an adaptor to recruit downstream signaling molecules that have DDs. MyD88 recruits IRAKs to form a complex called myddosome. Four different IRAK -like kinases have been identified (IRAK-1, IRAK-2, IRAK-4, IRAK-M). MyD88 interacts with IRAK 4, IRAK 4 phosphorylates IRAK 1. Auto phosphorylated IRAK 1 becomes fully functional. It then dissociates from the receptor complex. Fully functional IRAK 1 after dissociating from receptor complex activates ubiquitin E3 ligase TRAF 6 which is an ubiquitin protein ligase. Ubiquitination of TRAF 6 recruits TAK/TAB and IKK complexes. TAK is major complex that activates mitogen activated protein kinases (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) along with Janus kinase (JNK), extracellular signal regulated kinases (ERK), and p38 pathway. TLR signaling mainly activates p65/p50 heterodimer of NF-κB family which leads to the production of pro-inflammatory cytokines, chemokines and co-stimulatory molecules (7, 23, 34-37).

**2.2.1.1.1.2 MyD88 independent pathway/TRIF-dependent signaling pathway**

TRIF (TIR-domain-containing adapter-inducing interferon-β ) consists of N terminal domain, TRAF 6 binding motif, a TIR domain and RHIM domain (Receptor interacting protein (RIP) homotyping interaction motif**)**. RIP homotypic motif is essential for association with RIP 1. RIP 1mediates NF-κB activation. Signaling through TRIF leads to activation of transcription factors like NF-κB, IRF3 (Interferon regulatory factor 3), and activator protein 1 (AP 1). Phosphorylation activates C terminal regulatory domain of IRF3 which forms a dimer. After dimerization IRF3 is translocated to the nucleus. IRF 3 in nucleus recruits co-activators like p300 and CBP (cAMP-responsive-element-binding protein (CREB)-binding protein. These co-activators activate transcription of type-I IFN. Type I IFN activates IFN inducible genes (7, 23, 37-42).

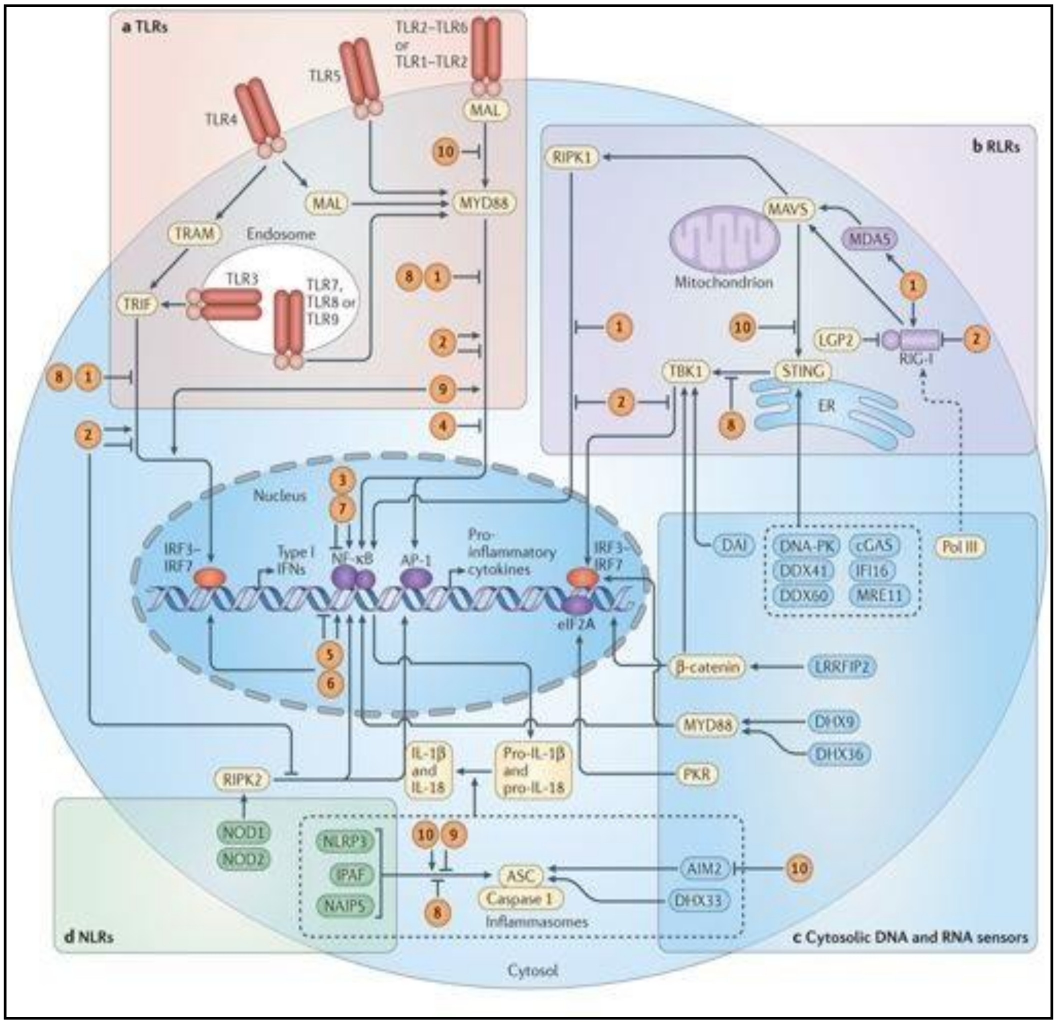


Fig 2: PRR signaling pathway along with regulator of signaling. Adapted from (43)

### 2.2.1.1.2 Negative regulation of TLR signaling

An excessive inflammatory cytokine produced during consistent TLR signaling may lead to endotoxic shock and systemic disorder. A negative regulation of TLR signaling occurs primarily through 3 major mechanisms. Dissociation of adaptor complexes, degradation of signal proteins, and transcriptional regulation all lead to negative regulation of TLR signaling. Molecules like suppressor of cytokine signaling 1 (SOCS1), IRAK M, MyD88 short (MyD88s), single immunoglobulin IL-1R-related molecule (SIGIRR) negatively regulate TLR signaling. IRAK M prevents the formation of IRAK1-TRAF complex by preventing dissociation of the MyD88-IRAK1-IRAK4 complex. SOCS1 proteins belong to E3 ubiquitin ligase and promote degradation of TRAF proteins. MyD88s is a transcript variant of MyD88 and owing to its inability to bind to IRAK4, prevents NF-κB activation. Regulation of gene expression through transcription regulation is also employed in negatively regulating TLR induced gene expression. miRNAs have emerged as a regulator of TLR signaling. miR-155, a TLR induced miRNA can suppress and enhance TLR signaling. miRNAs have emerged as fine tuners of TLR signaling (23, 44-46).

**2.2.1.2 NLRs**

Nucleotide-binding oligomerization domain (NOD) like receptors (NLRs) are cytoplasmic proteins involved in recognition of intracellular bacteria. There are more than 22 identified members of the NLR family in humans and more than 30 in mice. Two most extensively studied NLR subgroup includes NLR*C* and NLR*P*. NLR*C* are NOD proteins having N-terminal caspase recruitment domain (CARD), leucine rich repeats in C-terminus and nucleotide binding domain in between. In NLR*P* subgroup, CARD is substituted by Pyrin domain (47). NOD-1 and NOD-2 are two NOD proteins that recognize two different peptidoglycan fragments and are involved in pathogen recognition (48). Peptidoglycan (PGN) is a major constituent of gram-positive bacteria. In gram negative bacteria PGN is covered by a thick layer of lipopolysaccharide (LPS). PGN is composed of N-acetlyglucosamine (GlcNac) and N-acetylmuramic acid (MurNac) linked by β-(1-4) linkage (49). PGN’s role in producing an inflammatory response and stimulating immune response has been long known (50, 51). NOD-1/CARD4 recognizes peptidoglycan GlcNAc-MurNAc-L-Ala-γ-D-Glu-meso-DAP (GM-

TriDAP/iE-DAP) whereas NOD-2 recognizes muramyl dipeptide, MurNAc-L-AlaD-isoGln. NOD-1 and NOD-2 activate NF-κB by recruitment of receptor-interacting serine/ threonine kinase (RICK) leading to secretion of pro-inflammatory cytokines while type I interferons are secreted when IRF3/IRF7 dependent pathway is activated by these receptors (52-56). NOD protein can function as a mediator of innate immunity itself and also a modifier of innate immunity resulting from TLR stimulation (57).

**2.2.1.3 RLRs**

Retinoic acid inducible gene (RIG) like receptors (RLRs) are family of RNA helicases. RLRs include RIG-I, melanoma differentiating gene 5 (MDA5) and recently identified LGP2 (laboratory of genetics and physiology 2) proteins. Intracellular dsRNA is sensed by RIG-I, MDA-5. RIG-I senses blunt ended 5'phosphorylated dsRNA whereas MDA-5 recognizes long (>1000 nucleotide) dsRNA (58-60). Both RIG-I and MDA-5 are RNA helicases that have caspase recruitment domain (CARD) and helicase domain. Signal transduction after sensing of intracellular dsRNA is through CARD in both RIG-I and MDA-5. This results in the activation of IRF-3 and NF-κB and subsequent production of IFNs (type I, type III) and as well as pro-inflammatory cytokines like IL-6 and IL-8. LGP2 lacks CARD and is considered as a regulator of RIG-I and MDA-5 mediated immune response (61-65).

**2.2.1.4 ALRs**

Absent in melanoma protein 2 (AIM-2) is a member of a protein family called as PYHIN (pyrin and HIN200 domain containing). AIM2 is associated with dsDNA (double stranded DNA) induced inflammasome activation and interleukin-1β (IL-1β) production. DNA in the cytoplasm either during viral replication or delivered by immune complexes binds to AIM2. AIM 2 is an interferon inducible protein as it can bind to apoptosis related spec like protein (ASC) to form inflammasomes. Inflammasomes are multiprotein complex that can induce pyroptosis (caspase 1 dependent programmed cell death as means to combat intracellular pathogens by host cell) and pro-inflammatory cytokines like IL-1β (17, 66-69).

**2.2.1.5 OLRs**

2′–5′-oligoadenylate synthase (OAS) is a protein group that senses cytosolic dsRNA. Human OAS family consists of 4 IFN regulated genes OAS1, OAS2, OAS3, and OASL. OAS1, OAS 2, and OAS 3 can recognize cytosolic dsRNA and synthesize 2′–5′-oligoadenylate synthase which in turn activates RNase L which binds and degrades viral RNA. There is limited understanding of the mechanism of sensing dsRNA by OAS and RNase L binding to dsRNA (70-72).

**2.2.1.6 C-type lectin receptors (CLRs)**

C-type lectin receptors (CLR) are transmembrane receptors that bind to carbohydrates through carbohydrate binding domains (CRD). CLRs also include a protein that does not recognize carbohydrate ligands but has structurally similar C-type lectin domains (CTLDs). CLR activation can directly or indirectly induce intracellular signaling. Lectins like dectin-2, macrophage-inducible C-type lectin (Mincle) indirectly induce signaling by associating with immunoreceptor tyrosine-based activation motif (ITAM) containing adaptor molecules. During direct induction of signaling CLRs like Dectin 1 have ITAM like motif in the cytoplasmic region of the protein which they employ to induce signaling. In both cases, phosphorylated spleen tyrosine kinases are recruited. A series of signaling steps lead to activation of NF-κB, mitogen associated protein kinase (MAPK) which trigger cellular responses (73-75).

**2.3 Cytokines**

Cytokines are small non-structural proteins with molecular weight of 8-40,000 dalton. It is a biological function rather than consensus structural motif or amino acid sequence that groups cytokines into a different class (76). Cytokine comprises of a range of molecules that transmit intercellular signals. In the immune system, these orchestrate immune function by involving in the generation of an inflammatory response and restraining the inflammation (77, 78). Broadly the two major groups of cytokines are type I and type II cytokines. Type I cytokines signal through type I cytokine receptor. Type I cytokine have four α helical bundle and are further grouped into the short chain and long chain. Type I short chain cytokines are 15 amino acids long while long chain cytokines are 25 amino acid long. The IL-2 family of cytokine is grouped as short chain type I cytokine. IFN (IFN-α/β/γ) and IL-10 are grouped as type II cytokines (77, 79, 80). Based on immune function cytokines can be classified as pro-inflammatory and anti-inflammatory cytokines.

**2.3.1 Pro-inflammatory cytokines**

Predominantly secreted by activated macrophages, pro-inflammatory cytokines promote inflammation. These cytokines are also secreted by non-immune cells like fibroblasts, intestinal epithelial cells and endothelial cells (81).

Interferons (IFN) are pro-inflammatory cytokines predominantly produced to combat viral infections. There are three types of IFN, type I, type II, and type III. Type I and type III are involved in antiviral response while type II is involved in regulation of immune responses. Type I IFN binds to heterodimeric signaling complex composed of a single chain of IFNAR1 (IFN α/β receptor chain 1) and IFANR2 (IFN α/β receptor chain 2). Heterodimeric receptor complex for type I and type III IFN is present in almost all nucleated cells. Type II IFN (IFN-γ) produced mainly by immune cells binds to the tetrameric receptor complex composed of 2 subunits of IFNGR1 (IFN γ receptor 1) and IFNGR2 (IFN γ receptor 2). Type I and type II IFN activate both common and distinct STAT (signal transducer and activator of transcription) (82, 83).

Interleukin 1 was the first IL to be identified. Interleukin 1 (IL-1) family includes cytokines like IL-1α, IL-1β, IL-18, IL-33, IL-36 α, β, and γ. Cytokines belonging to IL-1 family promote the activity of innate immune cells like neutrophils, eosinophils, natural killer (NK) cells. IL-1 is an endogenous pyrogen that acts on hypothalamus-pituitary-axis to induce fever. Elevated body temperature increases leukocyte migration. IL-1α mediates early phase of sterile inflammation and the IL-1α precursor is fully functional. Unlike IL-1α, IL-1β precursor requires caspase 1 cleaving to transform into active cytokine. IL-1β is usually produced by hematopoietic cells, tissue macrophages, and dendritic cells. IL-1α is produced by epithelial cells lining gut, lungs, liver etc. Although both IL-1α and IL-1β act by binding to IL-1R1 (Interleukin 1 receptor 1), differences in their function is due to the difference in the source of origin. IL-1α has an amino acid sequence called nuclear localization sequence (NLS) that allows IL-1α to localize in the nucleus and act as transcription component. IL-1β lacks NLS. IL-1 can activate macrophages and epithelial cells and produce acute phase response (84, 85).

IL-6 is a pleiotropic cytokine and activates both T and B cells. It is mainly produced by macrophages and endothelial cells. IL-6 binds to membrane bound IL-6 receptor and associates with signaling glycoprotein gp130. Gp130 dimerization activates Janus kinase and ultimately leads to activation of MAP kinase. While limited number of cells express IL-6 R, an extensive number of cells express gp130. A soluble form of IL-6R is generated into circulation to which IL-6 binds. This IL 6 bound receptor complex can activate gp130 thus increasing the IL-6 spectrum. Apart from acute phase response IL-6 promotes differentiation of naïve $CD4^+$ T cells and thus links innate immune response with the adaptive immune system (T-helper cells) (86-88).

Tumor necrosis factor-α (TNF-α) is produced as type II transmembrane protein which is cleaved by TNF- α converting enzyme (TACE). TNF exerts biological function by binding with membrane bound TNF receptor that has cysteine rich repeats in the cytoplasmic domain. Cytokine of the TNF family exerts a biological effect by activation of the NF-κB pathway (89). TNF-α is involved in the anti-tumor response, apoptosis, cell survival and induction of inflammatory response (90). TNF- α is secreted by myeloid cells, antigen presenting cells, stromal cells, epithelial cells and activated T cells (91).

IL-8 is a pro-inflammatory cytokine that belongs to CXC chemokine family. IL-8 is neutrophil activating peptide and IL-8 acts as a chemoattractant for neutrophils, basophils and T cells. IL-8 acts through two receptors IL-8R A (CXCR1), and IL-8RB (CXCR2) (92, 93).

**2.3.2 Anti-inflammatory cytokines**

Anti-inflammatory cytokines are immune regulatory cytokines that check responses of pro-inflammatory cytokines. IL-1R antagonists, IL-4, IL-10, IL-11, IL-13 all act as anti-inflammatory cytokines (94).

IL-10 is an anti-inflammatory cytokine structurally related to IFN. Dysregulation of IL-10 leads to autoimmune disorders and immunopathies. Initially described as a cytokine produced from Th2 cells to check cytokine synthesis of Th1 cells, recent reports suggest that macrophages, dendritic cells also produce IL-10. IL-10 inhibits B7-1/B7-2 expression on monocyte and macrophages. B7-1/B7-2 are co-stimulatory molecules that activate $CD4^+$ T cells. IL-10 also inhibits secretion of pro-inflammatory cytokines and chemokines. IL-10 can thus limit T cell activation, inhibit production of pro-inflammatory cytokines and affect Th1 and Th2 responses (95-97).

### 2.4 Innate immune response at gastro-intestinal tract

Gastro-intestinal (GI) tract harbors largest number of microbiota as well as the largest number of immune cells for a given tissue. Gut microbiota educates immune cells and is essential for the development of a robust immune system. The host, in turn, needs to mount an effective immune response against invading pathogens and tolerance against commensals and food antigens. Thus, regulatory mechanism and barrier function of the GI tract is of utmost importance for appropriate host microbe interaction and homeostasis. A key to achieving this dynamic interaction is to segregate host tissue from gut microbiota. Intestinal epithelial cells (IECs) maintain homeostasis by providing a physical barrier and by sensing and responding to microbial stimuli (98-100).

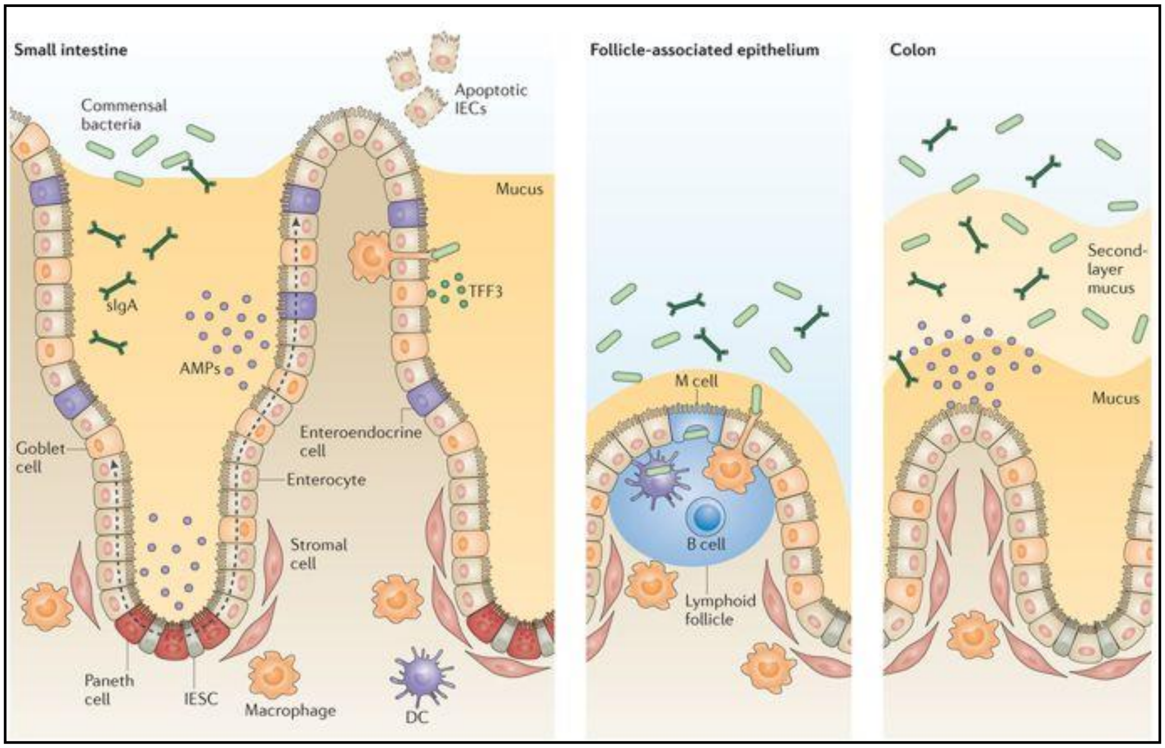


Fig 3: Schematic representation of the Intestinal Epithelial cell barrier. Adapted from (100).

GI tract is divided into four layers; mucosa, submucosa, muscularis propria, and serosa. Mucosa is the innermost layer and consists of epithelium, lamina propria, and muscular mucosae. Muscularis propria consists of an inner circular muscle layer and the outer longitudinal muscle layer.

The epithelium consists of different cell lineages originating from common stem cell progenitor. The epithelial layer is organized into crypts and villi. Pluripotent intestinal epithelial stem cells reside at crypts base. Enterocytes are most abundant cells in the intestinal epithelial layer and are involved in absorption of nutrients. Specialized secretory IECs are goblet cells that produce mucus, enteroendocrine cells that produce hormones, and Paneth cells that produce antimicrobial peptides like defensins. M cells (microfold cells) lack villi and are involved in transcytosis of antigen and thus help in antigen sampling (101, 102). Recent reports suggest that M cells can uptake specific antigen by using surface glycoprotein receptor. Thus M cells are capable of both specific and non-specific antigen uptake from intestinal lumen (103).

Mucin secreted from goblet cells acts as the first line of defense. Mucin deficient mouse develops colitis (104). Paneth cells are concentrated in the ileum and produce antimicrobial peptides like lysozymes, defensins, regenerating islet-derived protein-IIIγ (REGIIIγ). RegIIIγ is involved in host-microbial segregation in GI tract (105). Sensing of PAMPs by PRRs expressed in intestinal mucosa helps to mount an effective innate immune response against pathogens and induce tolerance to commensals. Such PRRs are expressed by intestinal epithelial cells and by mesenchymal cells like intestinal sub-epithelial myofibroblasts (ISEMFs). Unlike other body sites, IECs in intestine express PRRs that are involved in altered responsiveness. PRRs in other body sites are associated with induction of inflammatory response upon sensing of PAMPs. In intestine where the majority of microbes are commensals, PRRs are involved in dampening of immune response and maintaining tissue homeostasis (106, 107).

IECs express polymeric immunoglobulin receptor (pIgR). Secretory IgA (sIgA) produced by plasma cells binds to pIgR and this sIgA-pIgR complex is transcytosed to intestinal lumen. sIgA is essential to maintain intestinal homeostasis. Gut associated lymphoid tissue (GALT) and draining lymph nodes are essential for adaptive immune responses. These GALT are also part of the mucosal immune system. GALT consists of isolated lymphoid follicles or aggregated lymphoid follicles that collectively form Payer’s Patches. These sub-epithelial lymphoid aggregates reside in mucosa or submucosa. Follicle associated epithelium lies above these lymphoid aggregates. One of the follicles associated epithelium is M cell. Sub-epithelial dome region is rich in dendritic cells and dendritic cell processes antigens after transcytosis by M cells. Solitary isolated lymphoid follicles (SILT) are microscopic lymphoid aggregates which can range from cryptopatches to isolated lymphoid follicles (ILF). NOD1 signaling in stromal cells promotes intestinal SILT maturation (99, 100, 108).

Intestinal sub-epithelial myofibroblasts (ISEMFs) are mesenchymal cells residing beneath the intestinal mucosa (109-111). They also regulate the behavior of intestinal stem cells through intracellular mechanisms like Wnt, Bmp, Notch (5). ISEMF cells have been characterized as nonprofessional immune cells (112). They are also reported to be involved in the induction of peripheral tolerance in intestinal mucosa primarily through programmed death ligand-1 (PD-L1) mediated suppression of $CD4^+$ T cell activity (113, 114).

GI tract is involved in both induction of immune response and maintenance of tolerance by identifying pathogens from commensals microbes. This complex task requires complex interplay between the mucosal barrier and immune cells. Intestinal epithelial cells and intestinal sub-epithelial myofibroblasts by expression of PRRs mount selective immune responses and are key players of intestinal innate immune responses.

**2.5 RT-qPCR**

Polymerase chain reaction (PCR) is a revolutionary concept developed by Karry Mullis in 1980. The enzyme DNA polymerase adds nucleotides (dideoxynucleotides; dnTPs) complementary to given template. Since DNA polymerase can add nucleotide only to 3’-OH group, it requires short nucleotide sequences called primer sequence.

Changes in temperature allow for control over polymerase activity and primer binding. Conventional PCR could only detect the presence of a specific gene. Conventional PCR could not infer relative abundance of a gene in two samples. To overcome this, fluorescence-based chemistry was developed called as real time-PCR or quantitative PCR (qPCR). qPCR can obtain amplification data in real time. If complementary DNA produced by reverse transcribing of mRNA is used as a template for PCR, it is called as reverse transcriptase PCR. Fluorescence based real time reverse transcriptase PCR allows for quantification of steady state mRNA expression. SYBR green based qPCR is one of the widely used fluorescence-based PCR assay. SYBR green dye is a fluorescent dye that binds to double stranded DNA only. Fluorescence occurs only if SYBR green dye is bound to double stranded DNA. As the amplification occurs, fluorescence increases as more double stranded DNA are being formed. A sensor collects all the data which is expressed in terms of Ct (threshold cycle) values. Ct value is the number of PCR cycles required by fluorescent signal to overcome background signal. A lower Ct value indicates stronger gene expression. Since SYBR green dye can bind to any dsDNA, the specificity of the data is validated using a dissociation curve or melting curve. A dissociation curve is obtained at end of PCR process by first denaturing all products followed by annealing and dissociation. The first derivative of the dissociation curve assesses the homogeneity of the PCR product. Two major quantification approaches are employed for qPCR data. For absolute quantification, a standard curve using serial dilution of known RNA concentration or copy number is plotted against Ct values. Ct value of the unknown sample for the gene of interest is used to assess concentration using standard curve. In relative quantification, sample Ct values are normalized against the reference genes (115, 116).

## Chapter 3. Development and characterization of a stable bovine intestinal sub-epithelial myofibroblast cell line from ileum of a young calf.


### Abstract

Intestinal sub-epithelial myofibroblasts (ISEMFs) are mesenchymal cells that do not express cytokeratin but express α-smooth muscle actin and vimentin. Despite being cells with diverse functions there is a paucity of knowledge about their origin and functions primarily due to the absence of a stable cell line. Although myofibroblast in-vitro models for humans, mouse, and pig are available, there is no ISEMF cell line available from young calves. We isolated and developed an ileal ISEMF cell line from a 2-day old calf that expressed α-smooth muscle actin and vimentin but no cytokeratin indicating true myofibroblast cells. To overcome replicative senescence, we immortalized primary cells with SV40 large T antigen. We characterized and compared both primary and immortalized ileal ISEMF cells for surface glycan and Toll-like-receptors (TLRs) expression by lectin binding assay and real time-quantitative PCR (RT-qPCR) assay respectively. SV40 immortalization significantly decreased surface lectin binding for lectins GSL-I, PHA-L, ECL, Jacalin, Con-A, LCA, and LEL. Both cell types expressed TLR 1-9 and showed no significant differences in TLR expression. Thus, these cells can be useful in-vitro model to study ISEMF's origin, physiology, and functions.


**Keywords**: intestinal sub-epithelial myofibroblast, bovine, lectin, toll-like receptors.

### 3.1 Introduction

Intestinal sub-epithelial myofibroblasts are mesenchymal cells residing beneath the intestinal mucosa (109-111). Originally described by Kaye et al in 1968 (117) and later termed as myofibroblasts by Majno et al in 1971 (118), these cells have gained widespread interest due to their diverse functions ranging from wound healing (119), promotion of tumor progression (120), to their role in inflammatory bowel disease (IBD) (121). ISEMFs are important in the regulation of barrier function of the intestinal mucosa. They play a pivotal role in the development of the mucosal layer as they are involved in morphogenesis and cytodifferentiation of the intestinal mucosa (109, 110). Epimorphin/syntaxin 2, a mesenchymal protein expressed by ISEMF cells promotes morphogenesis of villi (122) and ISEMFs are also involved in restitution and differentiation of the epithelium by secreting stem cell factors, growth factors like transforming growth factor- β3 (TGF-β3), and amphiregulin (123, 124). They also regulate the behavior of intestinal stem cells through intracellular mechanisms like Wnt, bone morphogentic protein (Bmp), and Notch signaling (5). ISEMF cells have been characterized as nonprofessional immune cells (112) and nonprofessional antigen presenting cells (125). They also express Toll like receptors (TLRs) but their response to pathogen associated molecular patterns (PAMPs) have not been elucidated (126).

Myofibroblasts have characteristics intermediate between a fibroblast cell and a smooth muscle cell (111, 127). They are characterized mainly by the presence of alpha-smooth muscle actin (α-SMA) (123, 128) along with vimentin and negative to weak staining for desmin (112, 120, 129). Myofibroblast cell morphology transitions from discoid or polygonal to elongated with an increase in cell passage (111).

Limited understanding of ISEMFs owing to un-availability of stable ISEMF cell line has impeded studies on this cell type. Only a single bovine ISEMF cell culture study has been reported till date (130) and most of the other cell lines are either from rat (131-133), mouse (126, 134) or humans (5). In this study, we established and characterized a stable ileal ISEMF cell line from the 2-day old calf that demonstrates characteristics peculiar to myofibroblast cells.

### 3.2 Materials and Methods

#### 3.2.1 Establishment of primary ileal ISEMF cell culture

An animal protocol for the use of a calf for cell line development was approved by SDSU Institutional Animal Care and Use Committee (IACUC). Ileum from 2-day old, colostrum deprived Holstein male calf, was collected in supplemented Hanks Balanced Salt Solution (HBSS containing 1% streptomycin-penicillin, 5ug/ml of gentamycin, 2 mM of L-glutamine). The lumen was washed two times with warm phosphate buffered saline (PBS). Both ends of the ileal loop were ligated with silk suture after flushing the lumen with supplemented HBSS (HBSS-S). Lumen was filled with phosphate buffered saline (PBS) containing 1 mM dithiothreitol and incubated for 5-10 minutes in a water bath at $37^0$ C with constant shaking to remove mucus. To digest the intestinal tissue, loops were filled with warm ($37^0$ C) HBSS-S containing 300 units/ml of collagenase-type II (catalog number LS004176, Worthington Biochemical 130 Corporation, NJ, USA) and 0.24 units/ml of dispase (catalog number 50-100- 131 3345, Roche Diagnostics, IN, USA), and incubated at $37^0$ C in water bath with constant shaking for 15 minutes. The contents were discarded and the process of digestion with collagenase and dispase was repeated, but incubation time was increased from 15 minutes to 45 minutes. The content thus obtained were also discarded. The predigested epithelium and sub-epithelium were scraped with sterile scalpel blade after longitudinally opening the ileal loop. The contents thus obtained were incubated in HBSS containing 2.4 U/ml of dispase for 10 minutes and then centrifuged at 140 g for 3 minutes. The pellet was resuspended in 30 ml of DMEM (Dulbecco's modified eagle media) containing 2% sorbitol and centrifuged at 50 g for 3 minutes. The supernatant was collected and grown in T-75 flask containing DMEM-10 media. DMEM-10 media contained DMEM, 10 % fetal calf serum, 1 % non-essential amino acids, and 1 % penicillin-streptomycin. The cells attached and started showing myofibroblast like morphology and retained myofibroblast like morphology even at later passages when observed under Olympus IX70 phase contrast microscope.

