Characterization of Ovine Ileal Epithelial and Myofibroblast Cell Lines and their Role in Innate Immunity

Shaurav Bhattarai

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CHARACTERIZATION OF OVINE ILEAL EPITHELIAL AND MYOFIBROBLAST CELL LINES AND THEIR ROLE IN INNATE IMMUNITY

BY

SHAURAV BHATTARAI

A thesis submitted in partial fulfillment of the requirement for the Master of Science Major in Biological Sciences Specializing in Microbiology South Dakota State University 2020
THESIS ACCEPTANCE PAGE

Shaurav Bhattarai

This thesis is approved as a creditable and independent investigation by a candidate for the master’s degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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# TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................... viii

LIST OF TABLES .............................................................................................................. xi

ABSTRACT ....................................................................................................................... xii

1. Literature review ....................................................................................................... 1

   1.1 Innate immunity at the mucosal site ................................................................. 1

   1.2 Intestinal epithelial cells role in innate immunity ......................................... 6

       1.2.1 Mucosal barriers of the intestine ......................................................... 6

       1.2.2 Mediation role played by intestinal epithelial cells ......................... 7

   1.3 History of toll-like receptors ............................................................................ 9

   1.4 TLRs signaling pathway ................................................................................. 13

       1.4.1 MyD88 dependent pathway ............................................................... 19

       1.4.2 TRIF-dependent pathway .................................................................. 22

       1.4.3 Negative regulators of TLR signaling ............................................... 24

2. Establishment and Characterization of Ovine Ileal Epithelial Cell lines .......... 25

   2.1 Introduction ....................................................................................................... 26

   2.2 Materials and methods ................................................................................... 29

       2.2.1 Isolation and establishment of ovine ileal epithelial cell (OIEC) culture .......................................................... 29
2.2.2 Immunocytochemical staining of primary and hTERT immortalized epithelial cells ................................................................. 29

2.2.3 Human telomerase reverse transcriptase (hTERT)-mediated immortalization of cells ................................................................. 31

2.2.4 Confirmation of hTERT-mediated immortalization of ovine ileal epithelial cells ................................................................. 32

2.2.5 Study of cell growth kinetics ................................................................. 33

2.2.6 Barrier integrity ........................................................................... 33

2.2.7 Lectin binding assay ..................................................................... 37

2.2.8 Testing for mycoplasma contamination ........................................ 38

2.2.9 Statistical analysis ......................................................................... 38

2.3 Results ............................................................................................... 42

2.3.1 Primary OIEC and hTERT immortalized OIEC display epithelial phenotype ............................................................................ 42

2.3.2 Immortalization was confirmed by conventional PCR and immunocytochemistry ................................................................. 45

2.3.3 Primary and hTERT OIEC exhibits stable proliferation ability ...... 47

2.3.4 Cells were able to exhibit barrier integrity and express functional tight junction protein ........................................................................ 51

2.4 Glycosylation profile of Primary OIEC and hTERT-OIEC ............... 55

2.4.1 Cells were free from mycoplasma contamination .......................... 56

2.5 Discussion .......................................................................................... 56

2.6 Conclusion .......................................................................................... 62
3. Development and characterization of ovine ileal sub-epithelial myofibroblast (ISEMF) cells and studying its responses to various bacterial and viral ligands ................................................................. 63

3.1 Introduction................................................................................................................. 64

3.2 Materials and Methods.............................................................................................. 65

3.2.1 Isolation and establishment of primary sheep ileal sub-epithelial myofibroblast cell culture ................................................................. 65

3.2.2 Immortalization of ISEMF cells ........................................................................... 66

3.2.3 Confirmation of SV40 immortalization ................................................................. 67

3.2.4 Immunocytochemistry (ICC) of primary ISEMFs................................................. 68

3.2.5 Stimulation of Primary ISEMF cells with PRR ligands ....................................... 69

3.2.6 RNA extraction and reverse transcription .......................................................... 71

3.2.7 Real time quantitative PCR for studying changes in gene expression .................. 71

3.2.8 Statistical analysis of RT-qPCR data .................................................................. 75

3.3 Results....................................................................................................................... 76

3.3.1 Phenotypic characterization of the primary and SV40 immortalized ovine ISEMFs ......................................................................................... 79

3.3.2 Innate immune responses of primary ovine ISEMF cells after stimulating with bacterial ligands ................................................................. 80

3.3.3 Innate immune responses of primary ISEMF cells after stimulation with cytosolic and endosomal viral ligands ........................................ 81

3.4 Discussion .................................................................................................................. 94
3.5 Conclusion ........................................................................................................ 100

4. Conclusions and future directions.................................................................... 101

References............................................................................................................. 104
LIST OF FIGURES

Fig 1: Diagrammatic representation of mucosal barrier in the intestine................................. 4

Fig 2: Diagrammatic overview showing imbalance between mucosal barriers and gut microbes resulting to intestinal inflammation............................................................... 5