**3.2.2 Generation of SV40 immortalized ISEMF cell line.**

Passage 20, 0.5 X $10^6$ ISEMF cells were seeded into 3 wells of a 6-well tissue culture plate. Cells in the first well were kept as such. Cells on the second well were used for negative control, and cells in the third well were used for transfection with pSV3-neo plasmid (ATCC® 37150) vector containing SV40 Large T Antigen gene. For transfection, Lipofectamine® 2000 (catalog number 11668-027, Invitrogen, Carlsbad, CA) reagent and manufacturer's protocol were used. The lipofectamine-plasmid complex was added on to cells in the third well whereas only lipofectamine was added in cells on the second well. All three wells were incubated with serum-free OPTI-MEM® media for 12 hours followed by washing with 1X PBS. Cells were then grown in selection antibiotic G418 (catalog number 10131-035, Thermo Fisher, Waltham, MA, USA) for 7 days at a concentration of 2500 µg/ml of media. After antibiotic selection cells were grown on fibroblast media containing 500 µg/ml of G418 antibiotic.

### 3.2.3 Immunocytochemical (ICC) staining of primary and SV40 immortalized ISEMF cells

Monoclonal antibodies (mAbs) specific against epithelial, fibroblast, and smooth muscle cell markers were used to stain primary and SV40 immortalized cells using the protocol as described previously (135, 136). Briefly, cells cultured in T-25 or T-75 flasks were trypsinized using 0.05% of Trypsin EDTA (Corning®, reference number 25-052-CV) and counted using hemocytometer to prepare $10^6$ cells/ml suspension of primary ileal ISEMFs and SV40 immortalized ileal ISEMFs. Hundred microliter of cell suspension was used to prepare cytospins using a cytofuge (Cytospin 3; Thermo Shandon Inc. Pittsburgh, PA, USA). After air drying for 2 hours, the cytospins were fixed in acetone for 7 minutes. Slides were then washed with 1% PBS. Endogenous peroxidase was blocked by 7 minutes incubation of slides in blocking solution (0.3% hydrogen peroxide and 0.01% Sodium azide in PBS). To prevent further non-specific antibody binding, if any, slides were incubated with 1% goat serum for 20 minutes. An avidin-biotin blocking kit (catalog number SP2001, Vector Laboratories, Burlingame, CA, USA) was used to block endogenous biotin. Specific mAbs were used to detect the presence of cytokeratin, α-smooth muscle actin (α-SMA), desmin and vimentin proteins by immunocytochemistry. Anti-cytokeratin mAb (mAb) C6909 (IgG2a isotype), anti-vimentin mAb V5255 (IgM isotype), anti-alpha smooth muscle actin mAb A2547 (IgG2a) and anti-desmin mAb D1033 (IgG1) were used. Isotype-matched controls, mAbs M9269 (IgG1 isotype), M9144 (IgG2a isotype) and 196 M5170 (IgM isotype) were also used. Cells incubated with PBS alone served as negative control. All primary and isotype control mAbs were bought from Sigma-Aldrich (Sigma-Aldrich, St. Louis-MO). Slides were incubated for an hour with 100 µl (1 µg/ml) of the specific primary antibody or isotype controls. Slides were then washed and incubated with 100 µl (1:2000 dilution) of isotype-specific, biotinylated goat-anti mouse IgG2a, IgG1, and IgM antisera (Caltag Laboratories) for 30 minutes. Presence of specific protein in the ISEMFs was detected by incubating slides with the HRP-streptavidin solution for 30 min, followed by ready-to-use (RTU) diaminobenzene (DAB) substrate (Vector Laboratories, Burlingame, CA, USA). Hematoxylin was used as a nuclear stain. Images were taken with an Olympus BX53 upright microscope at 20X magnification.

### 3.2.4 Confirmation of SV40 immortalization by PCR

Genomic DNA from the primary (passage 22) and SV40 immortalized ISEMF cells (passage 22) was extracted using DNeasy Blood & Tissue Kit (Catalogue number 69504, Qiagen, Valencia, CA, USA). DNA concentration was quantified using Nanodrop 2000 (Thermo scientific). Hundred nanograms each of genomic DNA extracted from primary ISEMFs, SV40 immortalized ISEMFs, and pSV3-neo plasmid containing SV40 Large T Antigen gene were used to amplify SV40 gene using gene specific primers (Table 2) previously used by others (137). To amplify the gene of interest, we amplified the genomic DNA and pure plasmid using PCR. Taq PCR kit (catalog number E5000S, New England Biolabs, Ipswich, MA, USA) was used for PCR assay. The thermal profile used for PCR was; initial denaturation at $95^0$C for 5 minutes, followed by 30 cycles of annealing and extension at $95^0$C for 30 seconds, $55^0$C for 60 seconds, $72^0$C for 60 seconds, and the final extension at $72^0$C for 10 minutes. The amplicons were resolved in 2% agarose gel by running it at 85 volts for 45 minutes.

Table 2: Primer Sequences used for amplification of SV40 gene in ISEMF cells

| Gene | Primer sequence | Product size (bp) | Tm ($^{0}$C) |
|---|---|---|---|
| SV40 | Forward:<br>5′-AGCAGACACTCTATGCCTGTGTGGAGTAAG-3′<br>Reverse:<br>5′-GACTTTGGAGGCTTCTGGATGCAACTGAG-3′ | 751 | 55 |

**3.2.5 Confirmation of SV40 immortalization by indirect immunofluorescence assay**

Indirect immunofluorescence assay was also performed to further confirm the presence of SV40 protein in immortalized cells. Twenty-five thousand primary ISEMFs (passage 17) and SV40 immortalized ISEMF cells (passage 38, 18 passage after immortalization) were cultured in the chambered slide. For each cell type, two chambers were used, the first chamber for isotype control and second for confirmation of immortalization. After 24 hours, the cells in the slide were washed with 1X PBS, fixed and permeabilized using acetone and methanol mix (1:1, 250 µL/chamber) and incubated at -20$^{0}$C for 10 minutes. Cells were washed again with PBS, blocked for non-specific protein binding by incubating for 20 minutes at room temperature after addition of blocking solution (1% goat serum in 0.2% Triton X and 1% PBS). Cells were washed again and 250 ul (4ug/ml concentration) of either mouse IgG2a isotype control (mAb M9144, Sigma, St. Louis, MO, USA) or mouse anti-SV40 specific monoclonal IgG2a antibody (Santa Cruz Biotechnology, Dallas, TX, USA; sc-53488) was added on respective chambers. Cells were then incubated at 37$^{0}$C for an hour. After washing with

PBS, Alexa Fluor 488 conjugated goat anti-mouse IgG secondary antibody (1:200 dilutions, Invitrogen, USA, A-11001) was added and incubated in dark at $37^0$C for an hour. Cells were washed with PBS, equilibrated by addition of 2X saline sodium citrate (SSC) buffer for 5 minutes followed by washing with PBS. Then, 250 µl of propidium iodide (1uM, 1:1,000 dilution) was added and incubated for 5 minutes for nuclear staining of cells. Cells were then washed and mounted using permaflour mounting reagent. Images were taken using an Olympus BX53 upright microscope at 20X magnification.

**3.2.6 Lectin Binding Assay:**

The binding profile of 23 different lectins (Table 3) was identified in both primary and SV40 immortalized ISEMFs as per manufacturer's (Vector Laboratories, Burlingame, CA, USA) protocol. As per the protocol, cells were trypsinized and counted. Approximately $0.5X10^6$ cells were transferred to 96-well U bottom plate. Cells were incubated for an hour at $4^0$C with 100 µl of 10 µg/ml of specific biotinylated lectins. To show specificity of lectin binding, another well with the same number of cells was incubated with a same volume and concentration of lectin and its specific inhibitor solution. Cells were then washed three times with 1X PBS and centrifuged to pellet the cells. Cells were then incubated in dark with 5 µg/ml of streptavidin-FITC (1:200 dilutions) at $4^0$C for 30 minutes. Cells were then washed and resuspended in 200 µl of 1% paraformaldehyde. For each assay, a separate well containing cells and streptavidin-FITC was included as negative control. The percentage of cells staining with lectins was measured using FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA).

Primary ISEMF cells from passage 15-27 and SV40 immortalized ISEMF from passage 40 (20 after immortalization)- passage 47 (27 after immortalization) were used.

Table 3: Lectins and lectin inhibitors used for studying glycobiology of ISEMF cells

| **Lectins** | **Binding Specificity of Lectins** | **Inhibitors** | **Inhibitor concentration (mM)** |
|---|---|---|---|
| **1. Glucose/Mannose group** | | | |
| *Canavalia ensiformis* agglutinin (Con-A) | α-Man, α-Glc | α-methyl mannoside, α-methyl glucoside | 200 mM each |
| *Pisum Sativum* agglutinin (PSA) | α-Man, α-Glc | α-methyl mannoside, α-methyl glucoside | 200 mM each |
| *Lens culinaris* agglutinin (LCA) | α-Man, α-Glc | α-methyl mannoside, α-methyl glucoside | 200 mM each |
| **2. N-acteylgalactasomine group** | | | |
| *Griffonia simplicifolia* lectin I (GSL-1) | α-GalNAc, α-Gal | Galactose | 200 mM |
| Soybean (*Glycine maxi*) agglutinin (SBA) | α-GalNAc, | N-acetylgalactosamine | 100 mM |
| *Dolichohs biflorus* agglutinin (DBA) | α-GalNAc, | N-acetylgalactosamine | 100 mM |
| *Ricinus communis* agglutinin (RCA-120) | β-GalNAc, β -Gal | Galactose | 200 mM |
| *Sophora japonica* agglutinin (SJA) | β-GalNAc, | N-acetylgalactosamine | 100 mM |

| | | | |
|---|---|---|---|
| *Vicia villosa* agglutinin (VVA) | β-GalNAc, | N-acetylgalactosamine | 100 mM |
| **3. N-acetylglucosamine group** | | | |
| *Lycopersicon esculentum* (tomato) lectin (LEL) | β-GlcNAc | Chitin hydrosylate | 200 mM |
| *Solanum tuberosum* (potato) lectin (STL) | β-GlcNAc | Chitin hydrosylate | 200 mM |
| Wheat (*Triticum vulgaris*) germ agglutinin (WGA) | β-GlcNAc | Chitin hydrosylate | 200 mM |
| *Datura stramonium* lectin (DSL) | β-GlcNAc | Chitin hydrosylate | 200 mM |
| *Griffornia simplicifolia* lectin II (GSL-2) | α, β-GlcNAc | Chitin hydrosylate | 200 mM |
| Succinylated WGA (SWGA) | β-GlcNAc | Chitin hydrosylate | 200 mM |
| Peanut (*Arachis hypogaea*) agglutinin (PNA) | β -Gal | Chitin hydrosylate | 200 mM |
| *Erythrina cristagalli* lectin (ECL) | β -Gal, β-GalNAc | Lactose | 200 mM |
| *Artocarpus integrifolia* (Jacalin) | β -Gal | Galactose | 400 mM |
| **4. Fucose group** | | | |
| *Ulex europaeus* agglutinin I (UEA-1) | A-Fuc | No Inhibitor used | |

| | | | |
|---|---|---|---|
| **5. Oligosaccharide group** | | | |
| *Phaseolus vulgaris* erythroagglutinin (PHA-E) | Oligosaccharide | No Inhibitor used | |
| *Phaseolus vulgaris* Leucoagglutinin (PHA-L) | Oligosaccharide | No Inhibitor used | |
| **6. Sialic acid group** | | | |
| *Sambucus nigra* lectin (SNA) | NeuAcα(2,6)Gal | Lactose | 200 mM |
| *Maackia amurensis* lectin II (MAL-II) | NeuAcα(2,3)Gal | N-acetylneuraminic acid (NANA) | 200 mM |

### 3.2.7 Analysis of TLR expression in primary ISEMF and SV40 immortalized ISEMF by RT-qPCR

For analysis of TLR expression by primary and SV40 immortalized ISEMF cells, we used SYBR-green based real time-quantitative polymerase chain reaction (RT-qPCR) assay. RNA was extracted from cells using RNeasy Mini Kit (catalog number 74101, Qiagen, Valencia, CA, USA). RNA was quantified using Thermo Scientific™ NanoDrop 2000. One microgram of RNA was reverse transcribed to cDNA using TaqMan reverse transcription kit and kit protocol (TaqMan reverse transcription reagents, Applied Biosystems, catalog number N8080234). For RT-qPCR 2 µl of diluted cDNA (1:5 dilution), 1 µl each of forward and reverse primer (10 µM stock), 10 µl of RT² SYBR® Green/ROX qPCR mastermix (catalog number 330501, Qiagen, Valencia, CA, USA) and 6 µl of nuclease free water were added. The thermal profile used for amplification was: 2

minutes at $50^0$C; 10 minutes at $95^0$C; followed by 40 cycles of 45 seconds at $95^0$C, 30 seconds at $60^0$C and 30 seconds at $72^0$C. Ramping speed was set at $1.6^0$C/second. QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, NJ, USA) was used. Data were normalized using the housekeeping gene (Cyclophillin-A). Primer sequences previously used (138) for amplification of TLR 1-9 gene and Cyclophillin-A as housekeeping gene (139) are listed in Table 4. Primary ISEMF cells from passage 15-20 and SV40 immortalized cells from passage 33 (13 passage after immortalization) to passage 35 (15 passage after immortalization) were used for comparing TLR expression.

Table 4: Primer sequences of genes along with gene bank accession number used in analysis of TLRs expression of ISEMF cells

| | **Forward primer** | **Reverse primer** | **Accession number** |
|---|---|---|---|
| TLR 1 | CAT TCC TAG CAG CTA CCA CAA GCT | TGG GCC ATT CCA AAT AAG TTC T | NM_001046504 |
| TLR 2 | GGG TGC TGT GTC ACC GTT TC | GCC ACG CCC ACA TCA TCT | NM_174197 |
| TLR 3 | GGG CAC CTG GAG GTC CTT | TTC CTG GCC TGT GAG TTC TTG | NM_001008664 |
| TLR 4 | AGC ACC TAT GAT GCC TTT GTC A | GTT CAT TCC GCA CCC AGT CT | NM_174198 |
| TLR 5 | GTC CCC AAC ACC ACC AAG AG | GCG GTT GTG ACT GTC CTG ATA TAG | NM_001040501 |
| TLR 6 | TTT ACC CTC AAC CAC GTG GAA | GGG CCA AAG GAA CTG AAA AAC | NM_001001159 |
| TLR 7 | CAC CAA CCT TAC CCT CAC CAT T | GTC CAG CCG GTG AAA GGA | NM_001033761 |
| TLR 8 | TGT GTT TAG AGG AAA GGG ATT GG | TCT GCA TGA GGT TGT CGA TGA | NM_001033937 |
| TLR 9 | CAG TGG CCA GGG TAG TTT CTG | CCG GTT ATA GAA GTG ACG GTT GT | NM_183081 |
| Cyclophilin-A | CTTTCACAGAATAATTCCGGGATT | CAGTACCATTATGGCGTGTGAAG | BC105173 |

**3.2.8 Statistical Analysis:**

For comparison of differences in lectin binding between primary and SV40 immortalized ISEMFs, two tailed two sample unequal variance t-test was used. A p-value of less than 0.05 ($p<0.05$) was considered significant. For analysis of TLR expression in primary ISEMF, Ct values for TLR genes were normalized with housekeeping gene. The result was expressed as delta Ct (ΔCt). A lower ΔCt means stronger gene expression. A non-parametric test (Mann-Whitney U test) was used to compare normalized Ct (ΔCt) values (136). Mann-Whitney U test was applied using GraphPad Prism 7.04.

## 3.3 Results

### 3.3.1 Immunocytochemical (ICC) staining of primary and SV40 immortalized ISEMF cells

The primary ileal ISEMF cells were cultured up to 27 passages without any changes in their morphological characteristics. SV40 immortalized ISEMF cells were passaged up to 20 passage after immortalization and were stocked after this passage. Cells obtained after scrapping of enzymatically digested ileal tissue in culture started showing stellate to spindle shape typical of myofibroblast cells (Fig 4). Immunocytochemical (ICC) characterization of primary ISEMF (Figure 5) and SV40 immortalized ISEMF (Fig 6) cells showed positive staining for α-SMA (Fig 5C, and 6C) and vimentin (Fig 5B, and 6B), weak staining for desmin (Fig 5D, and 6D), and no staining for cytokeratin (Fig 5A and 6A). A weak staining indicates few cells expressing the markers while negative staining indicates complete absence of staining. The specificity of staining was demonstrated by negatively stained isotype contol for each marker.

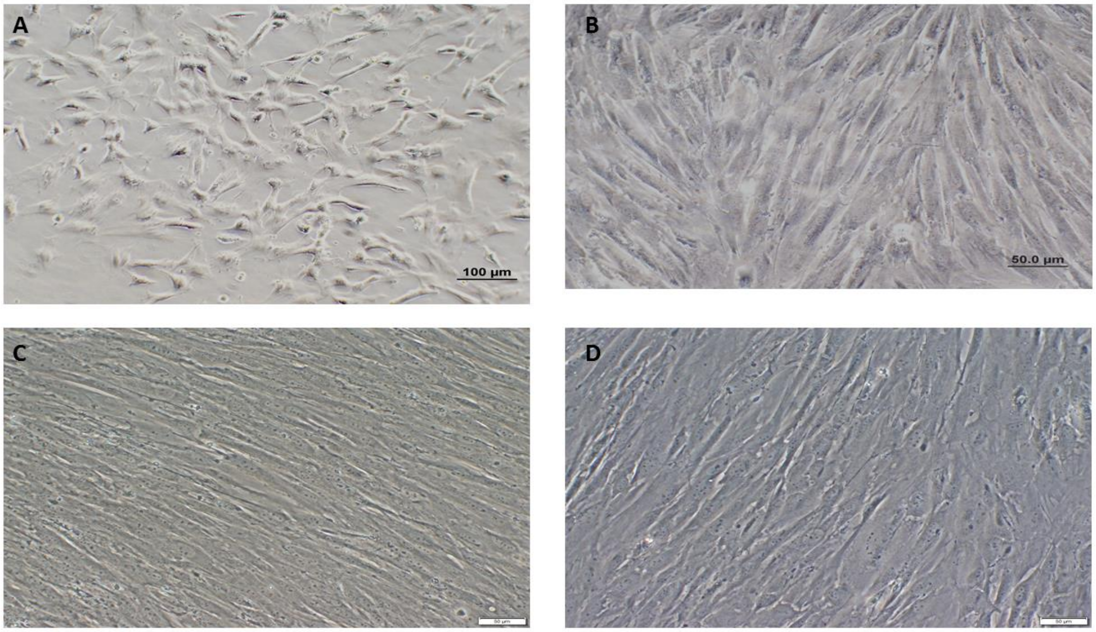


Fig 4: Phase contrast image of primary and SV40 immortalized ISEMF cells. **A**. Phase contrast image of primary ISEMF cells (cells obtained and cultured after scrapping of enzymatically digested ileal tissue). **B.** Phase contrast image of primary ISEMF cells (passage 1). **C** Phase contrast image of primary ISEMF cells (passage 15). **D.** Phase contrast image of SV40 immortalized ISEMF cells (passage 39, 19 passage after immortalization). Scale bar represents 100 µm for image **A** and 50 µm for rest of the images.

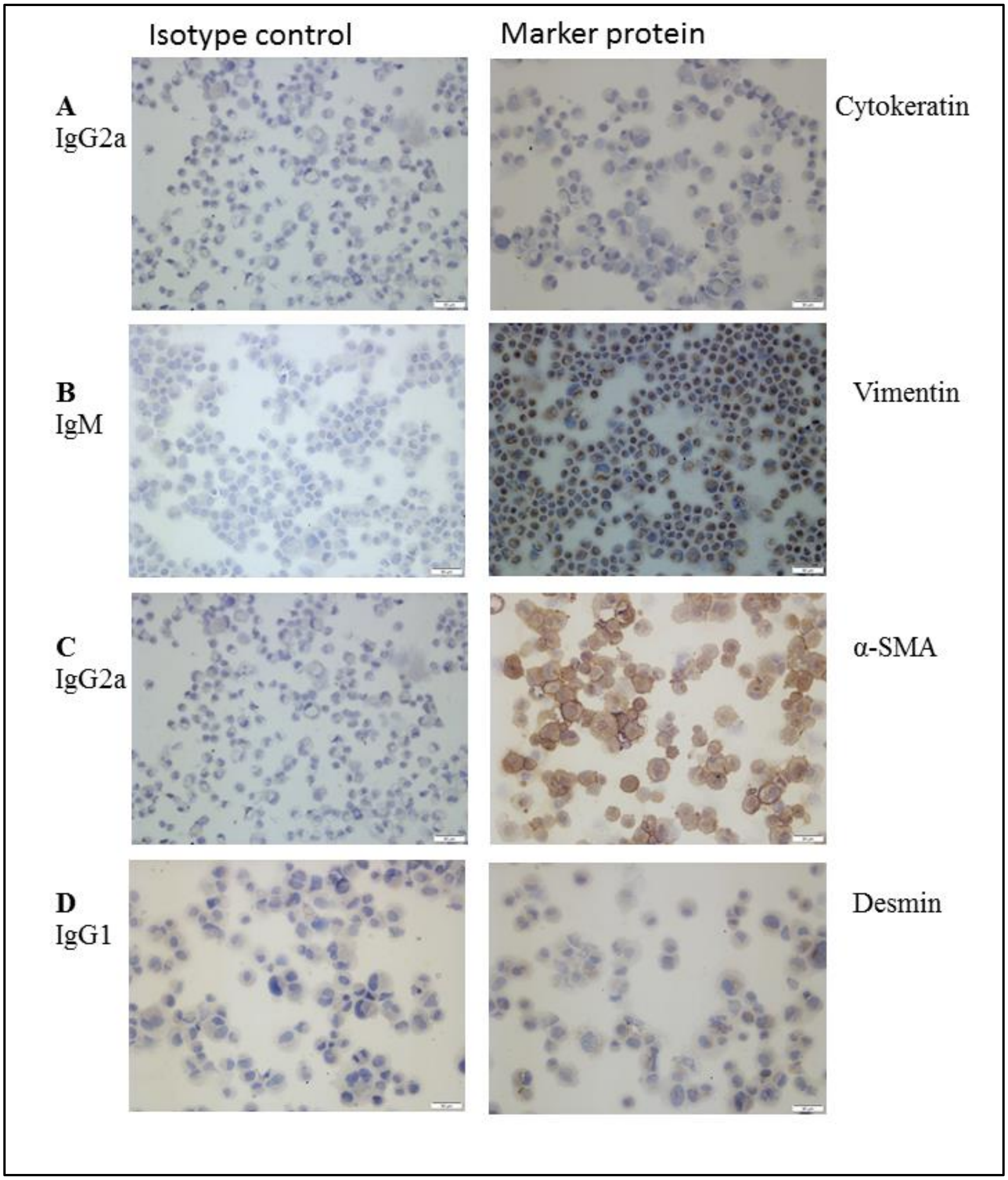
Isotype control
Marker protein
A
IgG2a
Cytokeratin
B
IgM
Vimentin
C
IgG2a
α-SMA
D
IgG1
Desmin

Fig 5: Immunocytochemistry of primary ISEMF cells. Images on right are specific isotypes, and images on left are for cell specific markers. Brown pigmented cells indicate positive staining **A**. IgG2a isotype control, and anti-cytokeratin-IgG2a Ab staining. **B**. IgM isotype control, and anti-vimentin-IgM-Ab. **C**. IgG2a-isotype control, and anti-α-SMA-IgG2a antibody. **D**. IgG1 isotype control, and anti-desmin- α-SMA-IgG2a antibody. Cells stained positive for α-SMA, and vimentin, weakly stained for desmin, and negative for cytokeratin. The scale bar at the bottom right of figure represents 50 μm length. Images are representative of 3 independent experiments.

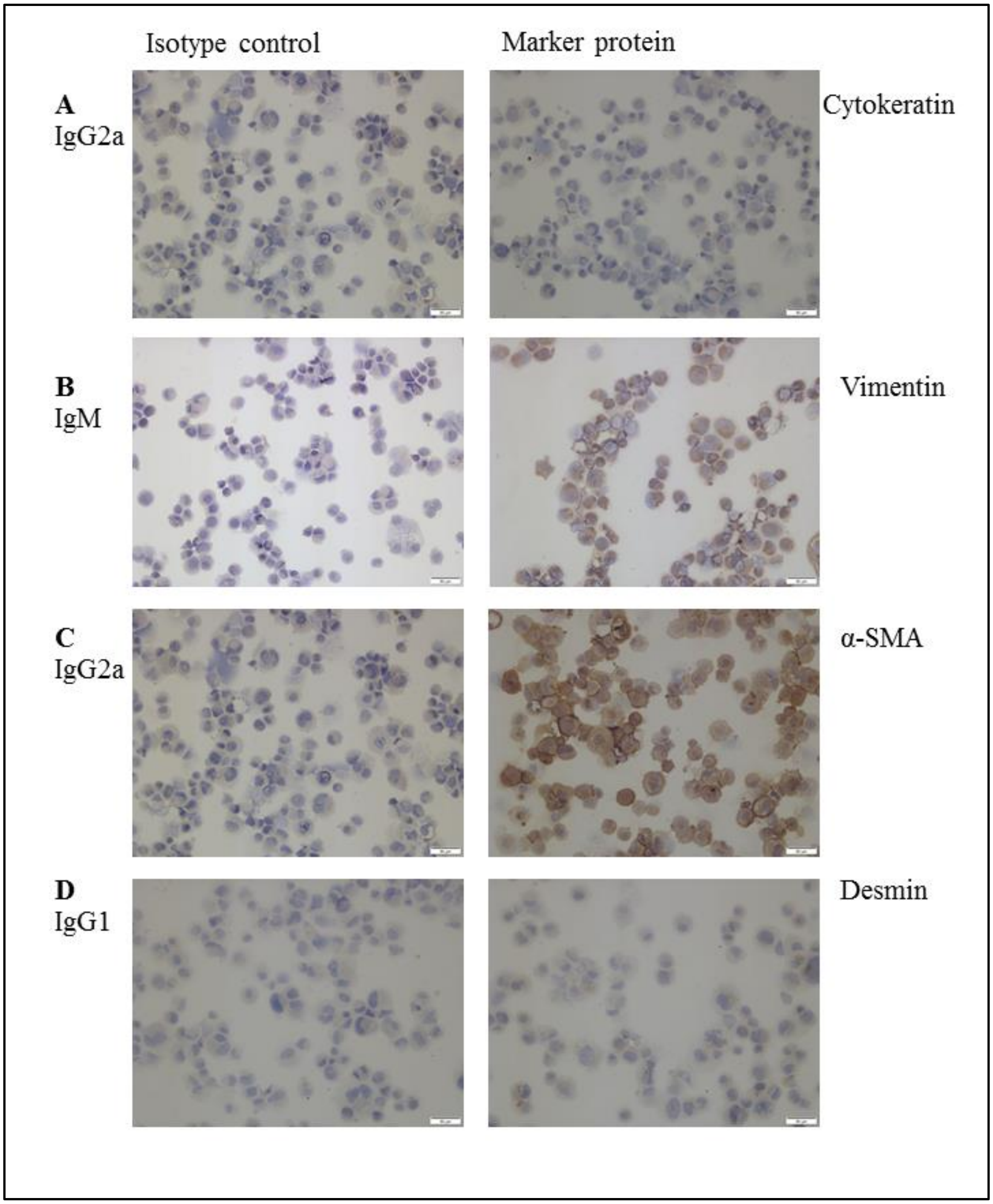
Isotype control
Marker protein
A
IgG2a
Cytokeratin
B
IgM
Vimentin
C
IgG2a
α-SMA
D
IgG1
Desmin

Fig 6: Immunocytochemistry of SV40 immortalized ISEMF cells. Images on right are specific isotype controls, and images on left are for cell specific markers. Brown pigmented cells indicate positive staining **A**. IgG2a isotype control, and anti-cytokeratin-IgG2a Ab staining. **B**. IgM isotype control, and anti-vimentin-IgM-Ab. **C**. IgG2a-isotype control, and anti-α-SMA-IgG2a antibody. **D**. IgG1 isotype control, and anti-desmin- α-SMA-IgG2a antibody. Cells stained positive for α-SMA, and vimentin, weakly stained for desmin, and negative for cytokeratin. The scale bar at the bottom right represents 50 µm length. Images are representative of 3 independent experiments.

### 3.3.2 Generation of SV40 immortalized ISEMF cells and confirmation of immortalization

After transfection of primary ISEMF cells with pSV3-neo plasmid, antibiotic selection with G418 resulted in SV40 immortalized ISEMF cells. To confirm the presence of the SV40 large T antigen gene, conventional PCR was performed, and product was resolved in 2 % agarose gel. Gel imaging confirmed the presence of the gene of interest in SV40 immortalized ISEMF cells (Fig 7), with pSV3-neo plasmid and SV40 immortalized ISEMF showing same amplified product size (751 bp) when amplified for SV40 large T antigen gene. Phase contrast microscopy showed SV40 immortalized ISEMFs cell resembled primary myofibroblasts in culture (Fig 4B).

An indirect immunofluorescence assay confirmed the presence of SV40 large T antigen protein in SV40 immortalized cells (Fig 8L). Isotype control and primary cells did not show fluorescence when stained with Alexa fluor-488 conjugated anti-SV40 large T antigen-IgG2a antibody (Fig 8C, 8F, and 8I).

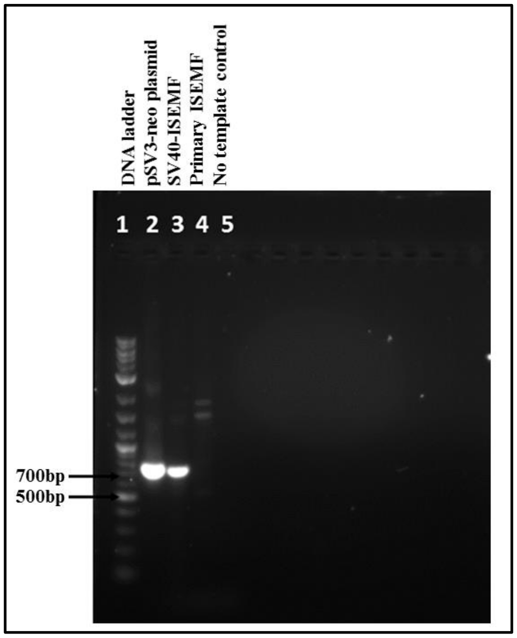


Fig 7: Gel image showing the presence of SV40 large T antigen gene in SV40 immortalized ISEMF cells. Lane 1 shows 2 log DNA ladder. Lane 2 shows pSV3-neo plasmid. Lane 3 shows SV40 large T antigen gene in SV40 immortalized ISEMF cells. Lane 4 shows absence of SV40 gene in primary ISEMF cells. Lane 5 shows no template control. The size of the amplified product is 751bp.

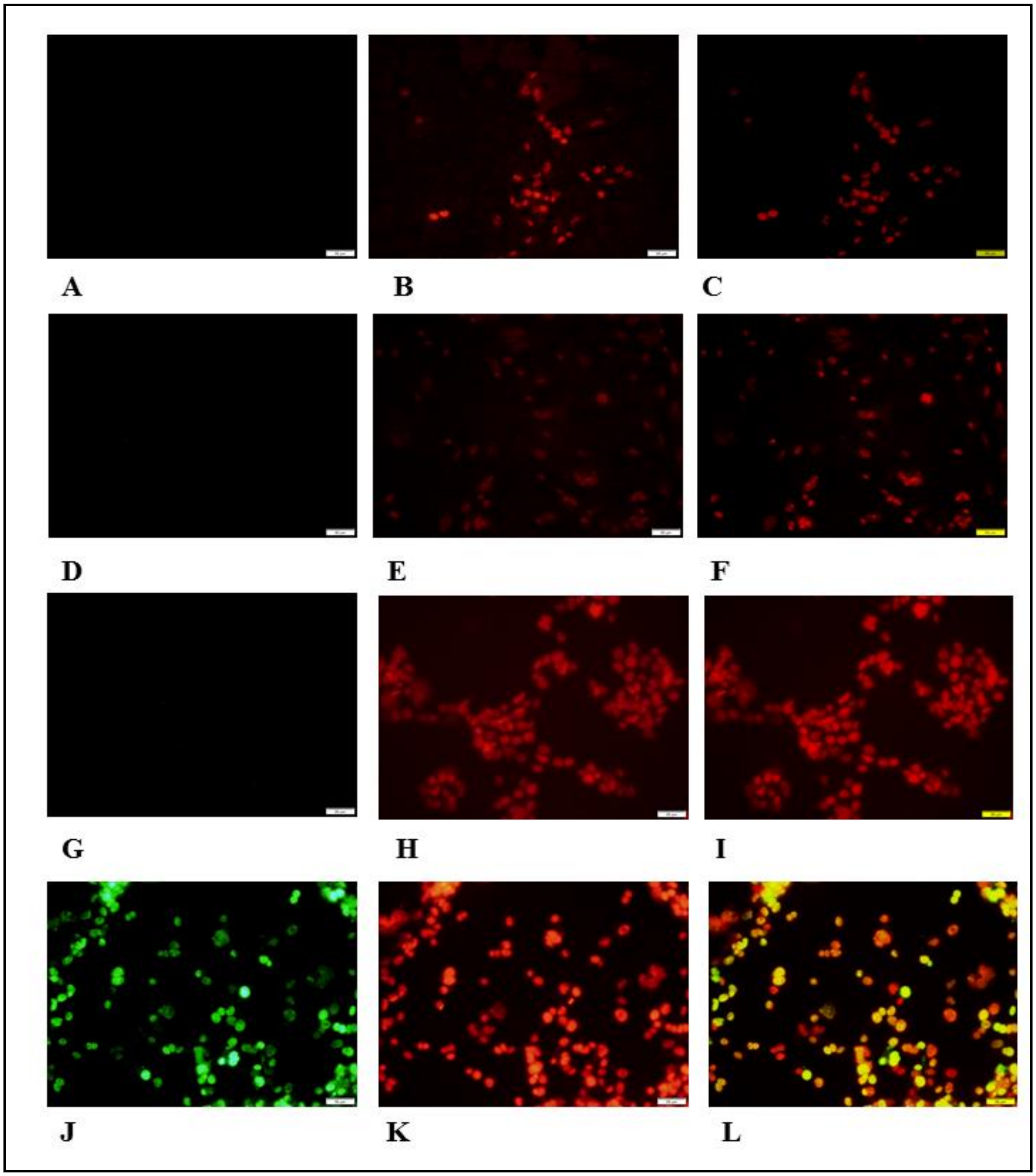

Fig 8: Immunofluorescence assay to confirm the presence of SV40 large T antigen protein in SV40 immortalized ISEMF cells. **A.** Primary ISEMF cells with isotype control. **B.** Primary ISEMF cells nuclear staining **C**. Composite image of A and B. **D.** Primary ISEMF cells with SV40 specific mAb and Alexa 488 conjugated secondary antibody. **E.** Primary ISEMF cells nuclear staining. **F.** Composite image of D and E. **G.** SV40 immortalized ISEMF cells isotype control. **H.** SV40 immortalized ISEMF cell nuclear staining **I.** Composite image of G and H. **J.** SV40 immortalized ISEMF cells with SV40 specific mAb and Alexa 488 conjugated secondary antibody. **K.** Primary ISEMF cells nuclear staining. **L.** Composite image of J and K. Images are representative of three experiments. Scale bar at bottom right represents 50 µm.

### 3.3.3 Lectin Binding assay

Out of 23 different lectins DBA, SJA, VVA, GSL-II, PSA, and UEA-I did not stain, or extremely low percentage of cells stained for these lectins in both cell types (primary and immortalized cells). Lectins RCA-120, LEL, GSL-I, STL, PHA-E, and PHA-L showed higher cell percentage of stained cells (>80%) in both cell types (Fig 9, and Fig 10). SV40 immortalization significantly decreased the percentage of cells staining for GSL-I ( 92.7± 1.17 for primary ISEMFs to 83.29± 2.07) for SV40 immortalized ISEMFs), LEL (91.44±5.4 for primary ISEMFs to 73.7± 6.19 for SV40 immortalized ISEMFs) and PHA-L (89.54±0.85 for primary ISEMFs to 72.02±5.82 for SV40 immortalized ISEMFs), Jacalin (62.18±11.72 for primary ISEMFs to 17.3±4.55 for SV40 immortalized ISEMFs), ECL (56.32±7.05 for primary ISEMFs to 18.09±5.4 for SV40 immortalized ISEMFs), Con-A (38.94±8.4 for primary ISEMFs to 4.65±2.4 for SV40 immortalized ISEMFs), and LCA (73.7±10.91 for primary ISEMFs to 24.34±8.2 for SV40 immortalized ISEMFs) (Figure 11). Most of the inhibitors reduced the percentage of cell stained for specific lectins in both primary and SV40 immortalized cells (Figure 9 and 10). For SNA, the inhibitor reduced the percentage of stained cells, but it was not a complete inhibition.

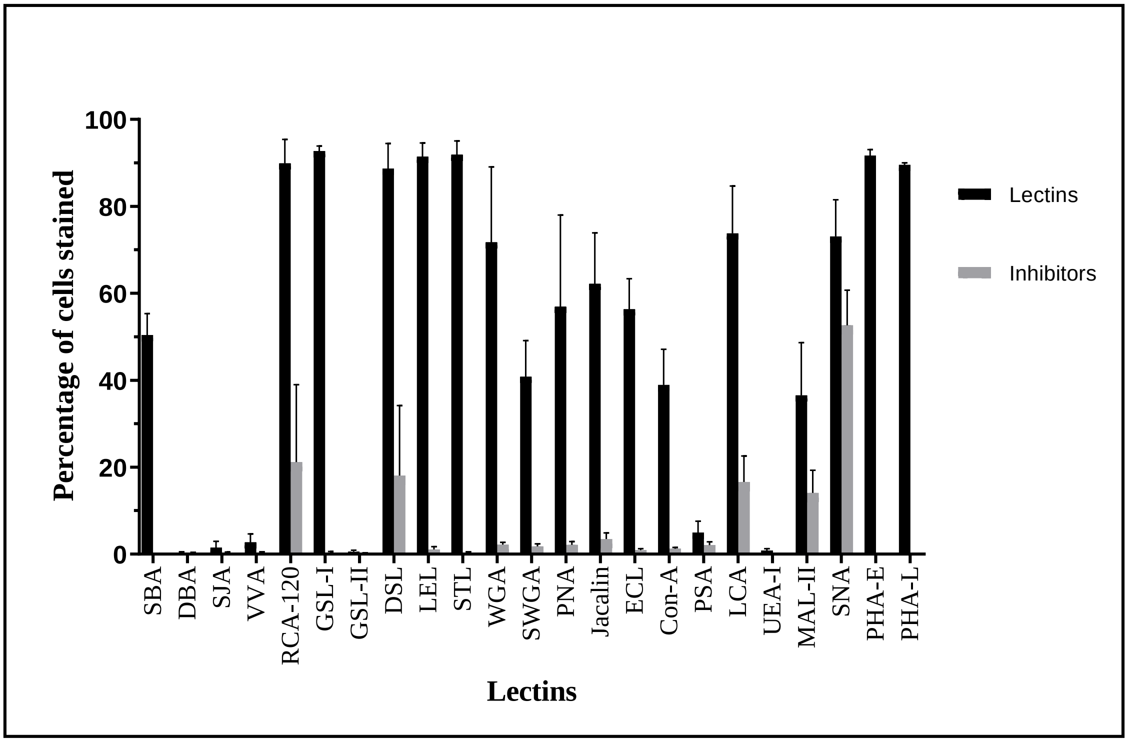


Fig 9: Lectin binding profile for primary ISEMF cells. Black bars show the percentage of cells staining for specific lectins and grey bars show inhibition of staining by specific inhibitors. Results are mean of three different experiments with error bars representing standard error of the mean (SEM).

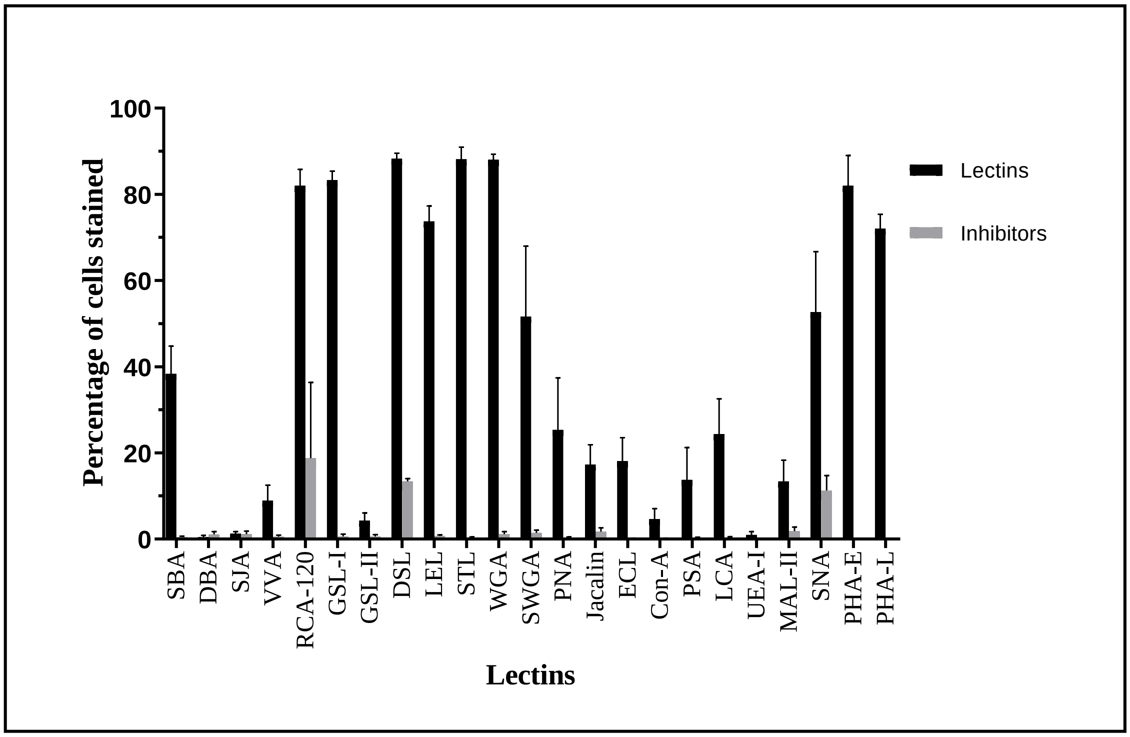


Fig 10: Lectin binding profile for SV40 immortalized ISEMF cells. Black bars show the percentage of cells stained for specific lectins and grey bars show inhibition of staining by specific inhibitors. Results are mean of three different experiments with error bars representing standard error of the mean (SEM).

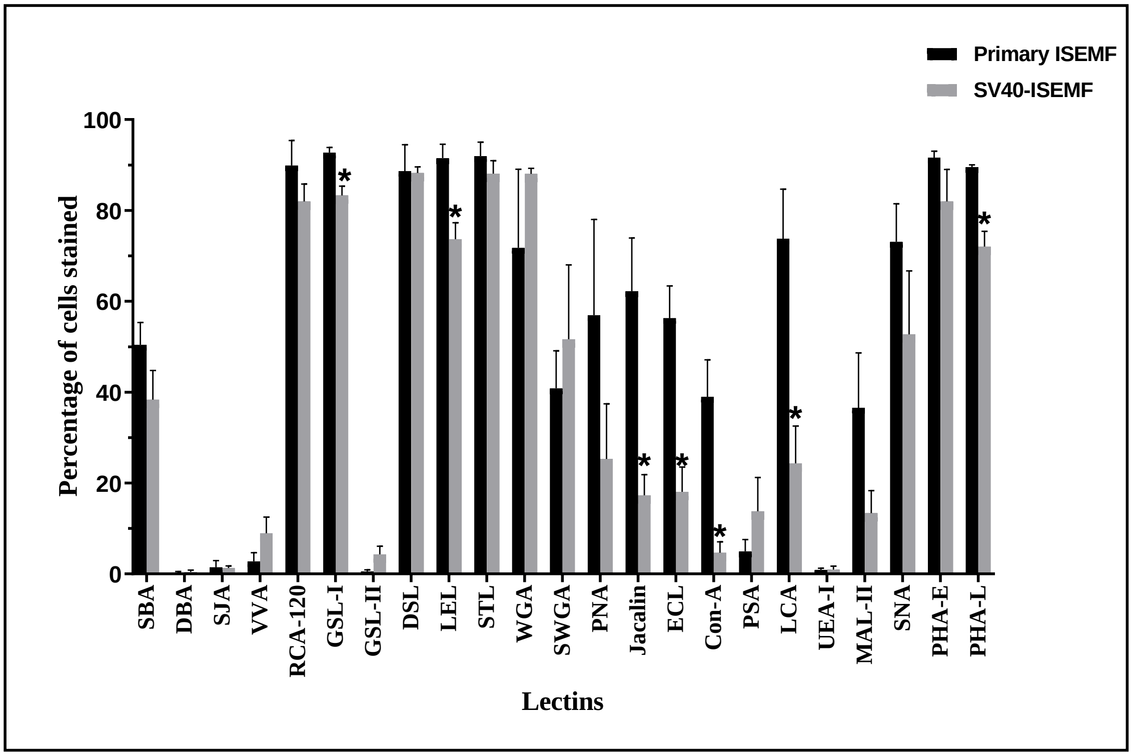


Fig 11: Comparison between primary and SV40 immortalized ISEMF for the percentage of cells stained for a given lectin. Significant differences in the percentage of cells stained for given lectin after immortalization are denoted by an asterisk (*). Results are mean of three different experiments with error bars representing standard error of the mean (SEM).

### 3.3.4 Analysis of TLR expression in primary ISEMF and SV40 immortalized ISEMF cells by RT-qPCR

Normalized Ct values (ΔCt) for primary and SV40 immortalized ISEMFs were analyzed for any differences using Mann-Whitey U test. The test showed no significant differences in TLR expression after immortalization (Figure 12). The Ct values for housekeeping gene Cyclophillin-A ranged from 19.15-20.18 for primary ISEMF cells, and 22.09-22.39 for SV40 immortalized ISEMF cells. All 9 TLRs (TLR 1-9) were expressed by both primary and SV 40 immortalized ISEMF. TLR 2, 3, 4 had a relatively stronger expression (Figure 12) than other TLRs in both cell types.

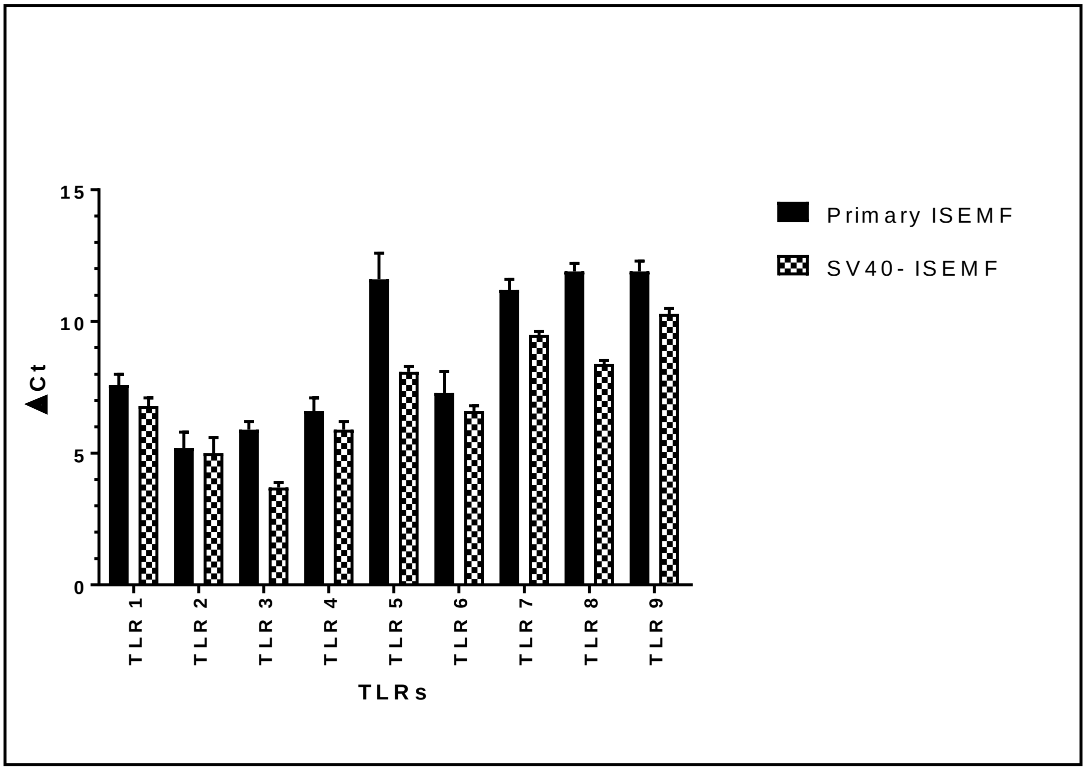


Fig 12: TLR 1-9 expression in primary and SV40 immortalized ISEMF cells represented by normalized Ct values (normalized against the Ct values of housekeeping gene Cyclophillin-A). A lower ΔCt indicates stronger gene expression. Mann-Whitney U test was applied to analyze the difference in ΔCt between primary and SV40 immortalized ISEMF. P-value <0.05 was considered significant. The data are representative of three independent experiments. Error bar represents standard error of the mean (SEM).

### 3.4 Discussion

ISEMF cells have diverse functions in wound healing, regulation of intestinal epithelial barrier function, maturation and differentiation of intestinal epithelium, orchestrating innate immune responses against invading pathogens, as well as role in the progression of tumors, and inflammatory bowel disease (119, 121, 123, 128, 140-144). Despite being cells with diverse functions, there is a paucity of knowledge about their origin (128) and function in fibrosis, inflammation, and repair of the intestinal mucosa (134, 145) primarily due to the absence of stable cell lines. Here, in this study, we successfully established and characterized a primary and SV40 immortalized ileal ISEMF cell lines from a 2-day old bovine male calf.

After isolation of intestinal sub-epithelial cells, they were cultured in fibroblast supporting DMEM-10 media (134). These cells showed spindle to stellate morphology on culture. On immunocytochemistry, these cells stained positively for cell specific markers like α-SMA and vimentin but showed no staining for cytokeratin and weak staining for desmin. There is a consensus understanding regarding the presence of α-SMA, vimentin and absence of cytokeratin in intestinal myofibroblast cells (109, 110, 112, 127, 141). The presence of myofibroblast specific markers along with polygonal morphology confirmed the cells cultured in this study as ileal ISEMFs.

Intestinal myofibroblasts like other diploid cells undergo replicative senescence due to shortening of telomerase (146) and thus can be cultured only for a finite number of passages known as Hayflick's limit (147). On reaching Hayflick's limit they undergo senescence and stop dividing. Previously established bovine colonic myofibroblastsmaintainedtheir phenotype and proliferative capacity until passage 11

(130). However, in this study we were able to maintain primary ileal cells up to 27 passages without losing proliferation and phenotypic characteristics. A possible explanation for this variation may be that unlike ileal myofibroblasts, colonic myofibroblasts need continuous stimulation with TGF-β to express myofibroblast marker α-SMA (148). The difference in age of animal (2-day old calf vs adult), site of cell isolation (ileum vs colon) and method of isolation could have resulted in this difference although further experiments need to be carried out to support these arguments. In two different studies, rat colonic myofibroblasts were successfully cultured up to 20 passages by Kawasaki et al (134) and only up to 9 passages by Pourreyron et al (149). One possible hypothesis for this discrepancy is that some cells can escape senescence spontaneously and acquire high proliferative capacity (134, 149).

To immortalize primary ileal ISEMF cells, we transfected them with pSV3-neo plasmid containing (Simian Virus) SV40 large T antigen gene. Large T antigen produces tumor protein that binds with transformation related protein 53 (TRP53), Rb (retinoblastoma ) proteins pRb, p107 and p130 resulting in suppression of cell cycle arrest, and apoptosis (150). T antigen protein also binds to hsc70 causing the cell to enter S-phase of the cell cycle (151, 152). In this study, we confirmed the presence of SV40 large T antigen gene in primary ileal ISEMFs by conventional PCR and confirmed the synthesis of SV40 large T antigen protein in cells by indirect immunofluorescence assay. SV40 immortalized ISEMFs retained myofibroblast morphology in culture and stained positive for α-SMA, vimentin, and negative for cytokeratin. Immortalization of primary cells by SV40 large T antigen has led to changes in expression of genes associated with immune function, antigen presentation, and metabolism in other transformed cells (153).

Therefore, we compared primary ISEMF cells and SV40 immortalized ISEMF in terms of changes in glycobiology and pattern recognition receptors (PRRs).

We first compared primary and immortalized cells for the differences in their lectin binding profile. Lectins are proteins that bind to carbohydrates, glycolipids or glycoproteins in a reversible and non-catalytic manner (154, 155). Based on the type of glycans lectins bind, lectins can be classified into various groups such as N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), glucose, mannose, galactose, fucose, sialic-acid specific lectins (156). In immune system two main lectin groups are important; sialic-acid binding immunoglobulin like lectins (siglec) and C-type (calcium dependent) lectins. Among many functions, siglecs are associated with leukocyte adhesion and leukocyte homing. C-type lectins are involved in pathogen recognition (157). C-type lectin receptors in cells have crucial functions in infection regulation, autoimmunity, and cellular homeostasis (158). Pathogenic bacteria like *Burkholderia* can use fucose-binding lectins in host recognition (159). Protozoan like *Entamoeba histolytica* uses Gal-lectin for adherence to host cell glycans, Gal or GalNac (160, 161). Similarly, mannose-binding lectins activate complement pathways, enhance the immune response in concert with TLR 2/6, and bind to glycans on various pathogens (162, 163). Low levels of serum mannose-binding lectins predispose to the risk of *Cryptosporidum* infections in children (164). Lectins are also used as a cancer biomarkers for certain cancers (165). Lectin binding is routinely used to study specific soluble and cellular glycans and glycoconjugates expressed by specific cell types (166). Since all eukaryotic cells have glycans in the form of glycolipids, and glycoproteins, there is emerging evidence of their roles in cell signaling and cell adhesions.

Out of 23 different lectins used in this study, RCA-120, LEL, GSL-I, STL, PHA-E, and PHA-L showed higher cell percentage of stained cells (>80%) in both cell types. Both primary and SV40 immortalized ileal ISEMF cells also stained fairly well for SNA, MAL-II, SBA, Con-A, PNA, ECL, Jacalin; however, they showed no staining to very faint staining for lectins UEA-I, SJA, VVA, GSL-II, DBA, PSA. SV40 immortalization decreased the percentage of stained cells for lectins LEL, PHA-L, GSL-I, Jacalin, ECL, Con-A, LCA, and PHA-L significantly. SV40 large T antigen mediated transformation of primary cells into immortalized cells is widely used and accepted method but it's associated effects on cell physiology have not been widely studied (167). The immortalized cells can have altered phenotypes than primary cells due to changes in karyotype or due to loss of genes associated with phenotype (168, 169). There have been few studies on changes in glycosylation before and after immortalization of cells and they have shown altered glycosylation in transformed cell lines (170). There is increasing evidence that surface glycans are linked with tumor progression primarily by altering the glycosylation process (171-177). This could possibly be an explanation for variation in altered glycans after immortalization. Studies on differences in surface glycan expression by normal and tumor transformed cell line has shown a decrease in PHA-L binding in tumor transformed cell line (178). Another possible reason could be the differences in passage number. In this study, SV40 immortalized cells were of late passage, whereas primary cells were of early passage. Studies on skin fibroblast cells have shown decrease sialylation in aged individuals and in late passage fibroblast cells (179, 180). Further study to identify a possible explanation for altered surface glycans in SV40 immortalized ISEMFs is required to elucidate underlying mechanisms. The first step in the pathogenesis of various bacterial and viral disease is interaction and binding of glycan-binding protein (lectins) on specific surface receptors (surface glycans) on host cell (181-184). Host cell uses these interactions to recognize pathogens and initiate an innate immune response (185, 186). Thus, identification of pathogen lectins and host glycan interactions could help in understanding disease pathogenesis (182). Expression of surface glycans from the sialic acid group by ISEMF cells represents a target for viral adhesions (187, 188). Various bacteria express adhesins. Some of the adhesins are expressed in the form of mannose-specific type-I fimbriae, and N-acetylglucosamine binding F-17 fimbriae (189). Expression of Mannose group glycans by ISEMF cells may indicate a possible target of adhesion with various bacteria. Thus, these cells can be a good model to study enteric bacterial and viral pathogenesis.

Historically, the role of stromal cells was thought to be limited to only a structural function, but a plethora of evidence has emerged that shows vital role of these cells in innate immune responses (190). ISEMF cells in mouse, rat, and human models have shown their role in innate immunity (112, 125, 126, 191). To identify the expression of toll-like receptors (TLRs) by primary ISEMF cells, RT-qPCR based mRNA expression assay was used. Primary ISEMFs expressed TLRs 1-9 illustrating their role in innate immunity in the intestine. To compare changes in TLR gene expression, if any, upon immortalization with SV40 large T antigen, again RT-qPCR assay was used to compare normalized Ct values ($\Delta$Ct ) for TLRs 1-9 of primary and SV40 immortalized ISEMFs. Statistical analysis showed no significant differences in gene expression between the two cell types.

TLR is one of the groups in PRR family that recognizes pathogen associated molecular patterns (PAMPs). These PAMPs are highly conserved across pathogens. Recognition of PAMPs by TLRs results in production of pro-inflammatory and anti-inflammatory cytokines by activation of MAPK/p38/JNK pathway, TAK1/NF-kB activation, and IRF3 or IRF7 pathways (17, 23, 43, 192). Breach in epithelial layer by invading pathogens may lead to activation of TLRs present in ISEMFs and consequently initiation of signaling pathways resulting in the release of cytokines and chemokines. Thus, ISEMFs are crucial in initiating an immune response against a pathogen that reaches sub-epithelial layers (193). In this study both primary and SV40 immortalized ISEMFs expressed various TLRs, another study needs to be carried out to evaluate the responses of ISEMFs to PRR ligands.

**3.5 Conclusion**

We were able to isolate, establish and characterize a bovine primary ileal ISEMF cell line. To delay replicative senescence, we immortalized this ISEMF cell line with SV40 large T antigen mediated transformation. Comparison of primary and SV40 transformed ileal ISEMFs showed morphology and cell markers specific for sub-epithelial myofibroblast cells. Study of cell surface glycan showed a decrease in lectin binding capacity for lectins GSL-I, PHA-L, LEL, Jacalin, ECL, Con-A, and LCA upon immortalization of ISEMFs. This decrease in lectin binding could be the result of SV40 immortalization of primary ISEMFs or due to use of late passage cells for SV40 immortalized ISEMF. We also analyzed expression of TLRs in both primary and SV40 immortalized ISEMFs. Both cell type expressed TLR 1-9 and showed no significant differences in TLR gene expression. So far to the author's knowledge, this is the first instance of development of stable primary and immortalized bovine ileal ISEMF cell lines from a young calf. These cell lines could be used to study a wide range of physiological functions of ISEMFs and their role in disease pathogenesis. We intend to use these cells in further studies to investigate their role in innate immunity and pathogenesis of enteric diseases.

# Chapter 4. Role of bovine intestinal sub-epithelial myofibroblasts in innate immune responses in the intestine

## Abstract

Intestinal sub-epithelial myofibroblasts (ISEMFs) support the growth and differentiation of intestinal epithelium. Further, their role as a generator of immune responses in the sub-epithelial intestinal compartment is emerging. We have isolated, developed and characterized a stable bovine ileal ISEMF cell line that expresses myofibroblast markers including α-smooth muscle actin, and vimentin. Assessed by real time-quantitative PCR (RT-qPCR) analysis, these cells expressed Toll like receptors (TLRs) 1-9. In this study, we investigated their responses to various pattern recognition receptors (PRRs) bacterial and viral ligands. Primary ileal ISEMF cells were stimulated with PRR ligands for 3 hours or 24 hours. The RT-qPCR assay was employed to analyze TLR and cytokine gene expression and quantified as fold expression changes. At 3 hours, lipopolysaccharide (LPS) downregulated TLR 1, 4, 7, and 9 expression while peptidoglycan (PGN) downregulated TLR 6 and 8. Similarly, flagellin (FLA) downregulated TLR 4, 5, 7, 8, and 9 at 3 hours. At 24-hours LPS down regulated TLR 4 and FLA downregulated TLR 6. At 3 hours, bacterial ligand γ-D-Glu-mDAP (iE-DAP) downregulated TLR 5 while muramyl dipeptide (MDP) and polyinosonic:polycytidylic acid (Poly I:C) downregulated TLR 1. Poly I:C complexed with lyovec (Poly I:C/lyovec) downregulated TLR 3 after 3-hours stimulation. We also analyzed cytokines expression by RT-qPCR after stimulation with various bacterial and viral ligands. Interleukin 6 (IL-6) was upregulated by LPS at 3 hours and 24 hours but downregulated by PGN at 24 hours. At 24-hours IL-1α was upregulated by PGN and Poly I:C/lyovec. TNF-α was downregulated by LPS at 24 hours while downregulated by FLA at 3 and 24 hours. Imiquimod upregulated TNF- α upon 24-hour stimulation. Anti-inflammatory cytokine IL-10 was downregulated by PGN upon 3-hour stimulation. As we observed changes in TLRs, pro-inflammatory and anti-inflammatory cytokine genes expression, we infered that bovine ISEMF cells responded to various bacterial and viral ligands. Thus, we conclude that bovine ileal ISEMF cells play a pivotal role in host defense against invading pathogens in the intestinal sub-epithelial compartment.

**Key-words**: intestinal sub-epithelial myofibroblasts, innate immunity, bovine, TLRs, cytokines

**4.1 Introduction**

Disruption of barrier function of intestinal epithelial cells exposes intestinal sub-epithelial compartment to numerous pathogens and leads to enteric inflammatory diseases (194). The intestinal sub-epithelial compartment is populated with mesenchymal cells and the interaction of surface epithelium and mesenchymal cells are crucial in the maintenance of barrier function (195). Intestinal sub-epithelial myofibroblasts (ISEMFs) are α-smooth muscle actin and vimentin positive mesenchymal cells (109-111) pivotal in regulating barrier function, in fibrosis and healing, in differentiation, restitution, and morphogenesis of epithelium (5, 6, 109, 110, 122, 124, 144). Mounting effective immune response against invading pathogen in the intestinal sub-epithelial compartment is essential and recent studies have shown that ISEMFs may be crucial in orchestrating innate immune responses against invading pathogens (123, 128, 190).

Toll like receptors (TLRs), a type of pattern recognition receptors (PRRs), are first to recognize and mount an effective innate immune response against invading pathogens (23, 196). Binding of pathogen associated ligands to TLRs induces MyD88 or TRIF dependent pathways leading to activation of NF-κB and MAPKs pathways and release of cytokine or chemokines (25). In murine and human models ISEMFs have been reported to express TLRs and respond to pathogen associated ligands by secreting cytokines and chemokines (19, 112, 126, 134, 193, 197). Pro-inflammatory cytokines are reported to be involved in cross-talk between intestinal epithelial cells and ISEMF cells (195).

Cytokines like IL-33 from intestinal sub-epithelial myofibroblasts stimulates intestinal stem cells and progenitor cells promoting differentiation of secretory intestinal epithelial cells (198). Thus, the study of the pattern of cytokine and chemokine production from ISEMF cells in response to pathogen associated TLR ligands can open new avenues to treat enteric diseases. Although ISEMFs express TLRs and NOD-like receptors (NLRs) their elucidated response to various pathogen associated molecular patterns (PAMPs) is still lacking (6, 126). ISEMF cells are suggested to enhance acquired mucosal immune response as they have emerged as non-professional antigen presenting cells (112, 125, 199). They are also reported to be involved in the induction of peripheral tolerance in intestinal mucosa primarily through programmed death ligand-1 (PD-L1) mediated suppression of $CD4^+$ T cell activity (113, 114). Thus, further studies to investigate the mechanism by which ISEMFs generate antagonistic responses is a must. This suggests emerging role of ISEMFs as focal immune cells in intestinal mucosal immunity.

We previously established and characterized stable bovine ISEMF cells that express α-SMA and vimentin. On RT-qPCR assay ISEMF cells expressed TLRs 1-9. In this study we investigated the response of ISEMF cells to various PRR ligands and subsequent changes in the expression of pro- and anti- inflammatory cytokines and chemokines.

## 4.2 Materials and Methods

### 4.2.1 Cell lines and culture conditions

Primary ISEMF cell line obtained from the ileum of the 2-day-old calf was used. Cells were grown in Dulbeco’s Modified Eagle Medium (DMEM)-GlutaMax™ (GIBCO) supplemented with 10 % fetal calf serum (FCS: Atlanta Biologicals, Lawrenceville, GA), pen-strep (100 IU/ml of penicillin and 100 ug/ml of streptomycin:

Invitrogen), and 1% of non-essential amino acids (HyClone 100X; GE Health Care Life Sciences). The supplemented media was named as myofibroblast media. In earlier study, on phase contrast microscopy, ISEMFs showed spindle shape typical of myofibroblasts morphology.  Immunocytochemistry showed the presence of α-SMA and vimentin confirming cells to be myofibroblasts. Moreover, the absence of cytokeratin indicated the absence of contamination with epithelial cells. Cells were grown in T75 flasks (75 $cm^2$, Corning) in a humid chamber ($37^0$C, 5% $CO_2$) until becoming confluent. Cells were detached and harvested using 0.05% Trypsin-EDTA (Corning; Manassas, VA). Half million cells were seeded into each well of six well tissue culture plate (Corning life sciences) for stimulating with PRR ligands. After 48 hours of incubation in a humid chamber, cells were washed three times with 1X phosphate buffered saline (PBS). Fresh media was added along with PRR ligands at a specific concentration in duplicates. A control was setup for each experiment. Each experiment was carried out in triplicates.

#### 4.2.2 PRR ligands for stimulation of ISEMF cells

ISEMF cells were stimulated with PRR ligands for 3- and 24-hours using end-time alignment method. Lipopolysaccharide (LPS: catalog number L6529-1mg) from *Escherichia coli* O55:B55 was used at 5 µg/ml concentration. Similarly, peptidoglycan (PGN: catalog number tlrl-pgnsa) from *Staphylococcus aureus* was used at 10 µg/ml and Flagellin (FLA: catalog number tlrl-stfla) from *Salmonella typhimurium* was used at 100 ng/ml. We also stimulated cells for 3-hours and 24-hours using ligands of cytosolic and endosomal PRRs. γ-D-Glu-mDAP (iE-DAP: catalog number tlrl-dap) was used at10 µg/ml, muramyl dipeptide (MDP: catalog number tlrl-mdp) at 10 µg/ml, polyinosonic:polycytidylic acid (Poly I:C: catalog number tlrl-pic) at 5 µg/ml, Poly I:C complexed with lyovec (Poly I:C/lyovec: catalog number tlrl-piclv) at1 µg/ml, and imiquimod ( catalog number tlrl-imq) at 5 µg/ml. All the ligands were bought from Invivogen, CA, USA. For stimulating cells with bacterial ligands, primary ISEMF cells from passage 15-20 were used. For stimulating cells with cytosolic and endosomal PRR ligands, cells from passage 18-24 were used.

**4.2.3 RNA extraction and cDNA preparation**

After 3 hours or 24 hours of incubation with ligands, cells were washed three times with 1X PBS. Cells were then trypsinized using 0.05% Trypsin-EDTA and centrifuged to form a pellet. RNA was extracted from pelleted cells using RNeasy Mini Kit (catalog number 74101, Qiagen, Valencia, CA, USA) and kit protocol. RNA was quantified using Thermo Scientific™ NanoDrop 2000. The RNA thus obtained was used to prepare cDNA. 1 µg of RNA was reverse transcribed to cDNA using TaqMan reverse transcription kit and kit protocol (TaqMan reverse transcription reagents, Applied Biosystems, catalogue number N8080234).

**4.2.4 Real time-quantitative PCR (RT-qPCR) for quantifying gene expression**

For RT-qPCR 2 µl of diluted cDNA (1:5 dilutions), 1 µl each of forward and reverse primers, 10 µl of RT² SYBR® Green/ROX qPCR mastermix (catalog number 330501, Qiagen, Valencia, CA, USA) and 6 µl of nuclease free water was added to bring total reaction volume to 20 µl. The thermal profile used for amplification was: 2 minutes at $50^0$C; 10 minutes at $95^0$C; followed by 40 cycles of 45 seconds (15 seconds for cytokine genes) at $95^0$C, 30 seconds at $60^0$C and 30 seconds at $72^0$C. Ramping speed was set at $1.6^0$C/second. QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, NJ, USA) was used. Data were normalized using the housekeeping gene (Cyclophillin-

A). Primer sequence previously used (138) for amplification of TLR 1-9 gene and Cyclophillin-A as housekeeping gene (139) are listed in Table 5. RT-qPCR was also used to identify any changes in pro-inflammatory and anti-inflammatory cytokines, after stimulation with PRR ligands. List of cytokine and chemokine primers (138, 200) is provided in Table 6.

Table 5: Primer sequence of genes along with gene bank accession number used in analysis of TLRs expression of ISEMF cells

| | **Forward primer** | **Reverse primer** | **Accession number** |
|---|---|---|---|
| TLR 1 | CAT TCC TAG CAG CTA CCA CAA GCT | TGG GCC ATT CCA AAT AAG TTC T | NM_001046504 |
| TLR 2 | GGG TGC TGT GTC ACC GTT TC | GCC ACG CCC ACA TCA TCT | NM_174197 |
| TLR 3 | GGG CAC CTG GAG GTC CTT | TTC CTG GCC TGT GAG TTC TTG | NM_001008664 |
| TLR 4 | AGC ACC TAT GAT GCC TTT GTC A | GTT CAT TCC GCA CCC AGT CT | NM_174198 |
| TLR 5 | GTC CCC AAC ACC ACC AAG AG | GCG GTT GTG ACT GTC CTG ATA TAG | NM_001040501 |
| TLR 6 | TTT ACC CTC AAC CAC GTG GAA | GGG CCA AAG GAA CTG AAA AAC | NM_001001159 |
| TLR 7 | CAC CAA CCT TAC CCT CAC CAT T | GTC CAG CCG GTG AAA GGA | NM_001033761 |
| TLR 8 | TGT GTT TAG AGG AAA GGG ATT GG | TCT GCA TGA GGT TGT CGA TGA | NM_001033937 |

| | | | |
|---|---|---|---|
| TLR 9 | CAG TGG CCA GGG TAG TTT CTG | CCG GTT ATA GAA GTG ACG GTT GT | NM_183081 |
| Cyclophilin-A | CTTTCACAGAATAATTC CGGGATT | CAGTACCATTATGGCGTG TGAAG | BC105173 |

Table 6: Primer sequence of genes along with gene bank accession number used in analysis of cytokine expression of ISEMF cells

| | **Forward primer** | **Reverse primer** | **Accession number** |
|---|---|---|---|
| IL-1α | CAG TTG CCC ATC CAA AGT TGT T | TGC CAT GTG CAC CAA TTT TT | NM_174092 |
| IL-1β | GAG CCT GTC ATC TTC GAA ACG | GCA CGG GTG CGT CAC A | NM_174093 |
| TNF-α | CGC ATT GCA GTC TCC TAC CA | GGG CTC TTG ATG GCA GAC A | NM_173966 |
| IL-6 | CCA CCC CAG GCA GAC TAC TTC | CCA TGC GCT TAA TGA GAG CTT | NM_173923 |
| IL-8 | TGC TCT CTT GGC AGC TTT CC | TCT TGA CAG AAC TGC AGC TTC AC | NM_173925 |
| IL-10 | AAGGTGAAGAGAGTCT TCAGTGAGC | TGCATCTTCGTTGTCATGT AGG | NM_174088 |

### 4.2.5 Statistical analysis for interpretation of RT-qPCR data

To compare the changes in TLR expression after ligand stimulation, double delta Ct (ΔΔCt) was calculated using the method previously described (201). Change in mRNA gene expression was calculated as 2-ΔΔCt. The method uses the following equation to calculate ΔΔCt:

ΔΔCt=ΔCt Treatment (Ct of reference gene Treatment-Ct Housekeeping gene Treatment)- ΔCt Control (Ct of reference gene Control-Ct Housekeeping gene Control).

A two tailed Student's t-test was then used to compare fold expression changes after treatment with ligands. A p-value of less than 0.05 ($p<0.05$) was considered significant. GraphPad prism 7.04 was used to prepare graphs. Data are expressed as a mean ± standard error of the mean.

**4.3 Results**

**4.3.1 Innate responses of ISEMF cells after 3-hours and 24-hours stimulation with bacterial ligands of surface PRRs**

LPS significantly downregulated TLR 1 (0.69±0.05, p=0.02), TLR 4 (0.52±0.08, p=0.03), TLR 7 (0.56±0.04, p=0.01), and TLR 9 (0.7±0.05, p=0.03) (Figure 13); however, PGN significantly downregulated TLR 6 (0.78±0.04, p=0.03), and TLR 8 (0.64±0.08, p=0.04) (Figure 14) gene expression at 3-hour time point. FLA downregulated TLR 4 (0.6±0.08, p=0.04), TLR 5 (0.51±0.06, p=0.01), TLR 7 (0.68±0.04, p=0.02), TLR 8 (0.55±0.06, p=0.02), and TLR 9 (0.6±0.01, p=0) (Figure 15) gene expression at 3-hour time point. Upon analysis of cytokine genes expressions, we found that LPS significantly upregulated IL-6 (7.63±0.89, p=0.02) (Figure 16), PGN downregulated TNF-α (0.56±0.05, p=0.01) and IL-10 (0.23±0.11, p=0.02) (Figure 17) while FLA only downregulated TNF-α (0.47±0.07, p=0.02) (Figure 18) gene expression at 3-hour time point.

After 24-hour stimulation, LPS downregulated TLR 4 (0.75±0.06, p=0.04) (Figure 13) and FLA downregulated TLR 6 (0.77±0.02, p=0.01) (Figure 15) genes expressions while PGN had no significant differences in TLR expression (Figure 14).

Upon analysis of cytokine genes expressions at 24-hour time point, we found that LPS significantly upregulated IL-6 (3.6±0.28, $p=0.01$) and downregulated TNF-α (0.53±0.05, $p=0.01$) (Figure 16) genes. After 24-hour stimulation, PGN significantly upregulated IL-1α (1.32±0.05, $p=0.02$) and downregulated IL-6 (0.94±0.01, $p=0.02$) (Figure 17) while FLA downregulated TNF-α (0.53±0.09, $p=0.04$) (Figure 18) genes expressions. Interestingly, no significant changes in the IL-8 gene expression were observed in response to LPS, PGN, and FLAs stimulation for 3 and 24 hours.

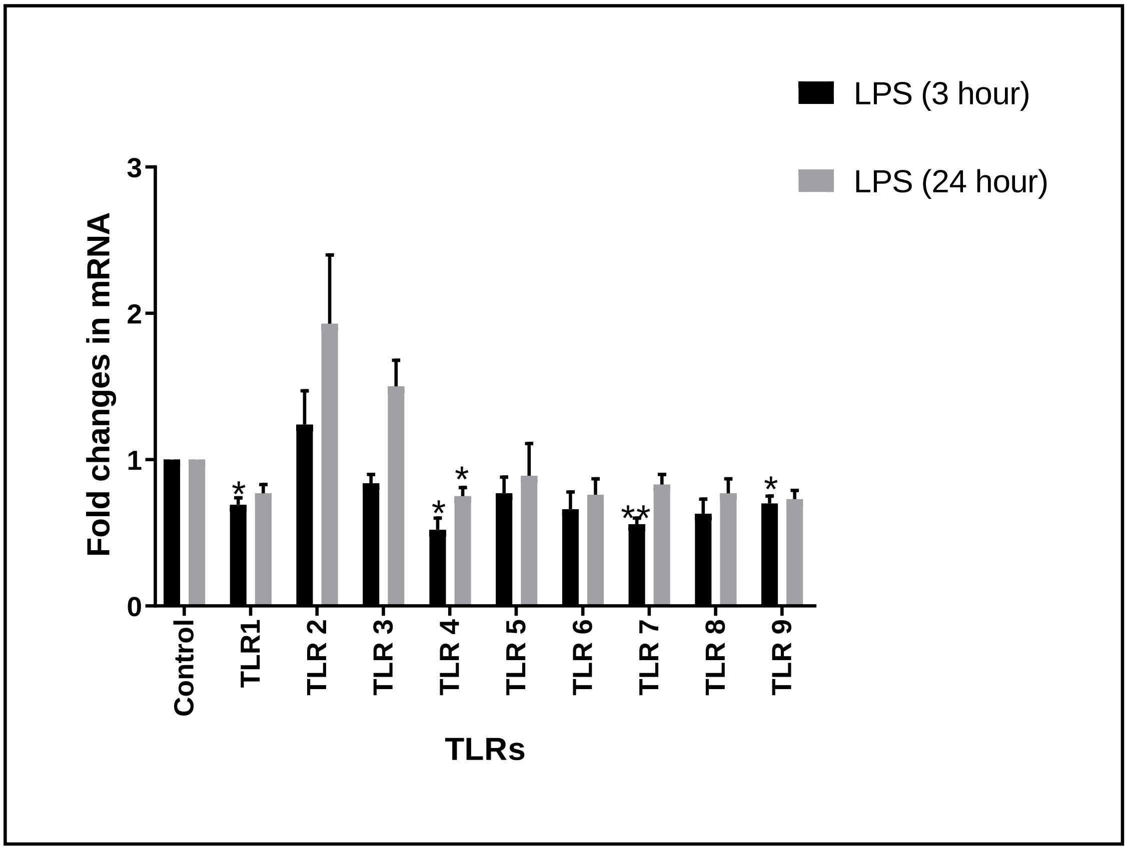


Fig 13: Fold changes in TLRs genes expressions in ISEMF cells upon stimulation with LPS. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after LPS treatment is denoted by an asterisk (*=p<0.05, **=p≤0.01).

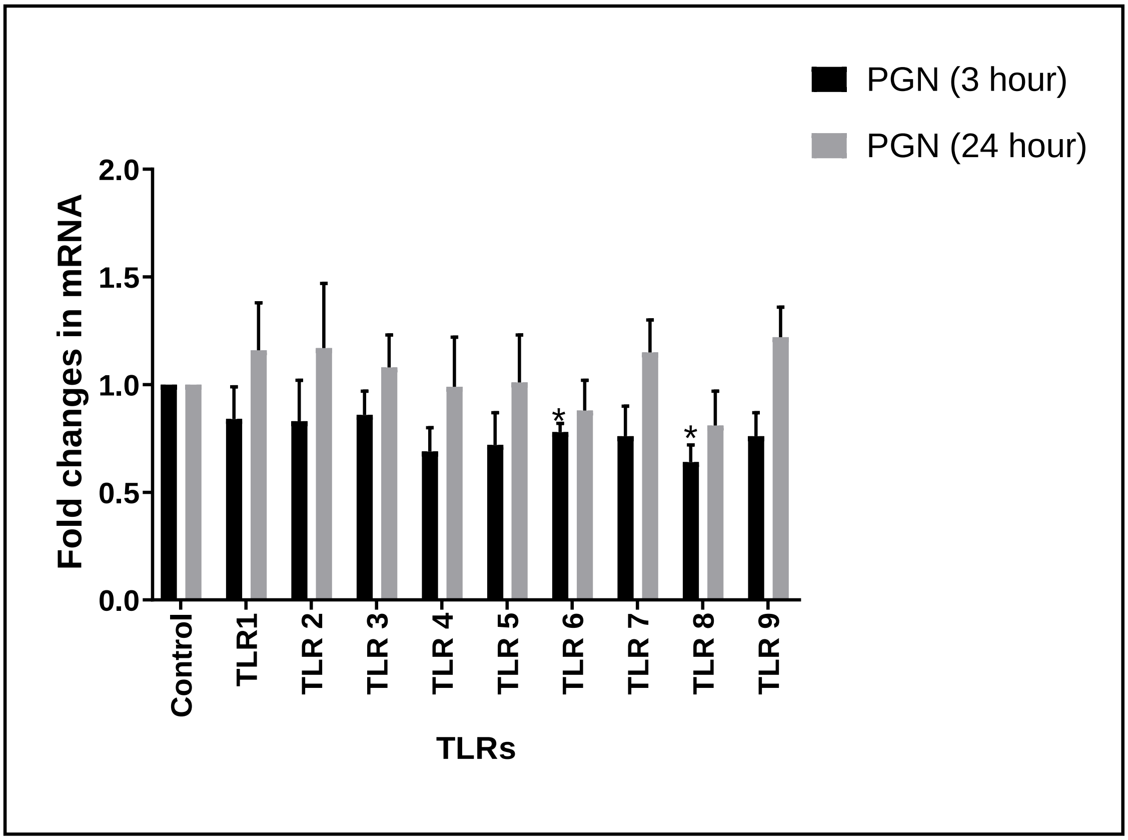


Fig 14: Fold changes in TLRs genes expressions in ISEMF cells upon stimulation with PGN. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after PGN treatment is denoted by an asterisk (*=$p<0.05$, **=$p\leq0.01$).

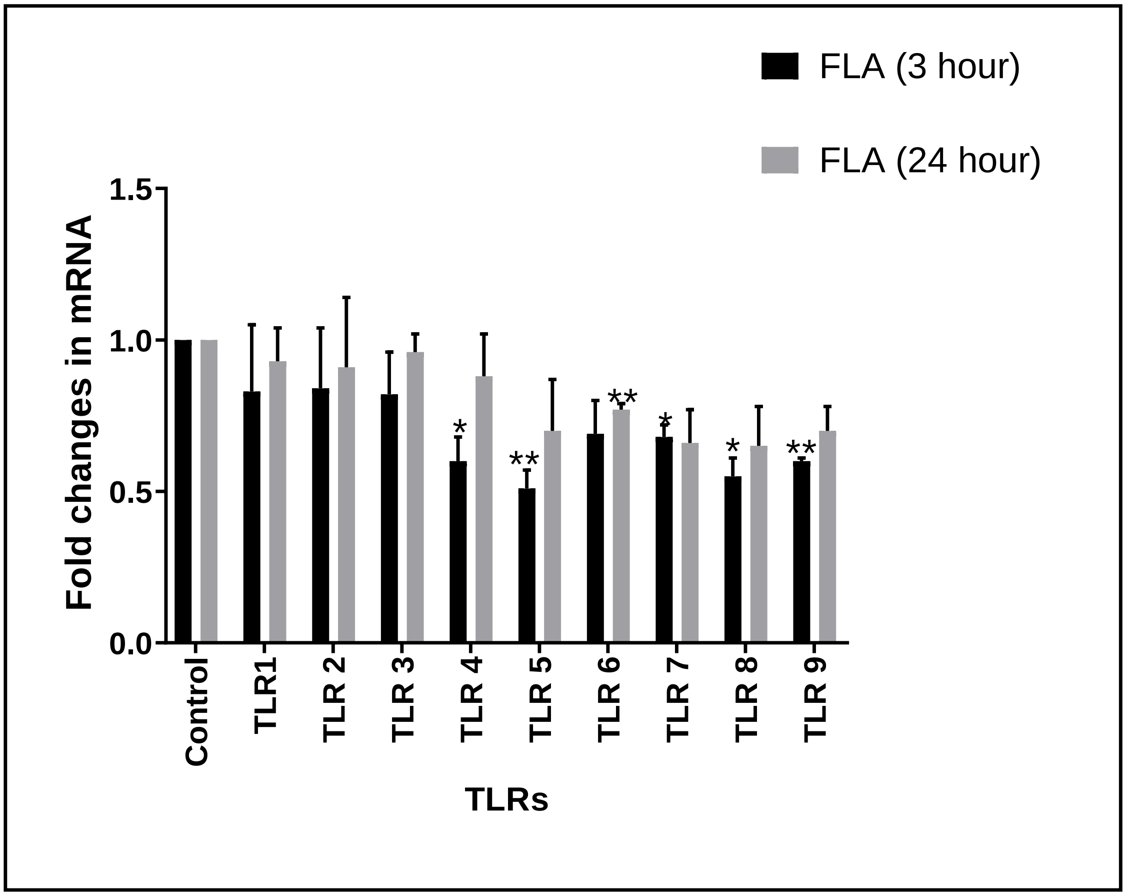


Fig 15: Fold changes in TLRs genes expressions in ISEMF cells upon stimulation with FLA. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after FLA treatment is denoted by an asterisk (*=p<0.05, **=p≤0.01).

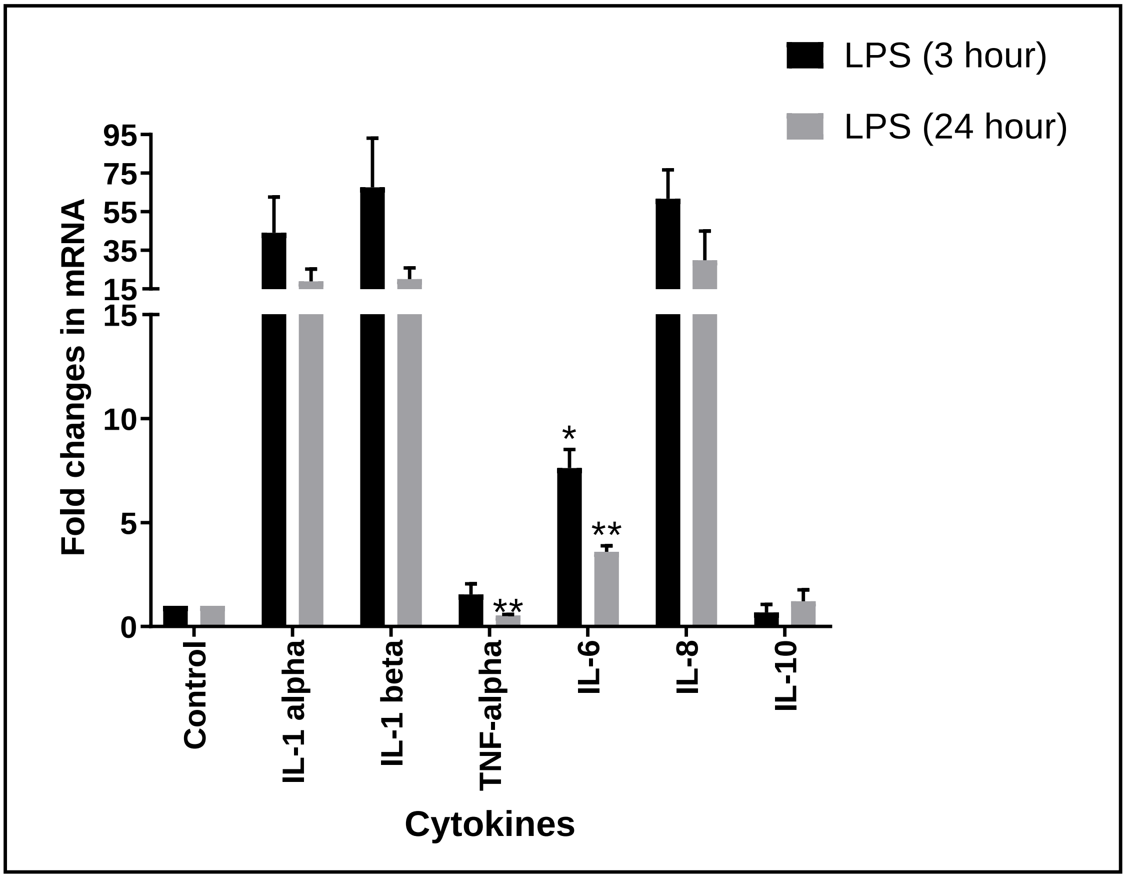


Fig 16: Fold changes in cytokines genes expressions in ISEMF cells upon stimulation with LPS. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after LPS treatment is denoted by an asterisk (*=$p<0.05$, **=$p\leq 0.01$).

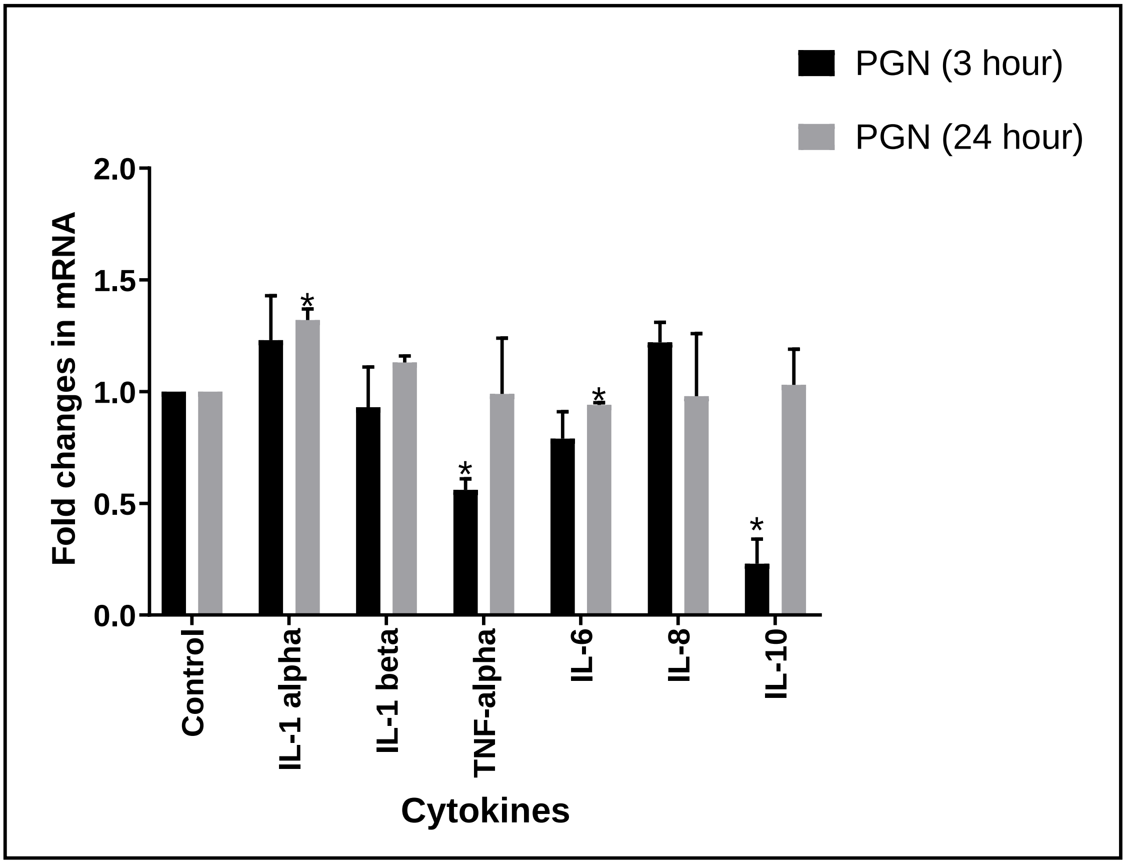


Fig 17: Fold changes in cytokines genes expressions in ISEMF cells upon stimulation with PGN. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after PGN treatment is denoted by an asterisk (*=p<0.05, **=p≤0.01).

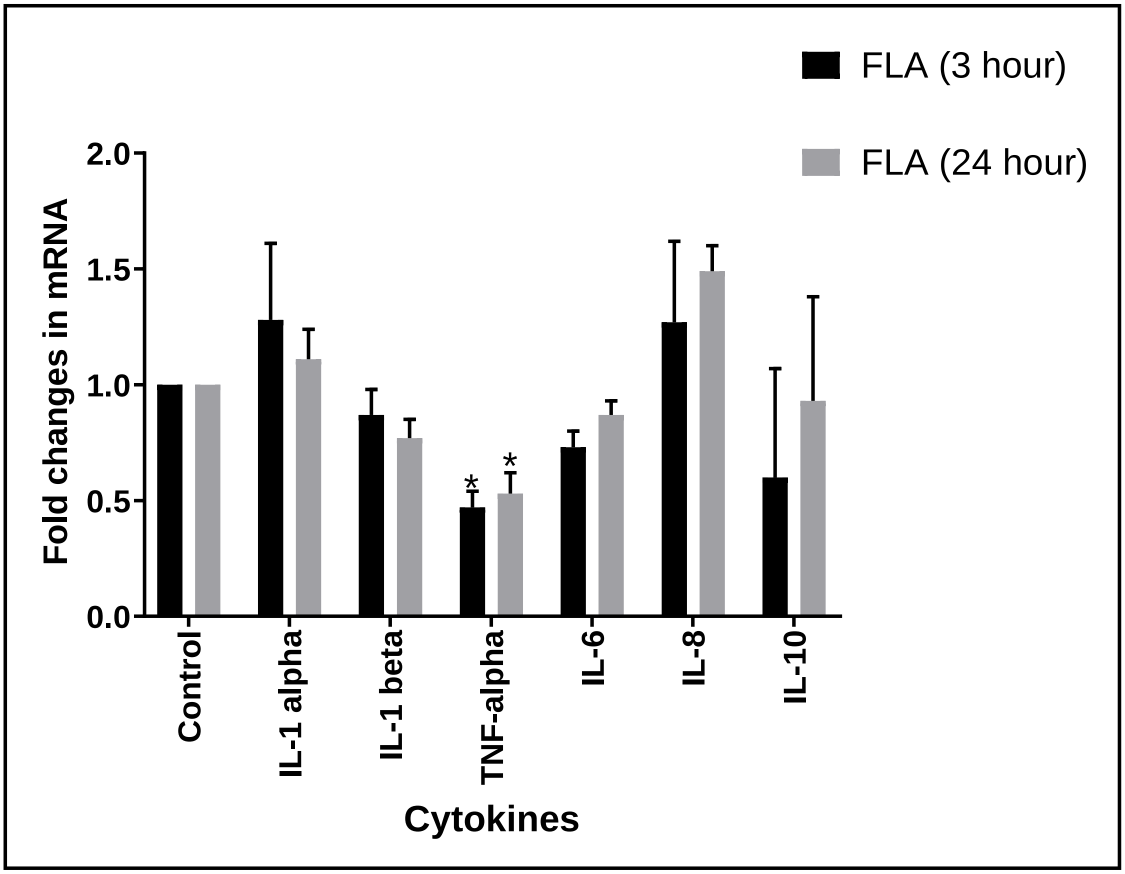


Fig 18: Fold changes in cytokines genes expressions in ISEMF cells on stimulation with FLA. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after FLA treatment is denoted by an asterisk (*=$p<0.05$, **=$p\leq0.01$).

**4.3.2 Innate responses of ISEMF cells after 3-hour and 24-hour stimulation with ligands of cytoplasmic and endosomal PRRs**

After 3-hour stimulation, iE-DAP, a ligand for NOD-1 significantly downregulated TLR 5 (0.76±0.04, p=0.02) (Figure 19), MDP, a ligand for NOD-2 downregulated TLR 1 (0.73±0.04, p=0.02) (Figure 20), Poly I:C, a ligand for TLR 3 downregulated TLR 1 (0.74±0.03, p=0.01) (Figure 21), and Poly I:C/lyovec, a ligand for RIG-I and MDA-5 downregulated TLR 3 (0.49±0.05, p=0.01) gene expression. Imiquimod, a ligand for TLR 7 and 8 did not alter expression of any of the 9 TLRs (Figure 23). None of the ligands for cytosolic and endosomal PRRs, after 3-hour stimulation, significantly altered the expression of cytokines investigated in this study (Figure 24-28). We could not detect expression of cytokine IL-10 after 3-hour stimulation with ligand MDP indicating downregulation of IL-10 gene expression (Figure 25).

Upon 24-hour stimulation, we observed no significant changes in TLR expression in response to any ligands of both cytosolic and endosomal PRRs (Figure 19-23). However, Poly I:C/lyovec significantly upregulated cytokine IL-1α (2.4±0.25, p=0.03) (Figure 27) and imiquimod significantly upregulated TNF-α cytokine gene expression (1.83±0.15, p=0.03) (Figure 28) after 24 hours of ligand stimulation. The expression of IL-10 gene remained undetected after both 3 hour and 24 hour stimulation indicating downregulation of IL-10 gene expression.

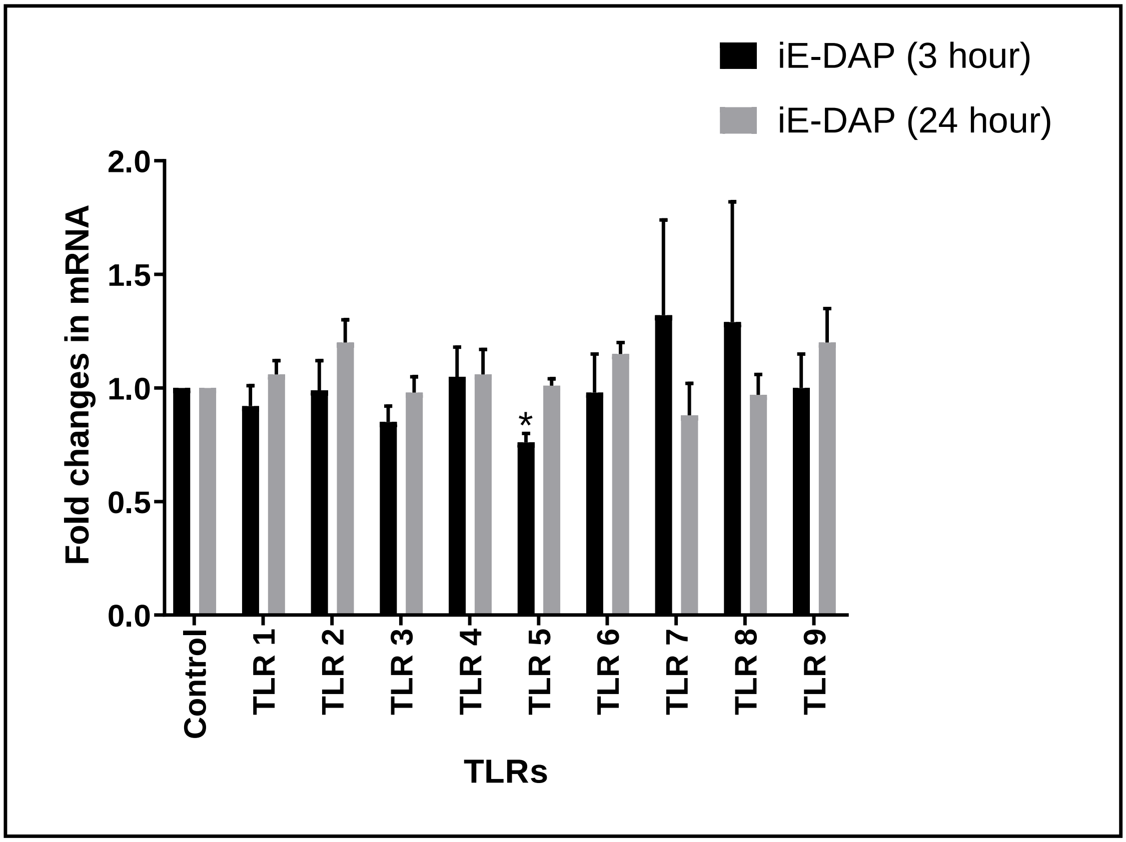


Fig 19: Fold changes in TLRs genes expressions in ISEMF cells upon stimulation with iE-DAP. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after iE-DAP treatment is denoted by an asterisk (*=p<0.05, **=p≤0.01).

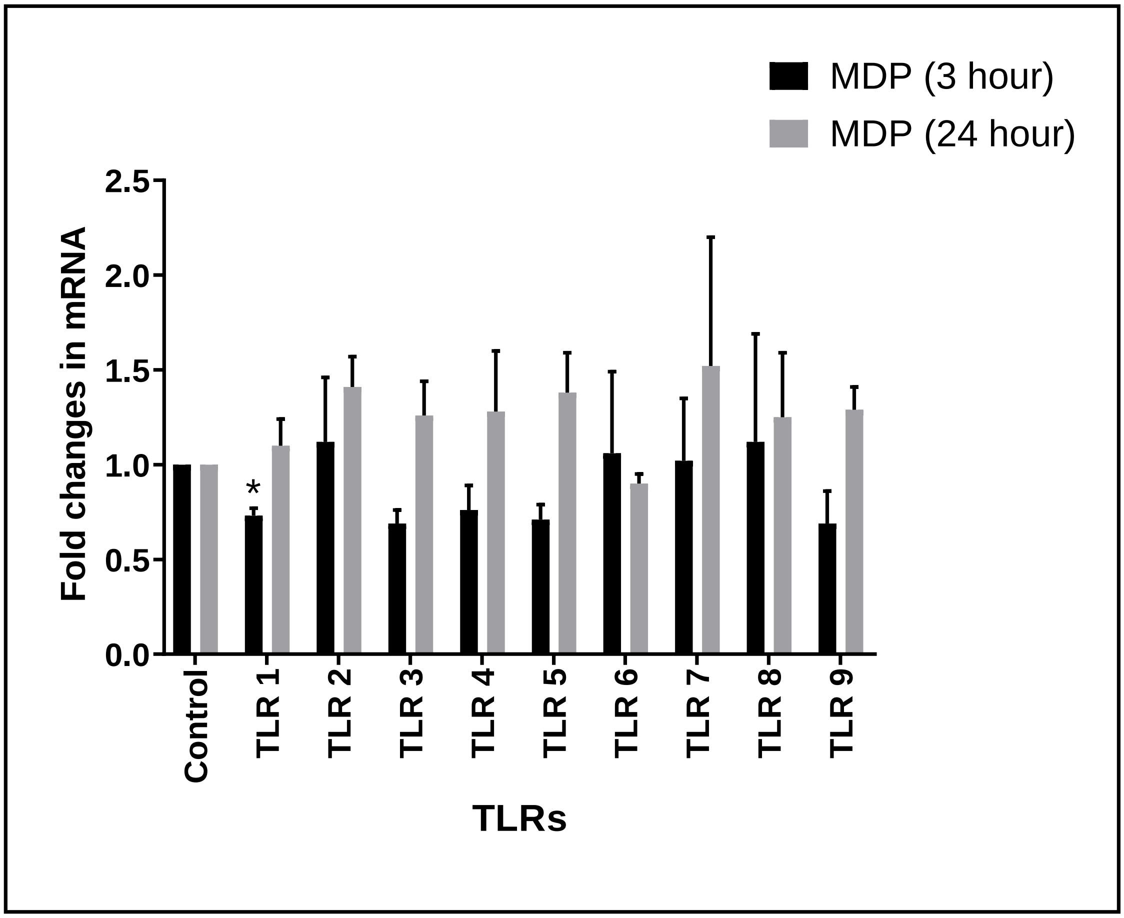


Fig 20: Fold changes in TLRs genes expressions in ISEMF cells upon stimulation with MDP. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after MDP treatment is denoted by an asterisk (*=p<0.05, **=p≤0.01).

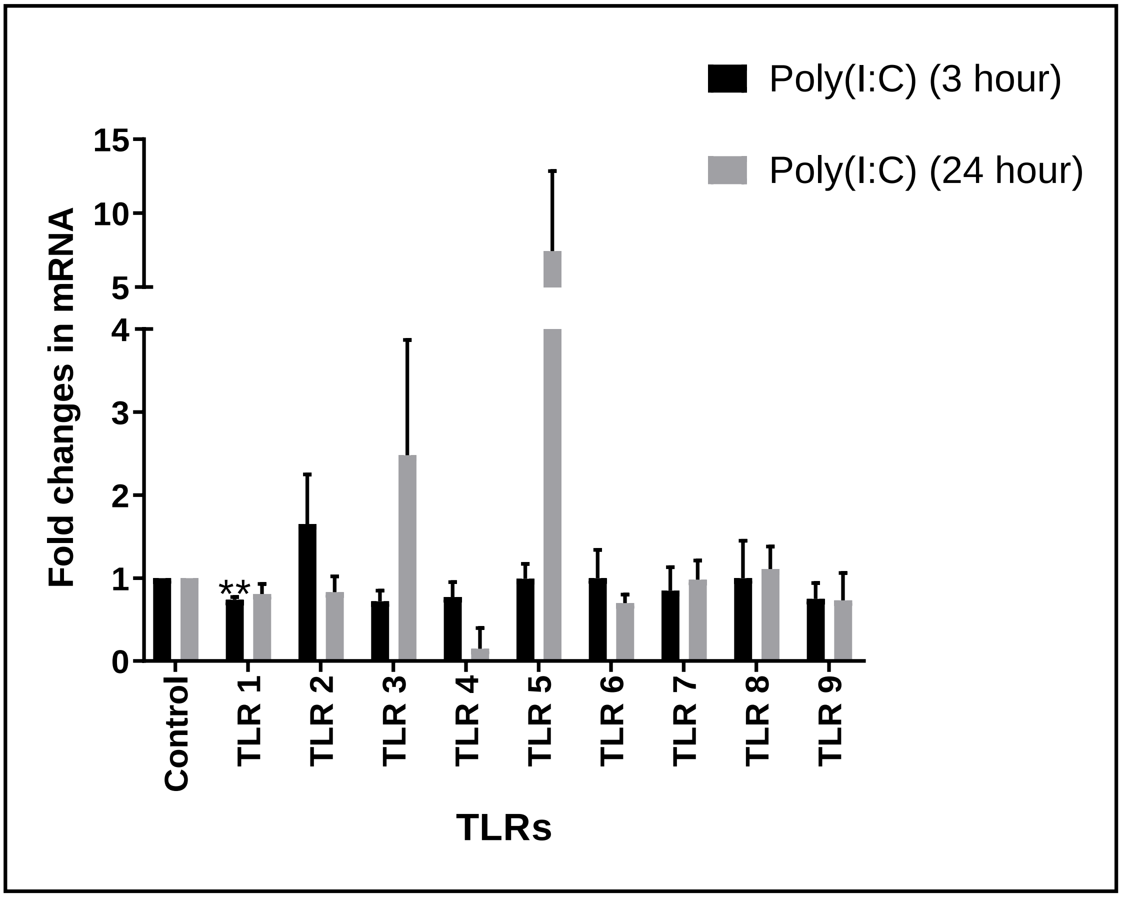


Fig 21: Fold changes in TLRs genes expressions in ISEMF cells upon stimulation with Poly (I:C). Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after Poly (I:C) treatment is denoted by an asterisk (*=p<0.05, **=p≤0.01).

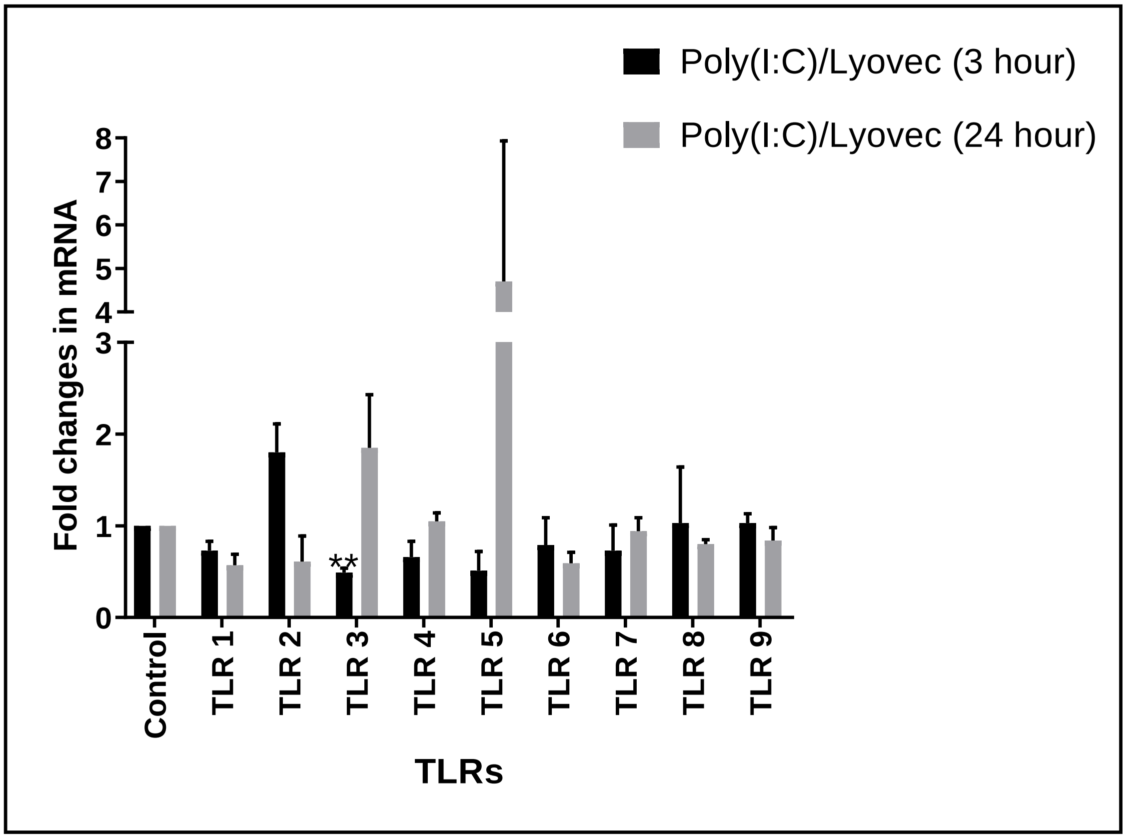


Fig 22: Fold changes in TLRs genes expressions in ISEMF cells upon stimulation with Poly (I:C)/Lyovec. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after Poly (I:C)/Lyovec treatment is denoted by an asterisk (*=$p<0.05$, **=$p\leq0.01$).

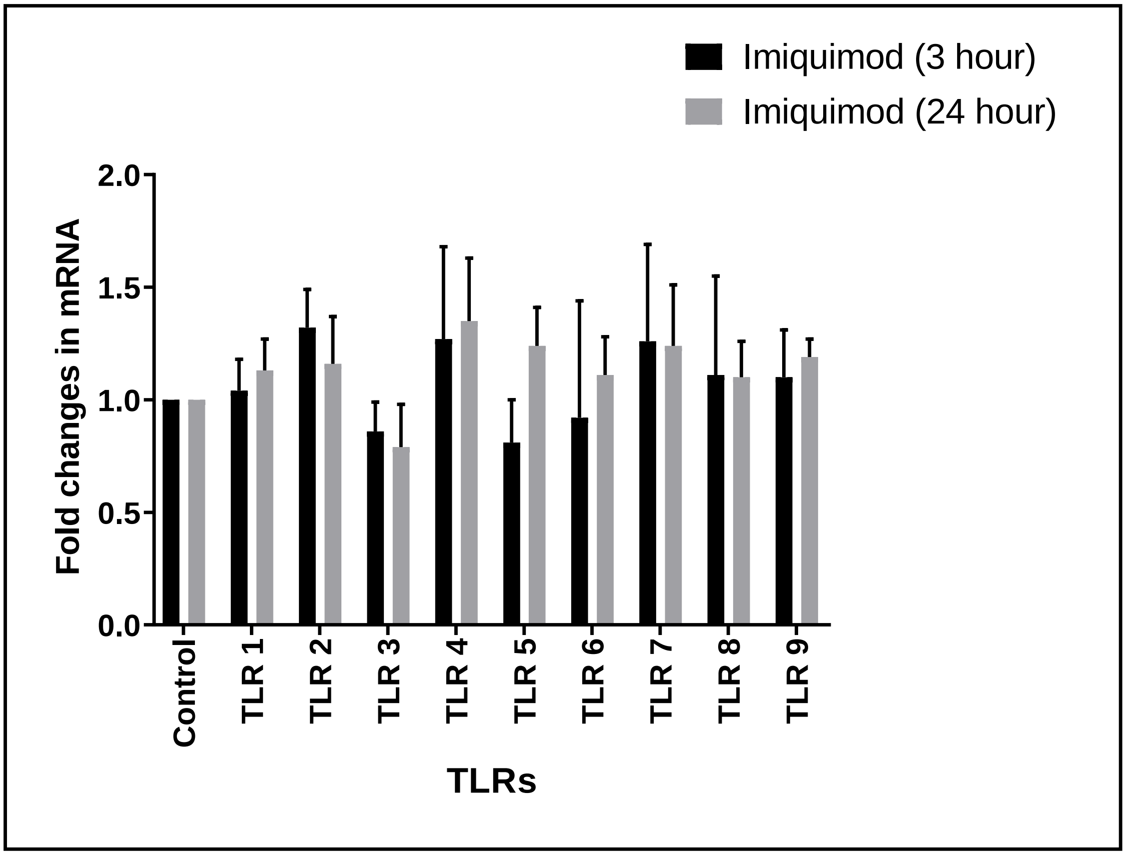


Fig 23: Fold changes in TLRs genes expressions in ISEMF cells upon stimulation with imiquimod. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after imiquimod treatment is denoted by an asterisk (*=$p<0.05$, **=$p\leq0.01$).

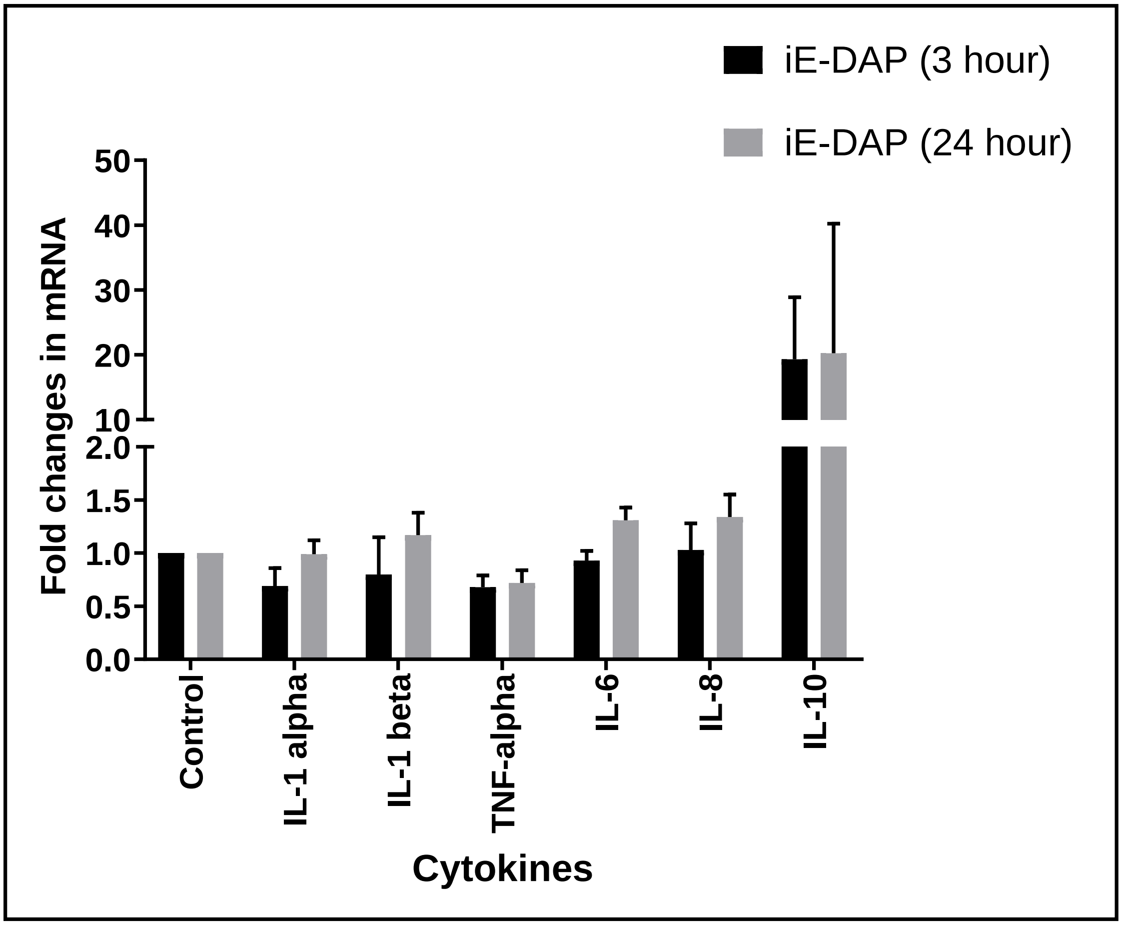


Fig 24: Fold changes in cytokines genes expressions in ISEMF cells upon stimulation with iE-DAP. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after iE-DAP treatment is denoted by an asterisk (*=$p<0.05$, **=$p\leq0.01$).

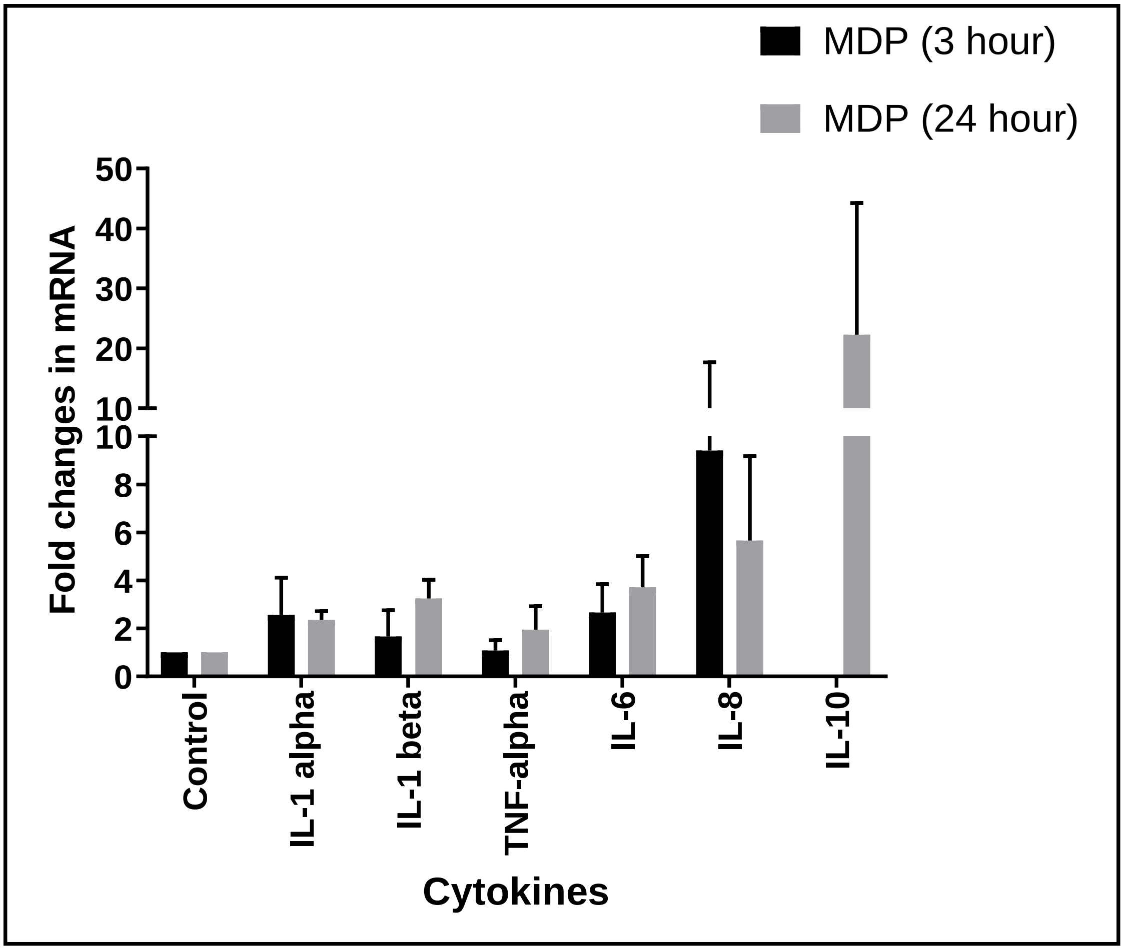


Fig 25: Fold changes in cytokines genes expressions in ISEMF cells upon stimulation with MDP. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after MDP treatment is denoted by an asterisk (*=p<0.05, **=p≤0.01).

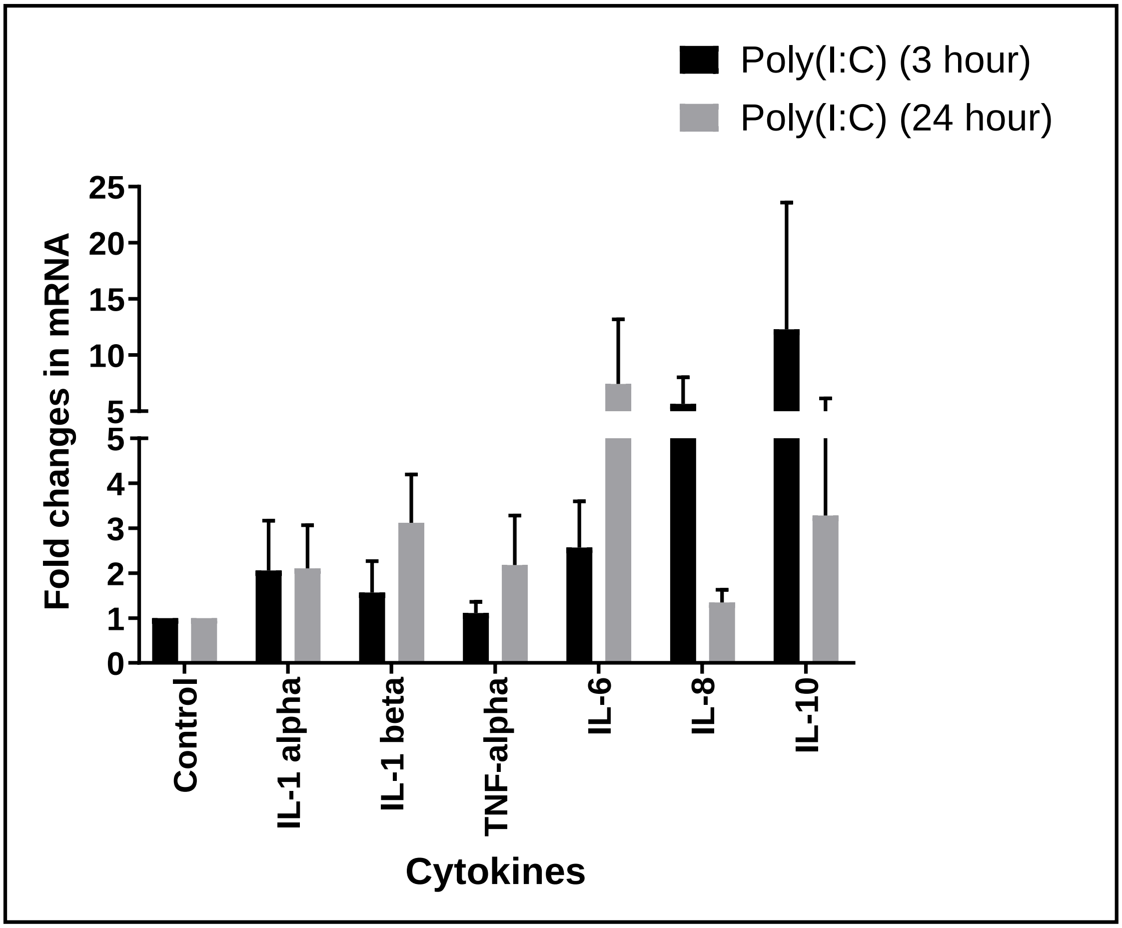


Fig 26: Fold changes in cytokines genes expressions in ISEMF cells upon stimulation with Poly (I:C). Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after Poly (I:C) treatment is denoted by an asterisk (*=p<0.05, **=p≤0.01).

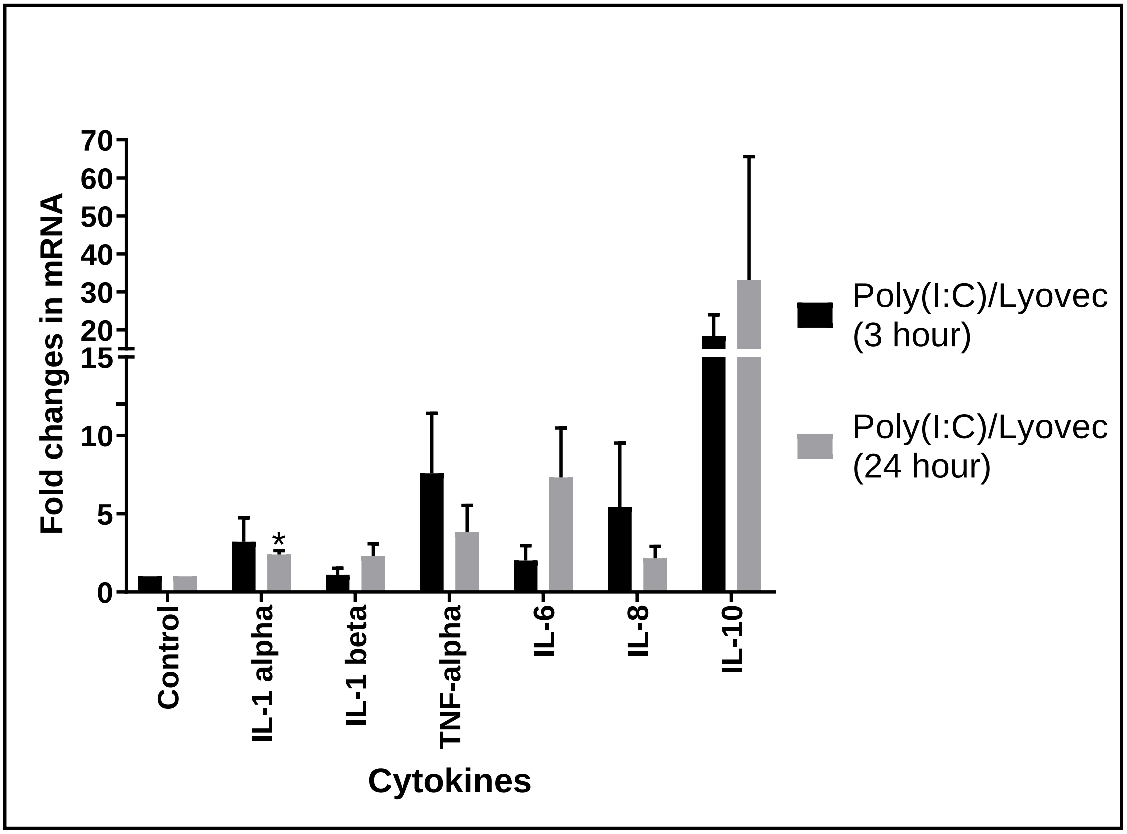


Fig 27: Fold change in cytokines genes expressions in ISEMF cells upon stimulation with Poly (I:C)/Lyovec. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after Poly (I:C)/Lyovec treatment is denoted by an asterisk (*=$p<0.05$, **=$p\leq 0.01$).

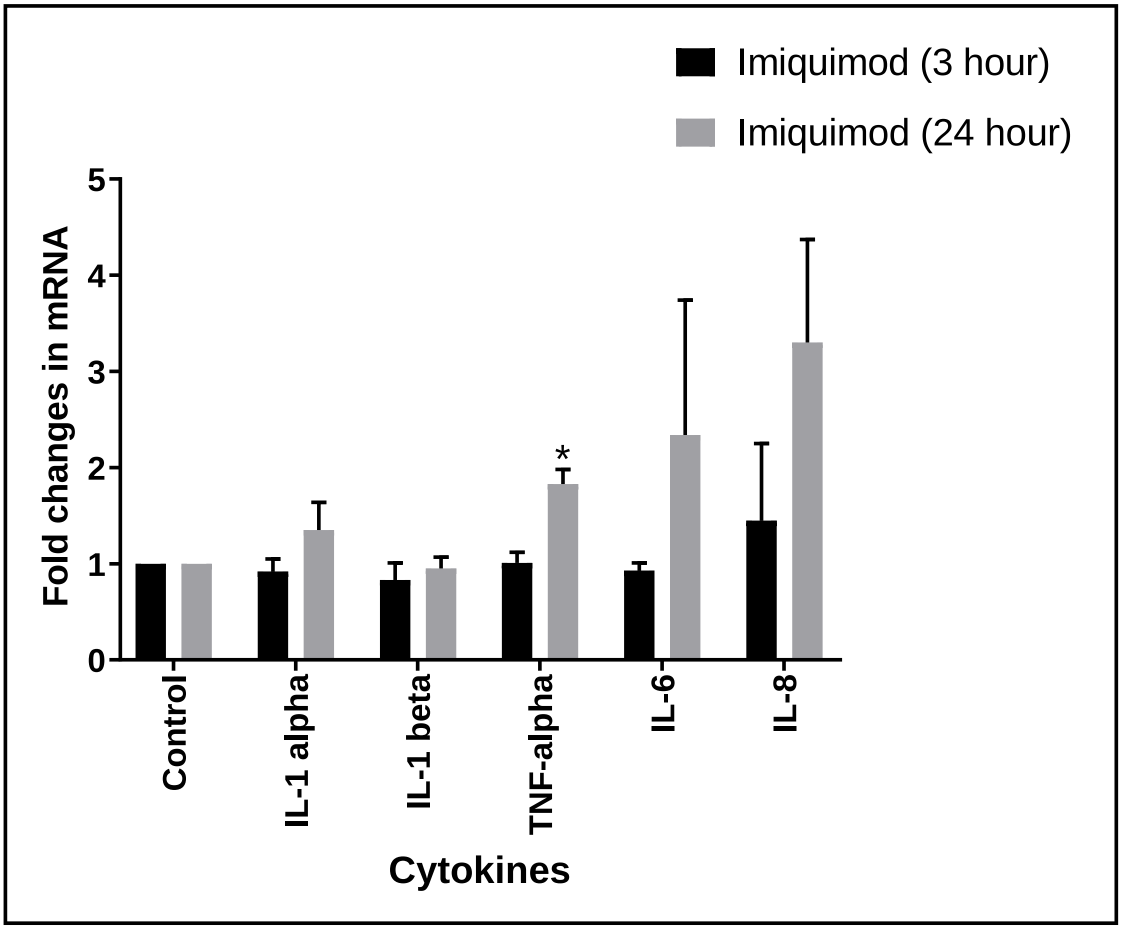


Fig 28: Fold changes in cytokines genes expressions in ISEMF cells upon stimulation with imiquimod. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after imiquimod treatment is denoted by an asterisk (*=p<0.05, **=p≤0.01).

**4.4 Discussion**

In this study bovine ileal ISEMF cells expressed various TLRs and responded to various bacterial and viral ligands of PRRs by changing the expression of various pro-inflammatory and anti-inflammatory cytokines (126). Inflammatory diseases of intestine or disruption of intestinal epithelium make sub-luminal compartment accessible to luminal antigens and pathogens to which ISEMFs respond by producing cytokines, chemokines, extracellular matrix components that modulate immune cells recruitment and activation (123, 202, 203). Cytokines and chemokines released in intestinal lumen or in the sub-epithelial compartment are key players in regulating barrier integrity (195, 204). Understanding cell specific response and intracellular mechanism that generates innate immune responses against enteric pathogens are crucial for the development of prophylaxis against such pathogens (126).

We first analyzed putative TLRs 1-9 expression in bovine ileal ISEMF cells. To identify if these cells respond to various pathogen associated molecular patterns and initiate any downstream signaling, we stimulated cells for either 3 hours or 24 hours with various ligands of PRRs. Stimulation for 3 hours mimicked early innate immune response while 24 hours mimicked late innate immune response. LPS significantly downregulated TLR 1, 4, 7, and 9 genes expressions after 3-hour stimulation and downregulated TLR 4 after 24-hour stimulation. LPS upregulated IL-6 gene expression at both 3 hours and 24 hours and downregulated TNF-α after 24-hour. The expression of TLR 4 to which LPS binds was downregulated by LPS stimulation (0.52 folds at 3 hours and 0.75 folds at 24 hours) and coincided with decreasing trend of IL-6 expression (7.63 fold at 3 hours and 3.6-fold at 24 hours) at 3 to 24 hours. LPS from gram negative bacteria is a potent immunostimulatory structure. LPS consists of endotoxin Lipid A, core oligosaccharide, and O-antigen. Lipid A is recognized by TLR 4 (205, 206). LPS recognition by TLR 4 requires accessory molecules. LPS binding protein binds to LPS allowing the association between LPS and co-receptor CD14 (monocyte differentiating antigen). CD-14 facilitates the binding of LPS to TLR 4/MD-2 complex (207, 208). Recognition of LPS by TLR 4 leads to signal transduction either by MyD88 (209) or TRIF pathway (210) ultimately leading to activation of transcription factors like NF-κB, AP-1, and IRF-3. Activation of transcription factors results in expression of pro-inflammatory cytokines like IL-6, IL-8, TNF-α, and type -I IFN (20, 211). Previous studies have shown that LPS stimulates expression of IL-6 in hepatic myofibroblasts (212). Murine intestinal myofibroblasts also expressed TLR 4 and demonstrated elevated levels of IL-6 when measured at 8-hour post stimulation with LPS (126). Continued activation of TLR 4 can lead inflammation induced damages and thus negative regulation needs to be in place. Radioprotective protein 105 (RP105), single immunoglobulin IL-1R-related molecule, IL1-RL1 protein negatively regulate TLR 4 signaling. LPS challenged mice that are deficient for RP105 showed elevated levels of TNF-α in serum (213). Thus, downregulation of TNF- α after 24-hour stimulation with LPS in this study could probably be the result of negative signaling. After TLR signaling, the LPS-TLR4-MD2 complex is endocytosed in endosome or lysosomes where degradation of TLR 4 occurs (214). This degradation can result in the termination of TLR 4 induced production of TNF (215). In human colonic myofibroblasts LPS altered the expression of TLR 2, 3, 4, 6, 7 indicating that LPS could alter expression of other TLRs apart from its specific receptor TLR 4 (193) also observed in this present study.

Peptidoglycan (PGN) is a major constituent of gram positive bacteria cell wall and is composed of N-acetlyglucosamine (GlcNac) and N-acetylmuramic acid (MurNac) linked by β-(1-4) linkage (49). PGN's role in inducing an inflammatory response and in stimulating innate immune responses has been long known (50, 51). TLR 2 knocked out mice revealed that TLR 2 is involved in recognition of PGN (216). In various cell models, PGN induced production of pro-inflammatory cytokines like IL-6, IL-1α/β, TNF-α (217). In this study, TLR 6 and TLR 8 were significantly downregulated at 3 hours along with downregulation of TNF-α and IL-10. IL-1α was upregulated while IL-6 was marginally downregulated after 24 hours with PGN stimulation. Unlike LPS stimulation, PGN stimulation did not alter TLR 2 expression at 3-hour or 24-hour time point. In earlier studies stimulation of rat intestinal myofibroblast cultures with cell wall polymers altered expression of cytokines like IL-1β and IL-6 (218) and mice corneal fibroblast cultures also responded to PGN treatment by altering expression of TLRs other than TLR 2 (219).

TLR 1 and TLR 6 are functionally co-related with TLR 2 in recognizing different classes of lipopeptides (220). Most of the studies co-relating PGN to TLR 2 used commercially obtained PGN from *Staphylococcus aureus.* This preparation is often co-purified with other cell wall components and pure PGN has failed to respond to TLR 2 in many experiments (221). The authors (221) even claimed that PGN sensing did not occur via TLR 2 which was later refuted (222).

Flagellin (FLA), a subunit of flagellum protein provides motility to the bacterium. Flagellin initially was considered as a virulence factor but subsequent in-vitro studies demonstrated its pro-inflammatory role (223, 224). Later it was demonstrated that recognition of flagellin by TLR 5 induced production of pro-inflammatory cytokines like TNF-α, IL-1β (225). In this study, 3- and 24-hours stimulation with flagellin downregulated TNF-α gene expression at both time points. TLR 4, 5, 7, 8, 9 genes expressions were downregulated at 3 hours while TLR 6 gene expression was downregulated at 24 hours. With three of these bacterial PRR ligands (LPS, PGN, and FLA) used in this study, we observed an alteration in expression of both cognate and non-cognate receptors. Recent studies have shown cross talk among these PRRs and that PRR ligand can overexpress or inhibit expression of other PRRs. Among multiple ligands, LPS showed a more pronounced effect on modulating expression of other PRRs, and PAMPs other than LPS downregulated the expression of TLR 4 (226). Triggering of single TLR when the specific ligand is recognized is insufficient to mount an effective innate immune response and thus triggering of other PRR family or multiple TLR may be required to mount a strong immune response. Often the synergism is between PRRs that mediate effector response through different signaling pathways (43, 227).

Nucleotide-binding oligomerization domain (NOD) proteins are cytoplasmic proteins involved in recognition of intracellular bacteria or their cell wall products. NOD proteins have N-terminal caspase recruitment domain (CARD), leucine rich repeats in C-terminus and nucleotide binding domain in between. NOD-1 and NOD-2 are two NOD proteins that recognize two different peptidoglycan fragments and are involved in pathogen recognition (48). NOD-1/CARD4 recognizes peptidoglycan GlcNAc-MurNAc-L-Ala-γ-D-Glu-meso-DAP (GM-TriDAP/iE-DAP) whereas NOD-2 recognizes muramyl dipeptide, MurNAc-L-AlaD-isoGln. NOD-1 and NOD-2 activate NF-κB by recruitment of receptor-interacting protein (RIP) 2 leading to secretion of pro-inflammatory cytokines while type I interferons are secreted when IRF3/IRF7 dependent pathway is activated by these receptors (52-56). In this study, iE-DAP significantly downregulated TLR 5 while MDP downregulated TLR 1 gene expression after 3-hour stimulation with no changes in cytokine expression. MDP alone has been shown to evoke weak immune stimulation (47, 228-231). However, addition of TLR agonists like LPS, lipoteichoic acid along with MDP has been shown to evoke a strong immune response (232, 233). TLR stimulation may promote internalization of MDP and iE-DAP which facilitates recognition by NOD proteins. NOD proteins also interact with other intracellular molecules such as GRIM-19, RIG-1, vimentin, RIPK2, NLRP1, that positively or negatively regulate NOD signaling pathways (47).

During viral replication, most of the viruses produce double stranded RNA (dsRNA) as replication intermediate. This dsRNA is sensed by PRRs present in the cell membrane, cytosol, and endosomes. TLR 3 is membrane receptor usually present in endosomes and recognizes dsRNA. Retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene -5 (MDA-5), and NLR pyrin domain 3 (Nlrp3) are present in the cytosol and associated with sensing of dsRNA (62, 234-237). Recognition of dsRNA by these receptors results in the production of type -I interferon (IFN) (238, 239). TLR 3 uses MyD88 independent pathway and uses toll/interleukin-1 receptor (TIR) domain-containing adaptor inducing IFN-β (TRIF) ultimately leading to induction of IFN-β (210, 240, 241). Poly (I:C) is the synthetic analog of dsRNA and is used to mimic viral infection in experimental conditions (242, 243). Recent reports have shown possible role of CD14 in internalizing extracellular dsRNA or poly (I:C) and delivering to TLR 3 located in the endosomal and lysosomal membrane (244). Apart from IFN, IL-6 induced from TLR 3 expression is reported to have a detrimental effect during infection with single stranded RNA viruses (245-247). Apart from IFN production, NF-κB activation also induces secretion of IL-32. IL-32 induces production of pro-inflammatory cytokines like IL-6, IL-8, TNF-α, IL-1β (241, 248). With ISEMF cells in this study, we observed downregulation of TLR 1 gene expression at 3-hour stimulation with poly (I:C) however; we did not observe any significant changes in cytokines and TLR 3 expression.

Poly (I:C) complexed with lyovec is Poly (I:C) complexed with a transfecting reagent that allows Poly (I:C) to be released into the cytoplasm. Accumulation of intracellular dsRNA during viral replication and subsequent induction of IFN production by the host cell is different from the IFN produced by sensing of extracellular dsRNA (249). Intracellular dsRNA is sensed by RIG-I, and MDA-5. Proteins RIG-I and MDA-5 belong to RIG-I like receptor (RLR) family. RIG-I senses blunt ended 5'phosphorylated dsRNA whereas MDA-5 recognizes long (>1000 nucleotide) dsRNA (58-60). Both RIG-I and MDA-5 are RNA helicases that have caspase recruitment domain (CARD) and helicase domain. Signal transduction after sensing of intracellular dsRNA is through CARD in both RIG-I and MDA-5. This results in activation of IRF-3 and NF-κB and subsequent production of IFNs (type I, and type III) and as well as pro-inflammatory cytokines like IL-6 and IL-8 (61-64). However, poly (I:C) complexed with transfecting reagent lyovec in this study downregulated TLR 3 gene expression and significantly upregulated IL-1α gene expression.

Imiquimod is a synthetic guanosine analog with antiviral and anti-tumor activity (250). Imiquimod is an immune response modifier that specifically activates TLR 7 signaling pathway (251). Through MyD88 signaling cascade imiquimod induces activation of transcription factors like NF-κB and MAPKs (mitogen activated protein kinases). Activation of these transcription factors leads to induction of IFN-α, IL-12, TNF-α, IL-6 and other cytokines (250, 252-255). In this study, ileal ISEMF cells responded to imiquimod by upregulation of TNF-α gene expression after 24-hours stimulation but no changes in expression of TLR genes were observed. Immune cells like phagocytes produce reactive oxygen species (ROS) during the microbial invasion. These ROS are highly reactive and induce oxidative damage to nucleic acids, lipids, and proteins. Guanosine and cytosine are more prone to undergo oxidative damage due to their electronic configuration. Such damaged guanosine may be sensed by TLR 7 and TLR 8 and produce necessary cytokines for activating immune cells (251, 256).

With most of the bacterial and viral ligands used in this study, we observed downregulation of TLRs. Some studies have suggested that TLR upregulation may favor entry of pathogens, especially in intestinal epithelium. In intestinal epithelium, TLRs upregulations is found to be associated with disruption of barrier function and thus may favor entry of pathogens (257). Thus, based on the findings of this study, intestinal myofibroblasts may also be involved in antiviral response and in activation of immune cells.

**4.5 Conclusion**

In this study, we investigated the putative expression of TLRs by bovine ileal ISEMF cells asthere has been a limited number of studies on the expression of PRRs by intestinal myofibroblast cells. To the author's knowledge, no studies on the role of bovine intestinal sub-epithelial myofibroblasts in innate immunity have been carried out so far. This study also analyzed the responses of bovine ISEMFs to various PAMPs and associated cytokines expression. This study did not analyze expression of NLRs and RLRs in ileal ISEMFs. This study only analyzed changes in gene expression in response to various PAMPs. This study is limited in scope as it did not analyze whether alterations in mRNA expressions were being carried out to protein level. No experiment to quantify cytokine level in cell culture supernatant was performed and changes in IFN gene in response to viral PAMPs was not analyzed. Despite these limitations, we demonstrated that bovine ileal ISEMF express TLRs 1-9 and respond to various bacterial and viral PAMPs. Based on our experiments we can conclude that bovine ISEMFs are involved in generating an innate immune response in the intestinal sub-epithelial compartment. Thus, this cell line can be used to accumulate knowledge of signal transduction in response to various bacterial and viral PAMPs. This bovine ISEMF cell line can be an excellent in-vitro model to study innate immune responses occurring at intestinal mesenchyma.

# Chapter 5. Role of bovine ileal epithelial cells in innate immune responses in the intestine

## Abstract

Intestinal epithelium plays important role not only in digestion but also in the maintenance of homeostasis in gastro-intestinal (GI) tract. It serves as a physical barrier in separating gut microbiota and lumen. There is a dynamic interaction among intestinal epithelial cells, intestinal mucosa and gut microbes. Knowledge of this interaction is essential for the better understanding of inflammatory and infectious enteric diseases where this delicate interaction is perturbed. Intestinal epithelial cells being equipped with pattern recognition receptors (PRRs) along with their proximity to gut microbiota play significant role in mounting innate immune responses to gut antigens and pathogens as well as in the maintenance of peripheral tolerance. We used cloned bovine-intestinal epithelial cell line (BIEC-c4) earlier developed from the ileum of the 2-day old calf to study putative expression of Toll like receptors (TLRs) and their responses to bacterial and viral pathogen associated molecular patterns (PAMPs). BIEC-c4 cells were stimulated with various PRR bacterial and viral ligands for 3 and 24 hours. The RT-qPCR assay was employed to analyze TLRs and cytokines gene expression and quantified as fold expression changes. At 3 hours, we observed no changes in TLR expression after stimulation of BIEC-c4 cells with the lipopolysaccharide (LPS), peptidoglycan (PGN), and flagellin (FLA). At 24-hour peptidoglycan PGN upregulated expression of TLR 3 and 9. LPS upregulated interleukin 8 (IL-8) and IL-10 at 3 hours while IL-6 and IL-8 were upregulated at 24 hours. FLA downregulated IL-1β gene expression at 3-hour after stimulation. Ligand γ-D-Glu-mDAP (iE-DAP) and muramyl dipeptide (MDP) upregulated TLR 9 expression at 3 and 24 hours after stimulation respectively. However polyinosonic:polycytidylic acid (Poly I:C) upregulated both TLR 8 and TLR 9 expression after 3 hours of stimulation. Poly I:C complexed with lyovec (Poly I:C/lyovec) and imiquimod did not alter expression of any TLRs. Overall, findings of this study suggest that theBIEC-c4 cells serve as a good in-vitro model to study immune responses specifically against bacterial pathogens.

**Keywords:** bovine intestinal epithelial cells, TLRs, cytokines, innate immunity

## 5.1 Introduction

Intestinal epithelium is important in digestion and nutrients uptake. It is also involved in the maintenance of homeostasis in gastro-intestinal (GI) tract. It serves as a physical barrier in separating gut microbiota and luminal content from intestinal sub-mucosa. The intestine is equipped with the largest arsenal of immune cells (204). Intestinal epithelial cells (IECs) play a significant role in inducing innate immune responses against invading pathogens. With both commensals and pathogens residing in the intestine, intestinal epithelial cells need to selectively mount an immune response against pathogens and develop tolerance against commensals. This dual task of maintaining tolerance as well as generating immune response surmounts unique challenge to the mucosal surface and specifically to intestinal epithelial cells (258).

IECs secrete antimicrobial peptides like defensins and calprotectins that have broad-spectrum anti-bacterial activity (259-261). IECs express pattern recognition receptors (PRRs) that sense various pathogen associated molecular patterns (PAMPs). IECs being equipped with PRRs along with their proximity to gut microbiota have been shown to play role in mounting an innate immune response as well as in the maintenance of peripheral tolerance (262-264). Toll like receptors (TLRs), a type of pattern recognition receptors (PRRs), are first to recognize and mount an effective innate immune response against invading pathogens (23, 196). Ten TLRs (TLR 1-10) have been reported in a bovine system with bovine intestinal epithelial cells expressing all ten of them (265-267). TLRs 1, 2, 4, 5, 6, 10 are expressed on the cell surface whereas TLR 3, 7, 8, 9 are intracellular and located in endosomes. Cell surface TLRs sense protein, lipid and lipopolysaccharide PAMPs while intracellular TLRs sense nucleotide PAMPs (7, 25, 268). Binding of pathogen associated ligands to TLRs induces MyD88 or TRIF dependent pathways leading to activation of NF-κB and MAPKs pathways and release of cytokines or chemokines (25). Cytokines like IL-17, IL-10, IL-22, IL-36, IL-6 upregulate JAK-STAT pathway leading to increased expression of target genes essential for epithelial regeneration, proliferation, barrier integrity, activation of the adaptive immune system and pathogen clearance. (269-273).

Development of stable intestinal epithelial cells that express PRRs and respond to PAMPs is pivotal in establishing an in-vitro model for studying enteric disease pathogenesis, signaling pathways and innate immune responses to pathogens. Cattles harbor enteric pathogens like enterotoxigenic *Escherichia coli*, *Salmonella*, *Campylobacter*, *Mycobacterium*, *Listeria*, *Leptospira* that cause huge economic losses to the livestock industry. Many of such zoonotic pathogens are equally important from a public health perspective (274-276). Development of host specific cell line helps in better understanding of disease pathogenesis and immune responses. There are limited number of stable primary cell lines available from the bovine intestine (265). Most of the intestinal cell lines are either from adult cattle or from fetal tissues. Unavailability of intestinal epithelial cell lines from young calves have hindered studies on enteric pathogens like bovine rotavirus, bovine coronavirus, and bovine viral diarrhea virus that infect young calves (135, 277).

Analysis of PRRs in the intestine, preferential activation of PRRs by pathogens, and cytokine signaling associated with PRR activation is essential for better understanding of gut immunity (264). In this study, we used an established and characterized cloned primary bovine intestinal cell line (BIEC-c4) from the ileum of the

2-day old calf which expressed TLR 1-9 as assessed by RT-qPCR assay. Here, we investigated the innate immune responses of BIEC-c4 cell line to various bacterial and viral PAMPs.

## 5.2 Materials and Methods

### 5.2.1 Cell lines and culture conditions

Cloned primary bovine intestinal epithelial cells (BIEC-c4) obtained from the ileum of the 2-day-old calf were used in this study. BIEC-c4 cells were grown in DMEM/F12 (Dulbeco's Modified Eagle Medium; GIBCO) media supplemented with 5% fetal calf serum (FCS: Atlanta Biologicals, Lawrenceville, GA), pen-strep (100 IU/ml of penicillin, 100 ug/ml of streptomycin: Invitrogen), 0.1% of mouse epidermal growth factor (EGF; Corning®, catalog number 4069007), and 0.1 % each of insulin, human transferrin and selenous acid (ITS; Corning®, catalog number 354351). The supplemented media was named as epithelial cell media. Cells were grown in T75 flasks (75 $cm^2$, Corning) in a humid chamber ($37^0C$, 5% $CO_2$) until becoming confluent. Cells were detached and harvested using 0.05% Trypsin-EDTA (Corning; Manassas, VA). Half million BIEC-c4 cells were seeded in each well of a six well tissue culture plate (Corning life sciences) for stimulating these cells with PRR ligands. After 48 hours of incubation in a humid chamber, cells were washed three times with 1X phosphate buffered saline (PBS). Fresh media was added along with PRR ligands at a specific concentration in duplicates. A negative control well was setup for each experiment. Each experiment was carried out in triplicates.

**5.2.2 PRR ligands for stimulation of BIEC-c4 cells**

BIEC-c4 cells were stimulated with PRR ligands for 3 and 24 hours using end-time alignment method. Lipopolysaccharide (LPS: catalog number L6529-1mg) from *Escherichia coli* O55:B55 was used at 5 µg/ml concentration. Similarly, peptidoglycan (PGN: catalog number tlrl-pgnsa) from *Staphylococcus aureus* was used at 10 µg/ml and Flagellin (FLA: catalog number tlrl-stfla) from *Salmonella typhimurium* was used at 100 ng/ml. Cells from passage 55 -62 were used for stimulating BIEC-c4 cells with bacterial ligands. We also stimulated cells (passage 32-42) for 3 hours and 24 hours using ligands of cytoplasmic and endosomal PRRs. γ-D-Glu-mDAP (iE-DAP: catalog number tlrl-dap) was used at10 µg/ml, muramyl dipeptide (MDP: catalog number tlrl-mdp) at 10 µg/ml, polyinosonic:polycytidylic acid (Poly I:C: catalog number tlrl-pic) at 5 µg/ml, Poly I:C complexed with lyovec (Poly I:C/lyovec: catalog number tlrl-piclv) at1 µg/ml, and Imiquimod (catalog number tlrl-imq) at 5 µg/ml. All PRRs ligands were bought from Invivogen, CA, USA.

**5.2.3 RNA extraction and cDNA preparation**

After 3 hours or 24 hours of incubation with ligands, cells were washed three times with 1X PBS. Cells were then trypsinized using 0.05% Trypsin-EDTA and centrifuged to form a pellet. RNA was extracted from pelleted cells using RNeasy Mini Kit (catalog number 74101, Qiagen, Valencia, CA, USA) and kit protocol. RNA was quantified using Thermo Scientific™ NanoDrop 2000. The RNA thus obtained was used to prepare cDNA. 1 µg of RNA was reverse transcribed to cDNA using TaqMan reverse transcription kit and kit protocol (TaqMan reverse transcription reagents, Applied Biosystems, catalog number N8080234).

### 5.2.4 RT-qPCR for quantifying gene expression

For RT-qPCR 2 µl of diluted cDNA (1:5 dilution), 1 µl each of forward and reverse primer, 10 µl of RT² SYBR® Green/ROX qPCR mastermix (catalog number 330501, Qiagen, Valencia, CA, USA) and 6 µl of nuclease free water was added. The thermal profile used for amplification was: 2 minutes at $50^0$C; 10 minutes at $95^0$C; followed by 40 cycles of 45 seconds (15 seconds for cytokine genes) at $95^0$C, 30 seconds at $60^0$C and 30 seconds at $72^0$C. Ramping speed was set at $1.6^0$C/second. QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, NJ, USA) was used. Data were normalized using housekeeping gene hypoxanthine phosphoribosyl transferase 1 (Hprt-1). Primer sequence previously used (138) for amplification of bovine TLR 1-9 gene, cytokine genes (138, 200) and Hprt-1 as housekeeping gene (278) are listed in Table 7 and 8. RT-qPCR was used to identify any changes in TLRs, pro-inflammatory and anti-inflammatory cytokines, after stimulation with PRR ligands.

Table 7: Primer sequence of genes along with gene bank accession number used in analysis of TLRs expression of BIEC-c4 cells

| | **Forward primer** | **Reverse primer** | **Accession number** |
|---|---|---|---|
| TLR 1 | CAT TCC TAG CAG CTA CCA CAA GCT | TGG GCC ATT CCA AAT AAG TTC T | NM_001046 504 |
| TLR 2 | GGG TGC TGT GTC ACC GTT TC | GCC ACG CCC ACA TCA TCT | NM_174197 |
| TLR 3 | GGG CAC CTG GAG GTC CTT | TTC CTG GCC TGT GAG TTC TTG | NM_001008 664 |
| TLR 4 | AGC ACC TAT GAT GCC TTT GTC A | GTT CAT TCC GCA CCC AGT CT | NM_174198 |
| TLR 5 | GTC CCC AAC ACC ACC AAG AG | GCG GTT GTG ACT GTC CTG ATA TAG | NM_001040 501 |
| TLR 6 | TTT ACC CTC AAC CAC GTG GAA | GGG CCA AAG GAA CTG AAA AAC | NM_001001 159 |
| TLR 7 | CAC CAA CCT TAC CCT CAC CAT T | GTC CAG CCG GTG AAA GGA | NM_001033 761 |
| TLR 8 | TGT GTT TAG AGG AAA GGG ATT GG | TCT GCA TGA GGT TGT CGA TGA | NM_001033 937 |
| TLR 9 | CAG TGG CCA GGG TAG TTT CTG | CCG GTT ATA GAA GTG ACG GTT GT | NM_183081 |
| Hprt-1 | GGATTACATCAAAGCA CTGAACA | CATTGTCTTCCCAGTGTCA ATT | NM_001034 035 |

Table 8: Primer sequence of genes along with gene bank accession number used in analysis of cytokines expression of BIEC-c4 cells

| | **Forward primer** | **Reverse primer** | **Accession number** |
|---|---|---|---|
| IL-1α | CAG TTG CCC ATC CAA AGT TGT T | TGC CAT GTG CAC CAA TTT TT | NM_174092 |
| IL-1β | GAG CCT GTC ATC TTC GAA ACG | GCA CGG GTG CGT CAC A | NM_174093 |
| TNF-α | CGC ATT GCA GTC TCC TAC CA | GGG CTC TTG ATG GCA GAC A | NM_173966 |
| IL-6 | CCA CCC CAG GCA GAC TAC TTC | CCA TGC GCT TAA TGA GAG CTT | NM_173923 |
| IL-8 | TGC TCT CTT GGC AGC TTT CC | TCT TGA CAG AAC TGC AGC TTC AC | NM_173925 |
| IL-10 | AAGGTGAAGAGAGTCT TCAGTGAGC | TGCATCTTCGTTGTCATGT AGG | NM_174088 |

**5.2.5 Statistical analysis for interpretation of RT-qPCR data**

To compare the change in TLR expression after ligand stimulation, double delta Ct (ΔΔCt) was calculated using the method previously described (201). Change in mRNA gene expression was calculated as 2-ΔΔCt. The method uses the following equation to calculate ΔΔCt:

ΔΔCt=ΔCt Treatment (Ct of reference gene Treatment-Ct Housekeeping gene Treatment)- ΔCt Control (Ct of reference gene Control-Ct Housekeeping gene Control).

A two tailed Student's t-test was then used to compare fold expression changes after treatment with ligands. A p-value of less than 0.05 ($p<0.05$) was considered significant. GraphPad prism 7.04 was used to prepare graphs. Data are expressed as a mean ± standard error of the mean.

## 5.3 Results

### 5.3.1 Responses of BIEC-c4 cells at 3-hour and 24-hour after stimulation with bacterial ligands of surface expressed PRRs

Three hours stimulation of BIEC-c4 cell with bacterial ligands LPS, PGN and FLA resulted in no significant changes in TLRs genes expressions (Figure 29-31). At 24-hour, PGN significantly upregulated TLR 3 (1.73±0.14, p=0.04), and TLR 9 (1.41±0.07, p=0.03) gene expression (Figure 30). LPS significantly upregulated cytokines IL-10 (2.42±0.2, p=0.02), and IL-8 (9.78±1.83, p=0.04) gene expression after 3-hour stimulation. At 24 hour, LPS also significantly upregulated IL-6 (3.58±0.15, p=0.00), and IL-8 (12.99±2.06, p=0.03) (Figure 32). PGN stimulation did not induce any significant changes in any cytokine gene expression at both 3 hours and 24 hour time points. FLA at 3-hour downregulated IL-1β (0.31±0.1, p=0.02) (Figure 34) gene expression.

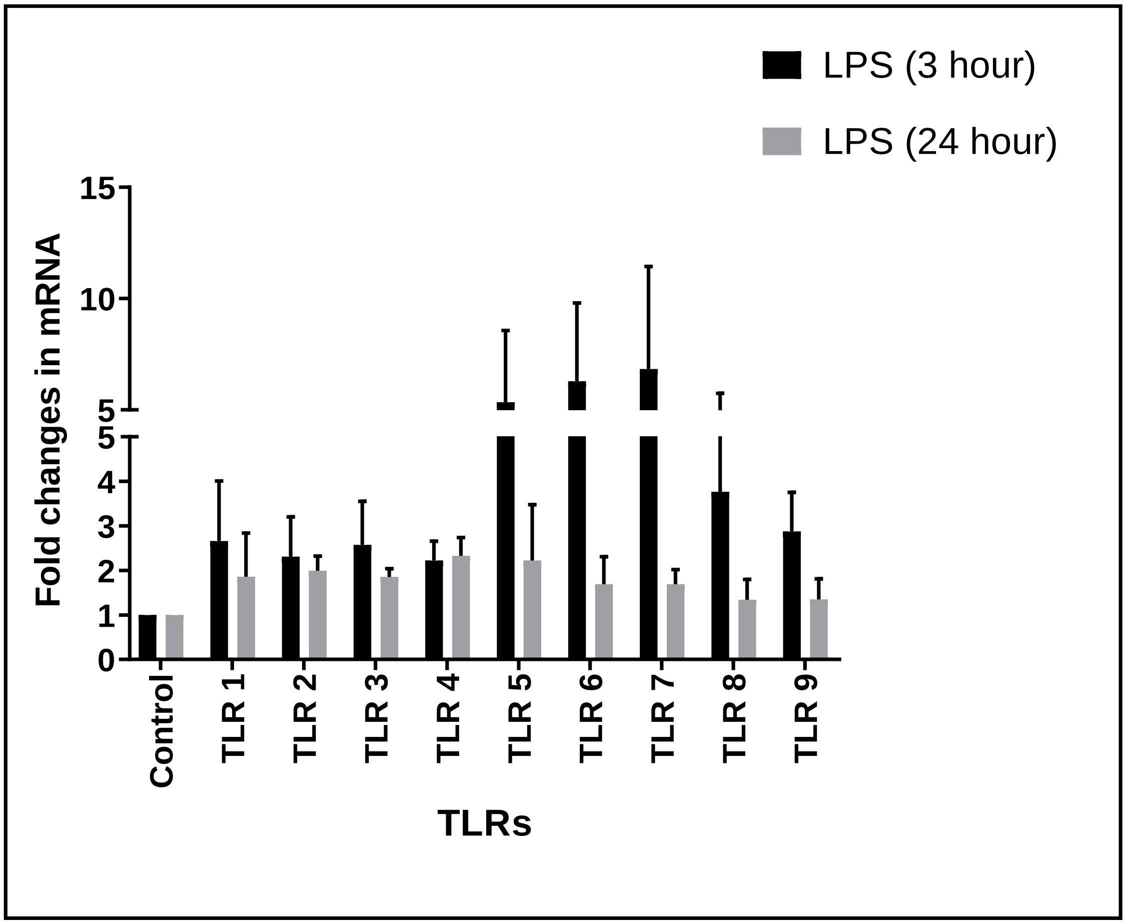


Fig 29: Fold changes in TLRs genes expressions in BIEC-c4 cells upon stimulation with LPS. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after LPS treatment is denoted by an asterisk (*=p<0.05, **=p≤0.01).

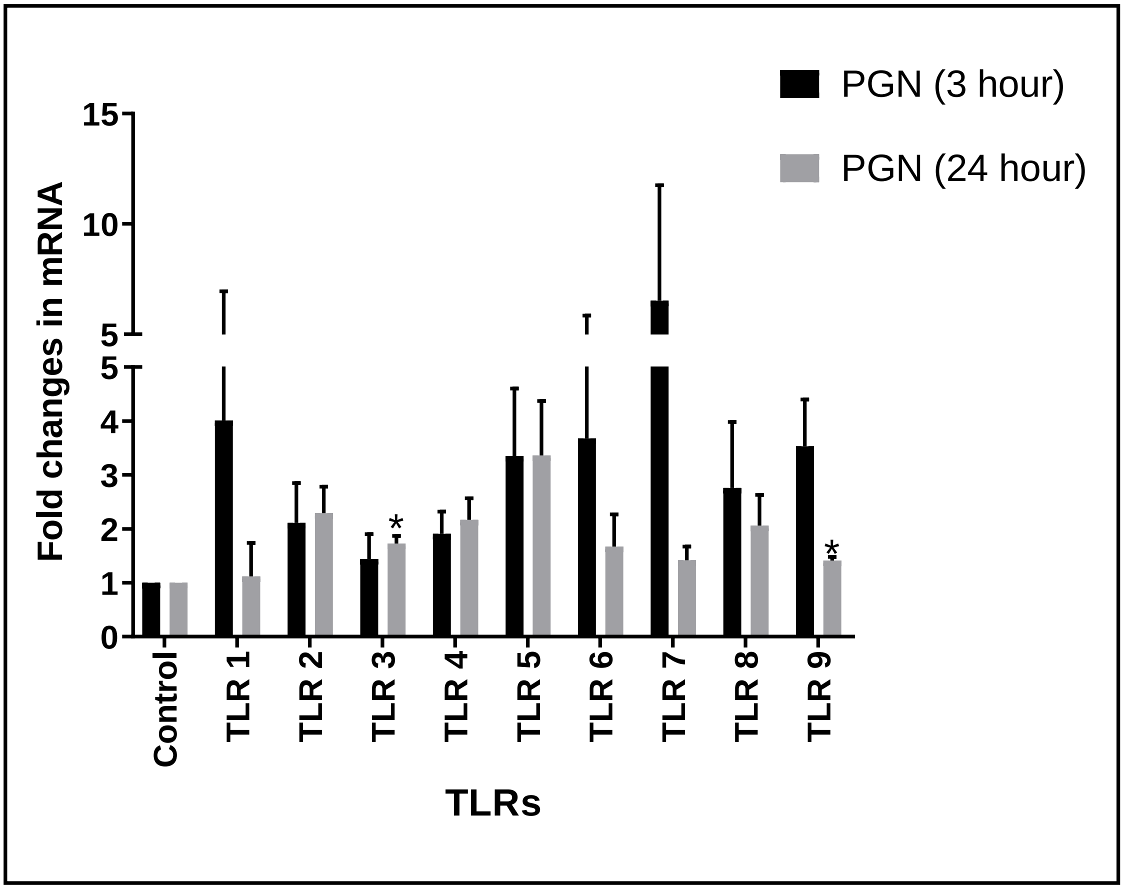


Fig 30: Fold changes in TLRs genes expressions in BIEC-c4 cells upon stimulation with PGN. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after PGN treatment is denoted by an asterisk (*=p<0.05, **=p≤0.01).

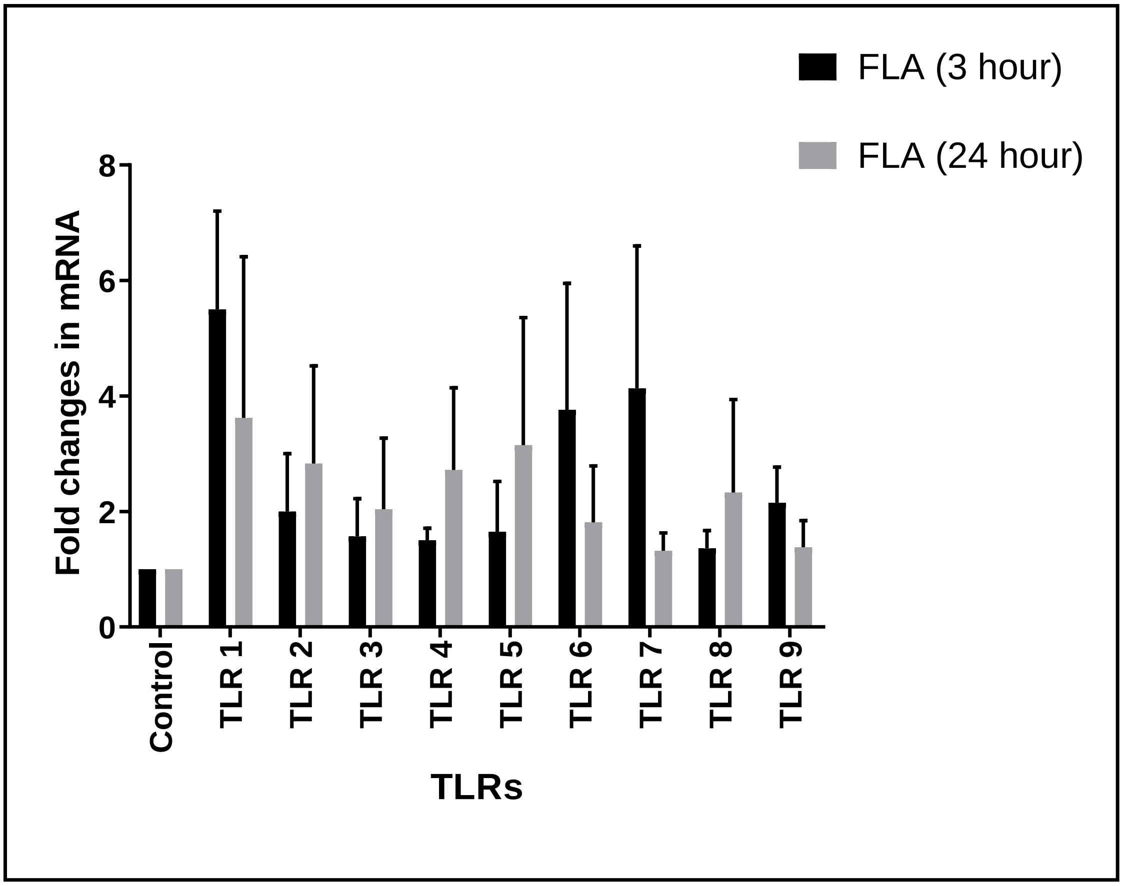


Fig 31: Fold changes in TLRs genes expressions in BIEC-c4 cells upon stimulation with FLA. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after FLA treatment is denoted by an asterisk (*=p<0.05, **=p≤0.01).

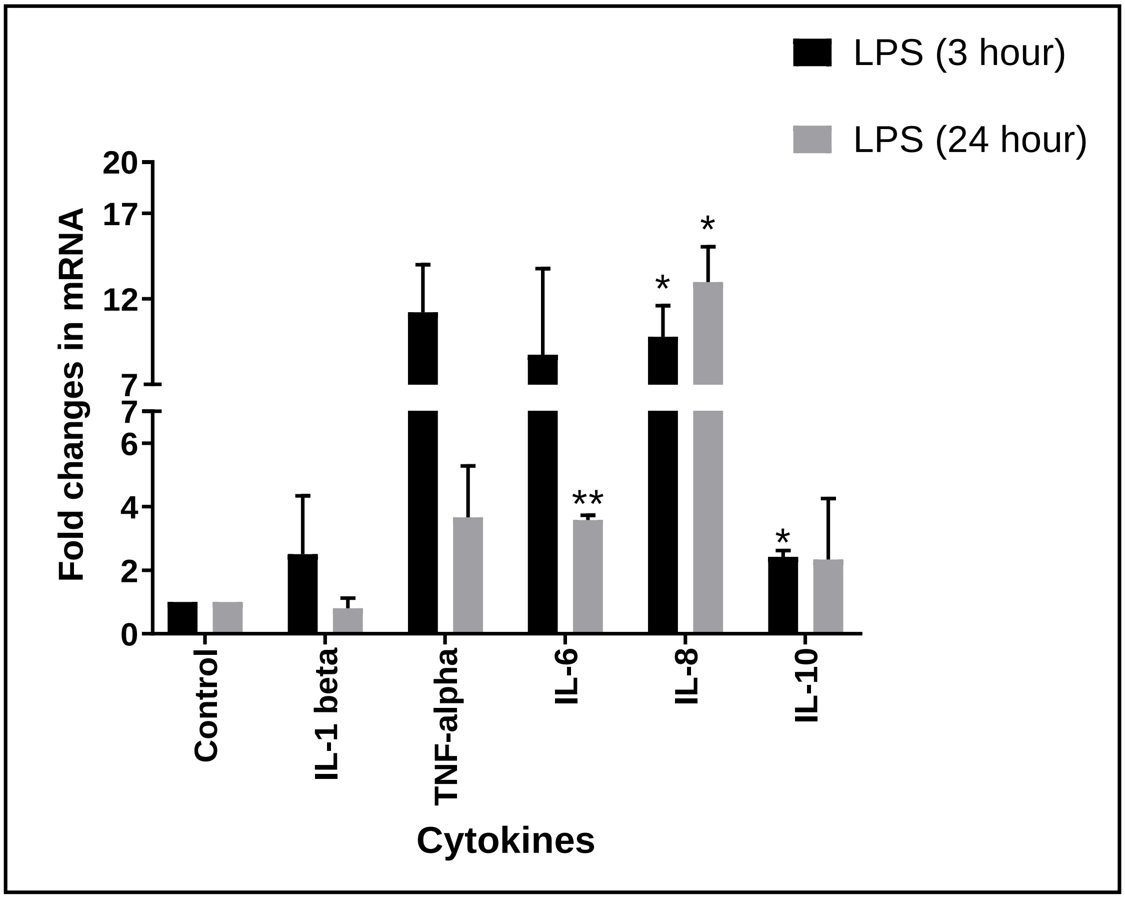


Fig 32: Fold changes in cytokines genes expressions in BIEC-c4 cells upon stimulation with LPS. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after LPS treatment is denoted by an asterisk (*=p<0.05, **=p≤0.01).

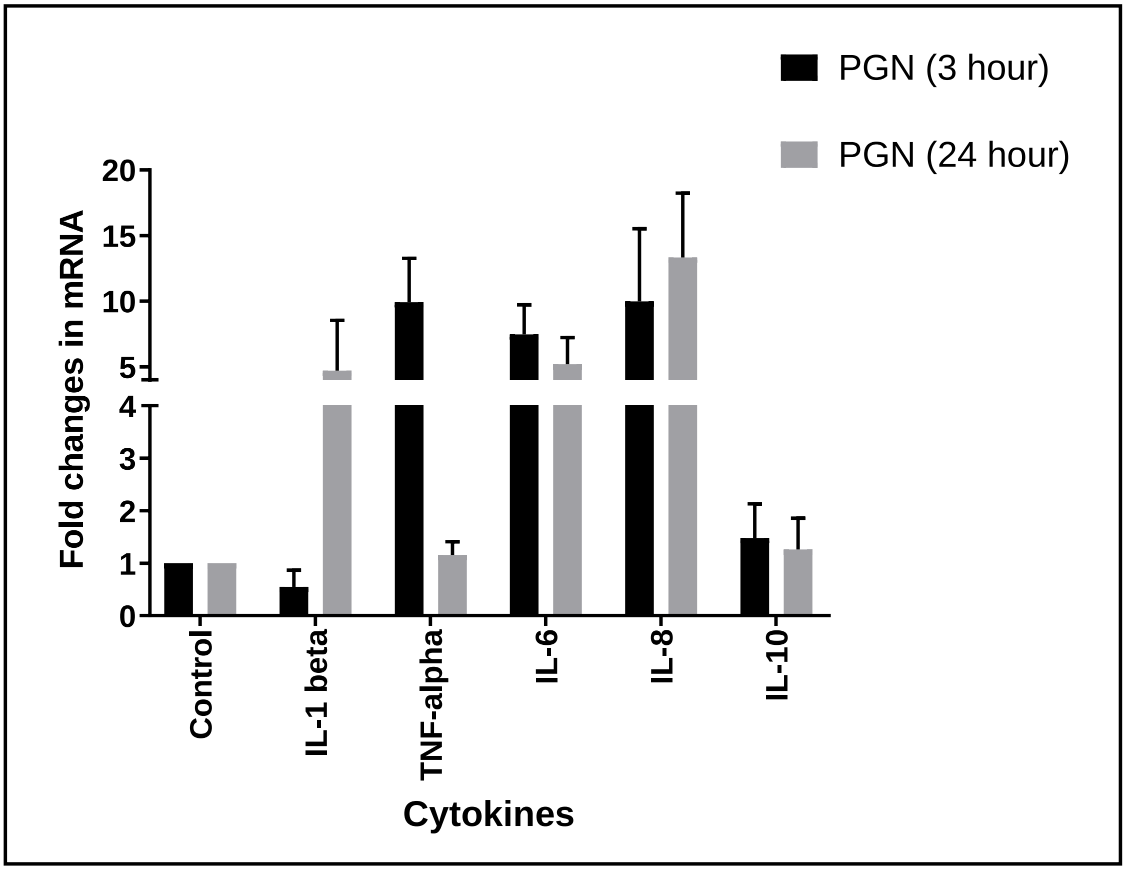


Fig 33: Fold changes in cytokines genes expressions in BIEC-c4 cells upon stimulation with PGN. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after PGN treatment is denoted by an asterisk (*=p<0.05, **=p≤0.01).

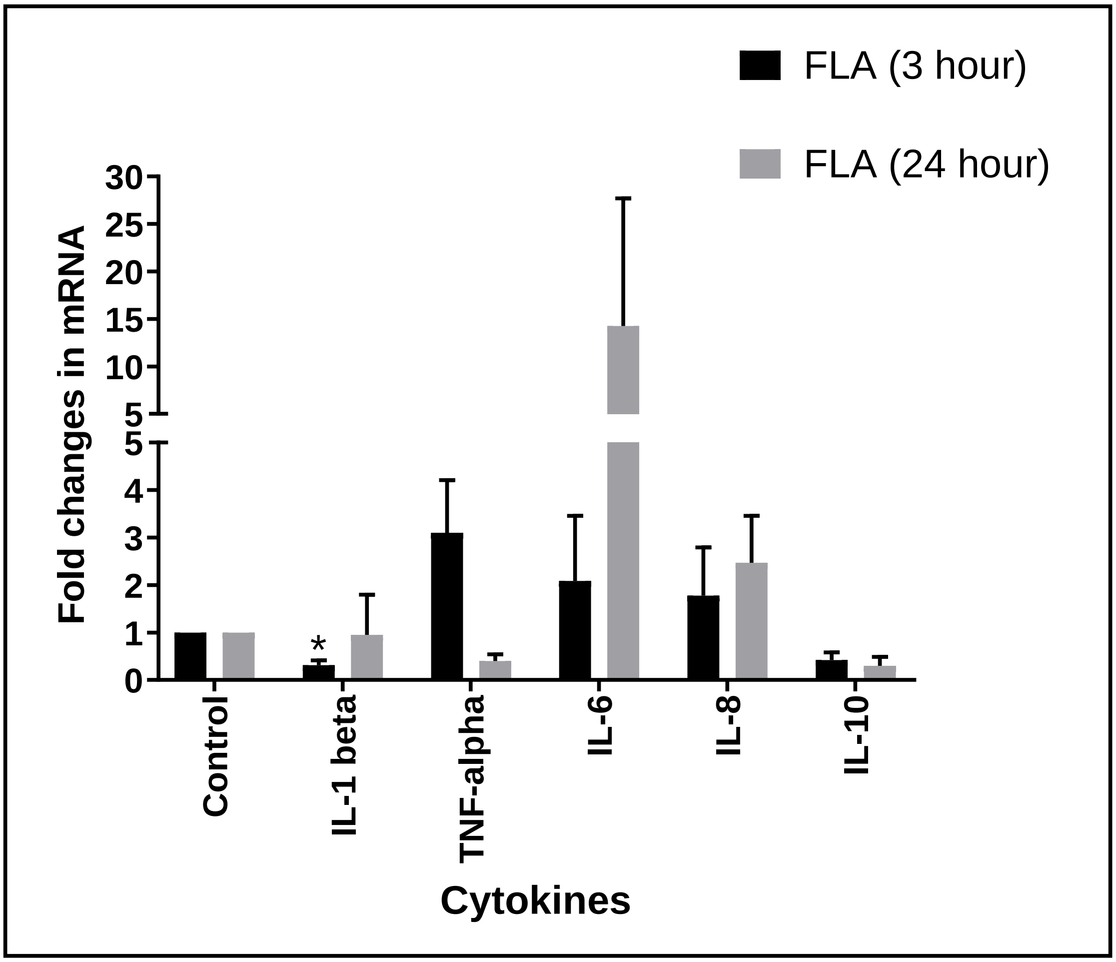


Fig 34: Fold changes in cytokines genes expressions in BIEC-c4 cells upon stimulation with FLA. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after FLA treatment is denoted by an asterisk (*=$p<0.05$, **=$p\leq0.01$).

### 5.3.2 The responses of BIEC-c4 cells at 3-hour and 24-hour after stimulation with ligands of cytoplasmic and endosomal PRRs

Out of various ligands of cytoplasmic and endosomal PRRs, only iE-DAP, MDP, and poly (I:C) altered the expression of TLRs in BIEC-c4 cells. iE-DAP stimulation at 3 hours significantly upregulated the expression of TLR 9 gene but expression of none of the other TLRs was affected (Figure 35). MDP after 24-hour stimulation upregulated TLR 9 (2.13±0.14, p=0.01) (Figure 36). Poly (I:C) after 3-hour stimulation upregulated TLR 8 (3.9±0.54, p=0.03), and TLR 9 (7.41±1.0, p=0.02) (Figure 37) gene expression. In general, we observed no alteration in TLRs expressions after 24 hours of stimulation with any ligands of cytoplasmic and endosomal PRRs except MDP (Figure 35-39).

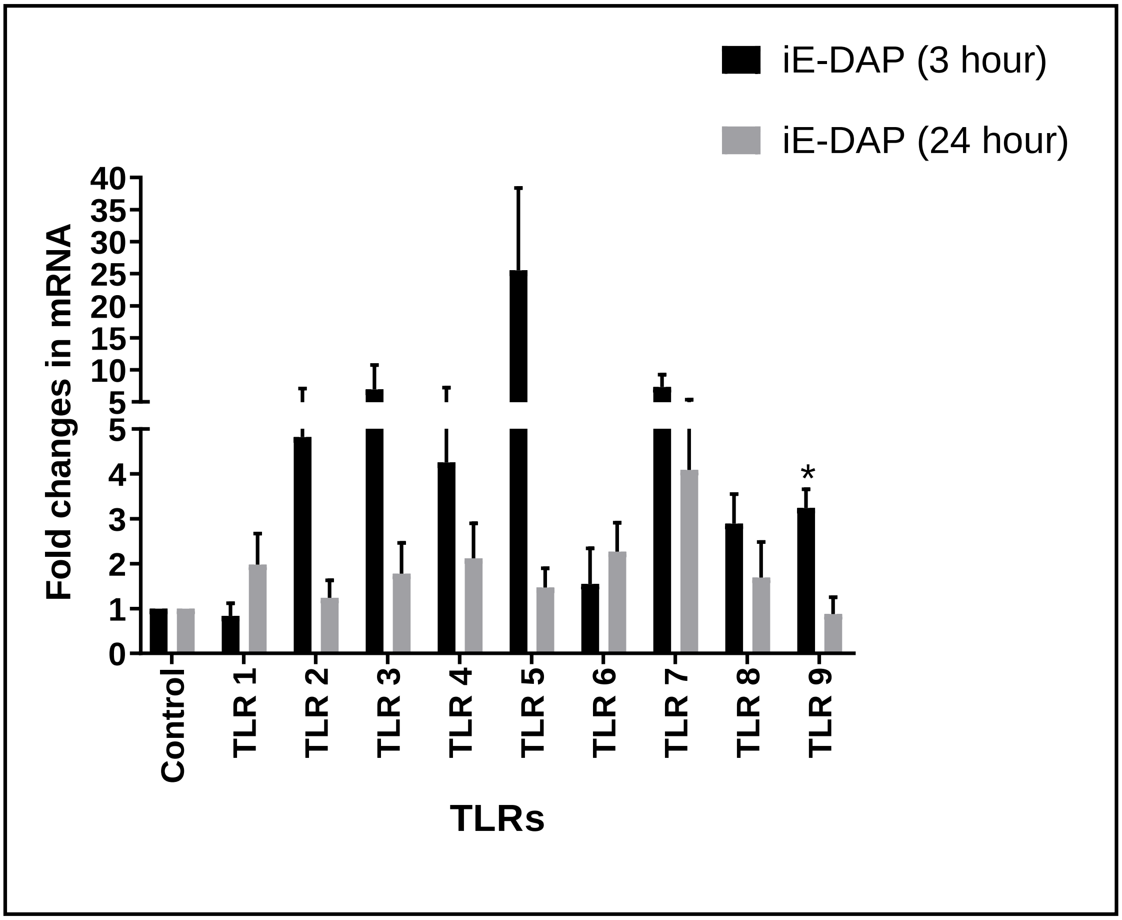


Fig 35: Fold changes in TLRs genes expressions in BIEC-c4 cells upon stimulation with iE-DAP. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after iE-DAP treatment is denoted by an asterisk (*=$p<0.05$, **=$p\leq0.01$).

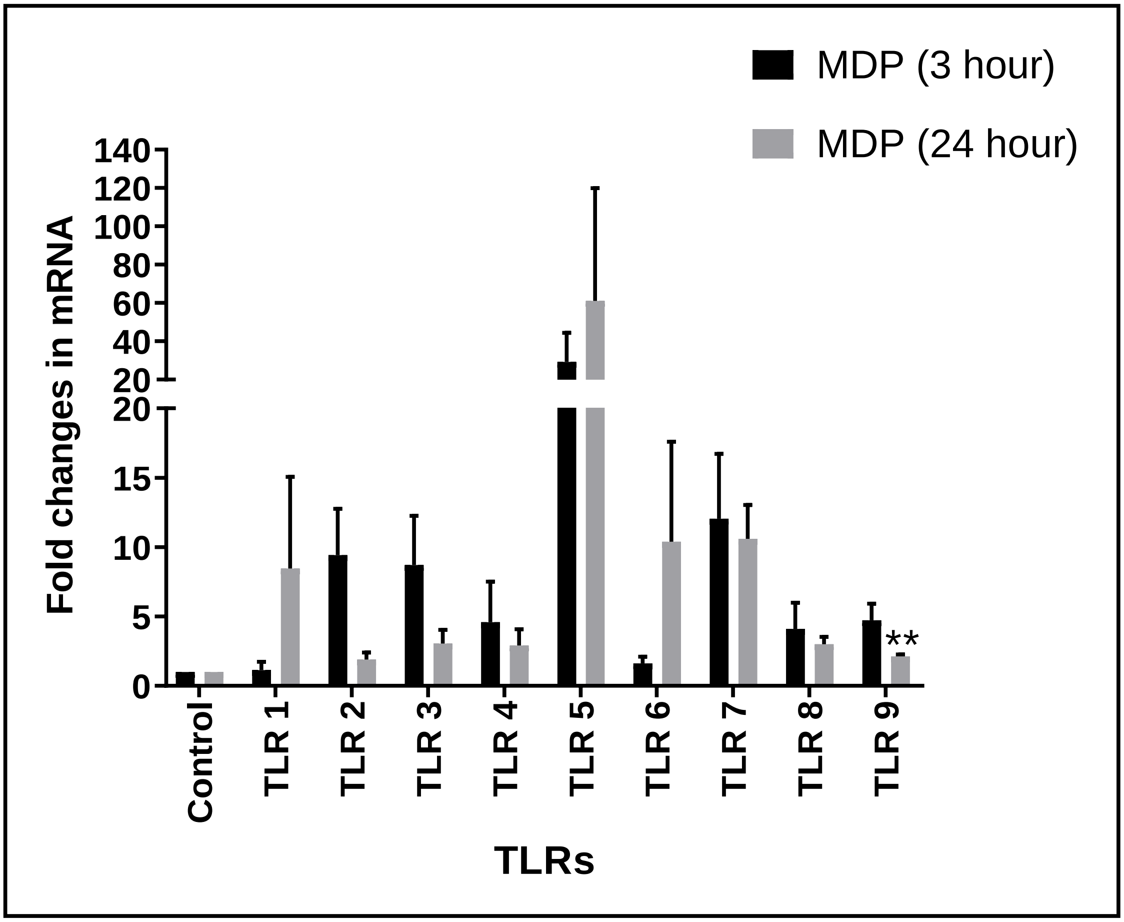


Fig 36: Fold changes in TLRs genes expressions in BIEC-c4 cells upon stimulation with MDP. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after MDP treatment is denoted by an asterisk (*=p<0.05, **=p≤0.01).

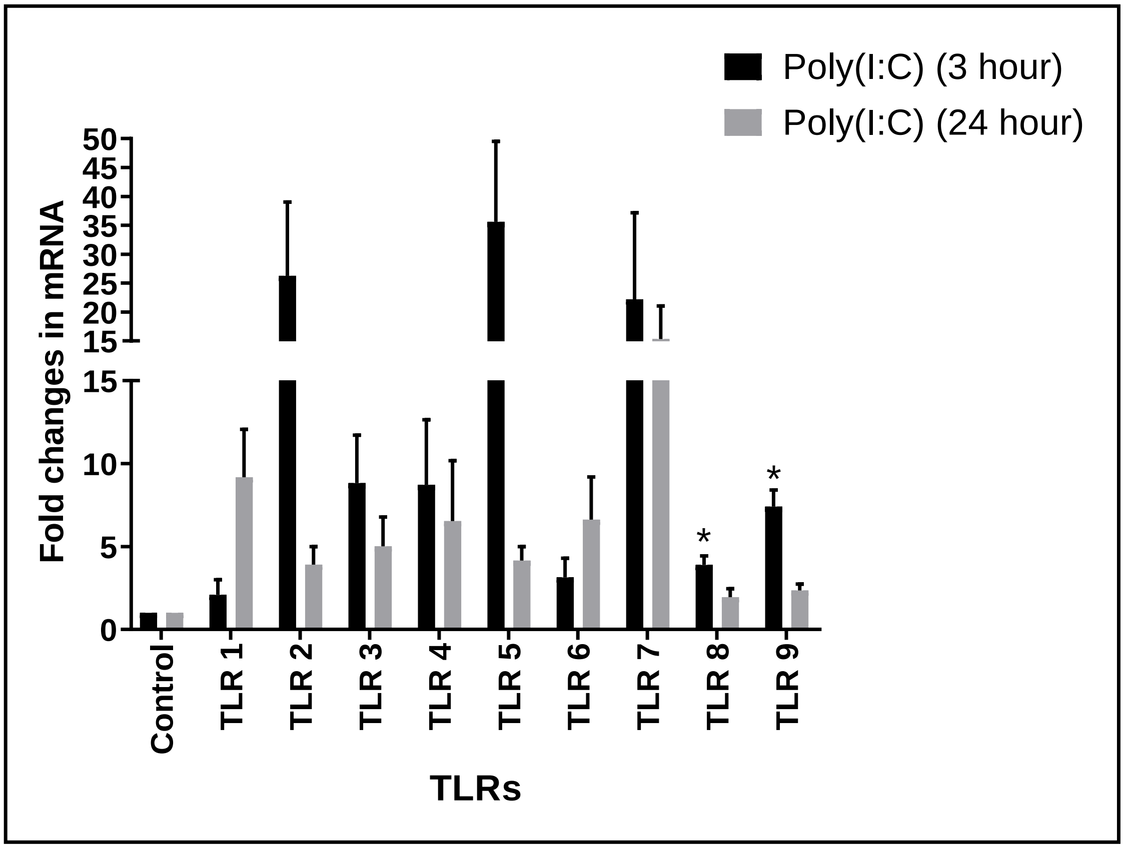


Fig 37: Fold changes in TLRs genes expressions in BIEC-c4 cells upon stimulation with Poly (I:C). Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after Poly (I:C) treatment is denoted by an asterisk (*=p<0.05, **=p≤0.01).

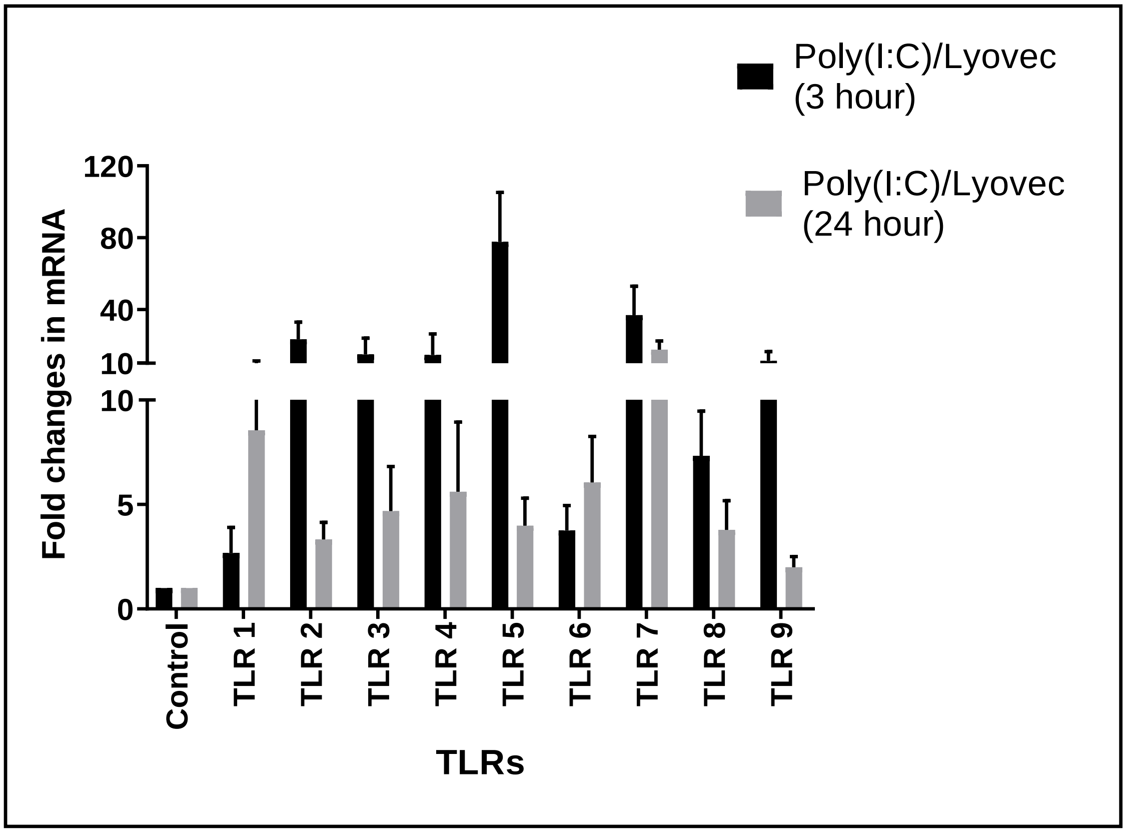


Fig 38: Fold changes in TLRs genes expressions in BIEC-c4 cells upon stimulation with Poly (I:C)/Lyovec. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after Poly (I:C)/Lyovec treatment is denoted by an asterisk (*=$p<0.05$, **=$p\leq0.01$)

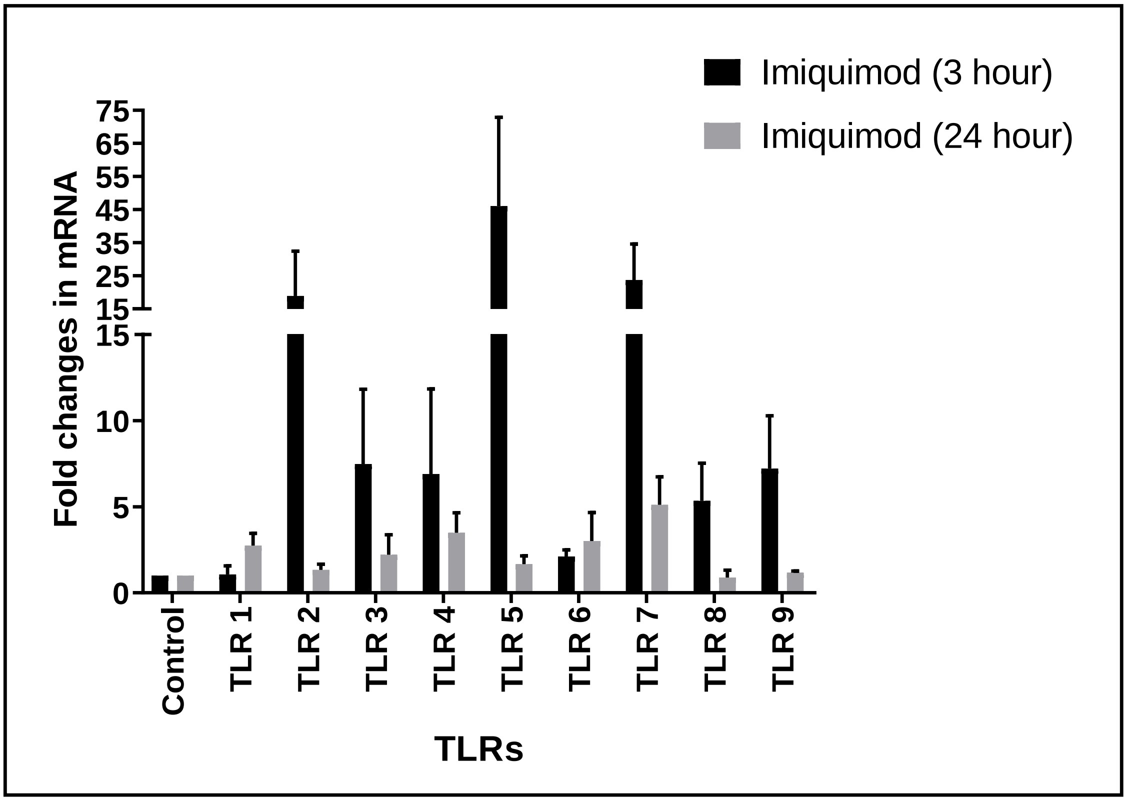


Fig 39: Fold changes in TLRs genes expressions in BIEC-c4 cells upon stimulation with imiquimod. Data are expressed as a mean of 3 independent experiments and error bar represents standard error of the mean. A significant difference in gene expression after imiquimod treatment is denoted by an asterisk (*=$p<0.05$, **=$p\leq0.01$).

**5.4 Discussion**

Host-specific in-vitro system that expresses PRRs and responds to PAMPs is essential for studying disease pathogenesis and host immune responses against pathogens. In this study, BIEC-c4 cells expressed various TLRs and responded to various bacterial associated ligands of PRRs by changing the expression of TLRs, pro-inflammatory and anti-inflammatory cytokines.

The intestinal epithelium is constantly exposed to the gut microbiota. There is a dynamic interaction between intestinal mucosa and gut microbes. Investigating these interactions is essential for better understanding of inflammatory and enteric diseases where this delicate interaction is perturbed (279). Intestinal epithelium should expediently detect pathogens from commensals and mount an effective immune response. PRRs especially TLRs, NLRs, RLRs recognize molecular patterns conserved across the pathogens. Pathogen sensing by PRRs results in activation of transcription factors and ultimately release of cytokines, chemokines and growth factors (280). Cytokines and chemokines released in intestinal lumen are key players in regulating barrier integrity (195, 204). Understanding cell specific responses and intracellular mechanisms that generate innate immune responses against enteric pathogens are crucial for the development of control methods against such pathogens (126).

In this study, we first analyzed putative TLRs 1-9 expression in BIEC-c4 cells. To identify if these cells respond to various pathogen associated molecular patterns (PAMPs) and initiate any downstream signaling, we stimulated cells for either 3 hours or 24 hours with various ligands of PRRs. Stimulation for 3 hours mimicked early innate immune responses while 24 hours point mimicked late innate immune responses. LPS did not alter expression of various TLRs genes at 3-hour or 24-hour stimulation. However, we observed upregulation of IL-8 and IL-10 genes at 3 hours and IL-6 and IL-8 at 24-hour after LPS stimulation. LPS from gram negative bacteria is a potent immunostimulatory structure. LPS consists of endotoxin Lipid A, core oligosaccharide, and O-antigen. Lipid A is recognized by TLR 4 (205, 206). LPS recognition by TLR 4 requires accessory molecules. LPS binding protein binds to LPS allowing the association between LPS and co-receptor CD14 (monocyte differentiating antigen). CD14 facilitates the binding of LPS to TLR 4/MD-2 complex (207, 208). Recognition of LPS by TLR 4 leads to signal transduction either by MyD88 (209) or by TRIF pathway (210) ultimately leading to activation of transcription factors like NF-κB, AP-1, and IRF-3. Activation of transcription factors results in expression of pro-inflammatory cytokines like IL-6, IL-8, TNF-α, and Type -I IFN (20, 211). Previous studies have shown that stimulation of bovine intestinal epithelial cells by *E. coli* PAMPs resulted in increased expression of pro-inflammatory cytokines like IL-6, IL-8, IL-1α, IL-1β (138, 265). Continued activation of TLR 4 can lead to inflammation induced damages and thus negative regulation needs to be in place. Radioprotective protein 105 (RP105), single immunoglobulin IL-1R-related molecule, and IL1-RL1 protein negatively regulate TLR 4 signaling. LPS challenged mice that are deficient for RP105 showed elevated levels of TNF-α in serum (213). After TLR signaling, the LPS-TLR4-MD2 complex is endocytosed in endosome or lysosomes where degradation of TLR 4 occurs (214). This degradation can result in the termination of TLR 4 induced production of TNF (215).

Peptidoglycan (PGN) is a major constituent of gram positive bacteria and is composed of N-acetlyglucosamine (GlcNac) and N-acetylmuramic acid (MurNac) linked by β-(1-4) linkage (49). PGN's role in producing an inflammatory response and stimulating immune response has been long known (50, 51). TLR 2 knocked out mice revealed that TLR 2 is involved in recognition of PGN (216). In various cell models, PGN induces production of pro-inflammatory cytokines like IL-6, IL-1α/β, TNF-α (217). In this study, TLR 3 and TLR 9 genes expressions were significantly upregulated at 24 hours after PGN stimulation although no changes in cytokine expression were observed. PGN stimulation did not alter TLR 2 expression at 3-hour or 24-hour time points and showed no significant changes in cytokine expression. In other in-vitro models, cells responded to PGN treatment by altering the expression of TLRs other than TLR 2 (219). TLR 1 and TLR 6 are functionally co-related with TLR 2 in recognizing different classes of lipopeptides (220). Most of the studies co-relating PGN to TLR 2 used commercially obtained PGN from *Staphylococcus aureus.* This preparation is often co-purified with other cell wall components and pure PGN has failed to respond to TLR 2 in many experiments (221). The authors (221) even claimed that PGN sensing did not occur via TLR 2 which was later refuted (222).

Flagellin (FLA), a subunit of flagellum protein, provides motility to the bacterium. Initially considered as a virulence factor, subsequent in-vitro studies demonstrated its pro-inflammatory role (223, 224). Later it was demonstrated that recognition of flagellin by TLR 5 induced production of pro-inflammatory cytokines like TNF-α, IL-1β (225). In this study, 3-hour and 24-hourstimulation of BIEC-c4 cells with flagellin did not alter expression of any TLRs but downregulated IL-1β at 3-hour time point. Recent studies have shown cross talk among various PRRs and that specific PRR ligand can overexpress or inhibit expression of other PRRs. Among multiple ligands, LPS showed a more pronounced effect on modulating expression of other PRRs, and PAMPs other than LPS downregulated the expression of TLR 4 (226). Triggering of single TLR when the specific ligand is recognized is insufficient to mount an effective innate immune response and thus triggering of other PRR family or multiple TLRs may be required to mount a strong immune response. Often the synergism exists between PRRs that mediate effector response through different signaling pathways (43, 227).

Nucleotide-binding oligomerization domain (NOD) proteins are cytoplasmic proteins involved in recognition of intracellular bacteria. NOD proteins have N-terminal caspase recruitment domain (CARD), leucine rich repeats in C-terminus and nucleotide binding domain in between. NOD-1 and NOD-2 are two NOD proteins that recognize two different peptidoglycan fragments and are involved in pathogen recognition (48). NOD-1/CARD4 recognizes peptidoglycan GlcNac-MurNac-L-Ala-γ-D-Glu-meso-DAP (GM-TriDAP/iE-DAP) whereas NOD-2 recognizes muramyl dipeptide, MurNac-L-AlaD-isoGln. NOD-1 and NOD-2 activate NF-κB by recruitment of receptor-interacting protein (RIP) 2 leading to secretion of pro-inflammatory cytokines while type I interferons are secreted when IRF3/IRF7 dependent pathway is activated by these receptors (52-56). In this study, iE-DAP upregulated expression of TLR 9 gene expression at 24 hours after stimulation in BIEC-c4 cells while MDP upregulated TLR 9 gene after 3-hour stimulation. MDP alone has been shown to evoke weak immune stimulation (47, 228-231). Addition of TLR agonists like LPS, lipoteichoic acid along with MDP has been shown to evoke a strong immune response (232, 233). TLR stimulation may promote internalization of MDP and iE-DAP which facilitates recognition by NOD proteins. NOD proteins also interact with other intracellular molecules that positively or negatively regulate NOD signaling pathways (47).

During viral replication, most of the viruses produce double stranded RNA (dsRNA) as replication intermediate. This dsRNA is sensed by PRRs present in the cytosol, and endosomes. TLR 3 is membrane receptor usually present in endosomes and recognizes dsRNA. Retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene -5 (MDA-5), and NLR pyrin domain 3 (Nlrp3) are present in the cytosol and associated with sensing of dsRNA (62, 234-237). Recognition of dsRNA by these receptors results in the production of type -I interferon (IFN) (238, 239). TLR 3 uses MyD88 independent pathway and uses toll/interleukin-1 receptor (TIR) domain-containing adaptor inducing IFN-β (TRIF) ultimately leading to induction of IFN-β (210, 240, 241). Poly (I:C) is the synthetic analog of dsRNA and is used to mimic viral infection in experimental conditions (242, 243). Recent reports have shown possible role of CD14 in internalizing extracellular dsRNA or poly (I:C) and delivering it to TLR 3 located in the endosomal and lysosomal membrane (244). Apart from IFN, IL-6 induced from TLR 3 expression is reported to have a detrimental effect during infection with single stranded RNA viruses (245-247). Apart from IFN production, NF-κB activation also induces secretion of IL-32. IL-32 induces production of pro-inflammatory cytokines like IL-6, IL-8, TNF-α, IL-1β (241, 248). In BIEC-c4 cells, we observed upregulation of TLR 8 and TLR 9 at 3-hour stimulation with poly (I:C). However, we did not observe any significant changes in TLR 3 expression.

Poly (I:C) complexed with transfecting reagent lyovec did not induce any significant changes in TLRs expressions in BIEC-c4 cells. Accumulation of intracellular dsRNA during viral replication and subsequent induction of IFN production by the host cell is different from the IFN produced by sensing of extracellular dsRNA (249). Intracellular dsRNA is sensed by RIG-I, MDA-5. RIG-I and MDA-5 belong to RIG-I like receptor (RLR) family. RIG-I senses blunt ended 5'phosphorylated dsRNA whereas MDA-5 recognizes long (>1000 nucleotide) dsRNA (58-60) Both RIG-I and MDA-5 are RNA helicases that have caspase recruitment domain (CARD) and helicase domain. Signal transduction after sensing of intracellular dsRNA is through CARD in both RIG-I and MDA-5. This results in activation of IRF-3 and NF-κB and subsequent production of IFNs (type I, and type III) and as well as pro-inflammatory cytokines like IL-6 and IL-8 (61-64).

Imiquimod is a synthetic guanosine analog with antiviral and anti-tumor activity (250). Imiquimod is an immune response modifier that specifically activates TLR 7 signaling pathway (251). Through MyD88 signaling cascade imiquimod induces activation of transcription factors like NF-κB, and MAPKs (mitogen activated protein kinases). Activation of these transcription factors lead to the induction of IFN-α, IL-12, TNF-α, IL-6 and other cytokines (250, 252-255). In this study, BIEC-c4 cells did not respond to imiquimod stimulation although they have been shown to express TLR 7 gene. Immune cells like phagocytes produce reactive oxygen species (ROS) during the microbial invasion. These ROS are highly reactive and induce oxidative damage to nucleic acids, lipids, and proteins. The Guanosine and cytosine are more prone to undergo oxidative damage due to their electronic configuration. The damaged guanosine may be sensed by TLR 7 and TLR 8 and produce necessary cytokines for activating immune cells (251, 256).

In this study, we studied the putative expression of TLRs by BIEC-c4 cells. There are limited number of studies on the expression of PRRs by the bovine ileal epithelial cell line. To the author's knowledge, no studies on the role of the bovine ileal epithelial cell line in innate immunity have been carried out. This study also analyzed the response of bovine ISEMFs to various PAMPs and associated cytokine expression but did not analyze expression of NLRs and RLRs genes. This study only analyzed changes in gene expression in response to various PAMPs. This study is limited in that it did not analyze whether alteration in mRNA expression was being carried out to protein level. No experiment to quantify cytokine levels in cell culture supernatant was performed and changes in IFN gene in response to viral PAMPs was not analyzed.  Despite these limitations, we demonstrated that bovine BIECs express TLRs 1-9 and respond to various bacterial PAMPs. These cells failed to respond to ligands of many cytoplasmic and endosomal PRRs. These cloned epithelial cells were homogenous in distribution and thus are not a true representative of tissue environment. These BIEC-c4 cells did not polarize, were spontaneously immortalized and did not allow replication of bovine rotavirus, bovine coronavirus and bovine viral diarrhea virus (unpublished data). We concluded that these are immature or undifferentiated epithelial cells. The BIEC-c4 clone could have arisen from intestinal stem cells and thus did not respond properly to ligands of cytoplasmic and endosomal PRRs. These cells were established from the 2-day old calf. Recent studies have shown that insufficient colonization by gut microbiota can lead to the defective immune system. Sufficient colonization by gut microbiota is essential for a fully functional immune system (281).

### 5.5 Conclusion

Based on this study we conclude that BIEC-c4 cells express TLRs 1-9 and respond to many bacterial PAMPs. Thus, these cell line can be used to accumulate knowledge of signal transduction in response to various bacterial PAMPs such as LPS, PGN and FLA. However, BIEC-c4 cell line did not respond to viral PAMPs. Differentiating these cells into more mature epithelial cells and analyzing their responses to ligands of cytoplasmic and endosomal PRRs can help decide their relevance as an in-vitro model. However, these cell line can be a good in-vitro model to study enteric bacterial pathogens.

**Chapter 6: Conclusions and future directions**

We successfully developed primary ileal myofibroblast cultures from the 2-day old bovine calf. These ileal intestinal sub-epithelial myofibroblasts (ISEMFs) showed phenotypic characteristics typical of myofibroblasts. On immunocytochemistry ISEMFs demonstrated the presence of α-smooth muscle actin and vimentin. But absence of cytokeratin which confirmed the presence of pure myofibroblast cells. Since primary cells can be grown for finite passages, we immortalized primary ileal ISEMFs using SV40 large T antigen. Glycobiology of primary ISEMF cells and immortalized ISEMFs showed differences for some lectins. TLR expression analysis showed no differences between primary and immortalized ISEMFs.

Earlier we had established primary bovine ileal epithelial cells (BIEC-c4) in our lab. Both primary ISEMFs and primary BIEC-c4 cells were from same calf and same ileal segment. In this study, we analyzed if both BIEC-c4 and ISEMF cells respond to various PAMPs. On analysis, both BIEC-c4 and ISEMF responded to bacterial PAMPs while only ISEMF mainly responded to ligands of cytoplasmic and endosomal PRRs.

Based on our finding we concluded that bovine ISEMFs can be a good model to study innate immune responses occurring at sub-epithelial compartment. Primary ISEMF cells can also be used to study PRRs signaling pathways. ISEMF cells have emerged as a mediator of diverse functions. ISEMFs are involved in wound healing, regulation of barrier function of the intestinal epithelium, differentiation and maturation of epithelium and in generating innate immune responses occurring at sub-epithelial compartment. ISEMF cells developed and characterized in our lab can be a good model to study intestinal inflammatory disease pathogenesis as well.

BIEC-c4 cells responded only to bacterial PAMPs. BIEC-c4 cells established in our lab was a cloned cell line. BIEC-c4 cells behaved like stem cells as they did not polarize on culture and kept growing for more than 100 passages without immortalization. Since they responded to bacterial PAMPs, we concluded that these cells can be used to study enteric bacterial disease pathogenesis.

Since our findings were based on RT-qPCR assay, further studies to corroborate these findings at protein levels are essential. Western blot to detect changes in TLR proteins upon stimulation with PAMPs can bolster the findings. Cytokine ELISA of cell supernatants after stimulation with PAMPs can further support our data. Bacterial invasion assay on this BIEC-c4 cells and subsequent analysis of TLRs expression could mimic in-vivo conditions. Transforming immature BIEC-c4 cells to more mature and differentiated epithelial cells expressing tight junction proteins should be carried out. A 2D co-culture of primary ISEMF and BIEC-c4 cells to investigate ISEMFs role in maturation and differentiation of intestinal epithelial could be next project using these cells.

Overstimulation of TLRs often leads to excessive cytokine production which can be detrimental to host. A detailed understanding of key signaling molecules involved in TLR signaling in these cells as a model can be beneficial in developing therapeutic strategies of various infectious diseases.

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