Fig 3: Detail timeline history of TLRs and different milestones of TLR research........ 12

Fig 5: Intracellular TLR signaling pathway and graphic illustration of TLR trafficking..23

Fig 6: Ovine ileal epithelial cells (OIEC) demonstrate normal epithelial cell morphology under phase-contrast microscope................................................................. 39

Fig 7: Immunocytochemical (ICC) staining of primary-OIEC confirms the epithelial origin of the cells.. ........................................................................................................ 40

Fig 8: Immunocytochemical (ICC) staining of hTERT-OIEC confirms the epithelial origin of the cells. ........................................................................................................ 41

Fig 9: Gel image reveals presence of hTERT gene in hTERT-OIEC.............................. 43

Fig 10: Immunocytochemical (ICC) staining shows presence of hTERT protein in hTERT-OIEC........................................................................................................... 44

Fig 11: Growth curve of primary-OIEC and hTERT-OIEC........................................ 46

Fig 12: Mean population doubling time (PDT) of primary-OIEC and hTERT-OIEC... 47

Fig 13: TEER reading of primary-OIEC and hTERT-OIEC over a period of 10 days. 48

Fig 14: Immunofluorescence revealed expression of tight junction protein in primary-OIEC. .................................................................................................................. 49

Fig 19: Primary-OIEC and hTERT-OIEC were free of mycoplasma contamination.... 55

Fig 20: Phase contrast image of primary and SV40 immortalized ovine ISEMF cells... 76
Fig 21: Immunocytochemical staining of primary ovine ileal ISEMF cells confirms the mesenchymal origin of the cells. ................................................................. 77

Fig 22: Gel image reveals presence of SV40 gene in SV40 immortalized ovine ISEMF cells. ................................................................. 78

Fig 23: Change in gene expression profile of different TLRs in ovine ISEMF cells after LPS stimulation................................................................. 82

Fig 24: Change in gene expression profile of MyD-88, NF-Kb, and different cytokines in ovine................................................................. 83

Fig 25: Change in gene expression profile of different TLRs in ovine ISEMF cells after PGN stimulation................................................................. 84

Fig 26: Change in gene expression profile of MyD-88, NF-Kb, and different cytokines in ovine ISEMF cells after PGN stimulation. ................................................................. 85

Fig 27: Change in gene expression profile of different TLRs in ovine ISEMF cells after flagellin stimulation. ................................................................. 86

Fig 28: Change in gene expression profile of MyD-88, NF-Kb, and different cytokines in ovine ISEMF cells after flagellin stimulation................................................................. 87

Fig 29: Change in gene expression profile of different TLRs in ovine ISEMF cells after imiquimod stimulation................................................................. 88

Fig 30: Change in gene expression profile of MyD-88, NF-Kb, and different cytokines in ovine ISEMF cells after imiquimod stimulation................................................................. 89

Fig 31: Change in gene expression profile of different TLRs in ovine ISEMF cells after poly I:C stimulation. ................................................................. 90
Fig 32: Change in gene expression profile of MyD-88, NF-Kb, and different cytokines in ovine ISEMF cells after poly I:C stimulation. ................................................................. 91

Fig 33: Change in gene expression profile of different TLRs in ovine ISEMF cells after poly I:C with lyovec stimulation................................................................. 92

Fig 34: Change in gene expression profile of MyD-88, NF-Kb, and different cytokines in ovine ISEMF cells after poly I:C with lyovec stimulation. ........................................ 93
LIST OF TABLES

Table 1: Details on TLR’s cellular location and their ligands.................................16

Table 2: Lectins and inhibitors used for studying surface glycans of Primary-OIEC and hTERT-OIEC........................................................................................................................................35

Table 3: Primers used to amplify SV40 large T antigen gene in SV40 immortalized ovine ISEMF cells. ........................................................................................................................................67

Table 4: Details of ligands used in the stimulation of primary ovine ISEMF cells........70

Table 5: Details of primers used in studying TLRs and cytokine expressions in primary ovine ISEMF cells........................................................................................................................................73
ABSTRACT
CHARACTERIZATION OF OVINE ILEAL EPITHELIAL AND MYOFIBROBLAST CELL LINES AND THEIR ROLE IN INNER IMMUNITY
SHAURAV BHATTARAI

2020

Development of continuous cell lines has been an indispensable tool for studying disease pathogenesis at cellular and molecular level. Intestinal epithelial cells serve as a barrier between the luminal contents and internal tissue and face unique challenge in maintaining homeostasis. Any form of disruption in the uppermost intestinal epithelium exposes underlying intestinal subepithelial myofibroblast (ISEMF) cells to the luminal content, which results in the generation of immune responses from ISEMF cells. Our main goal was to establish and characterize intestinal epithelial and myofibroblast cell lines from a 3-day old sheep. ISEMF cells play significantly important role in wound healing, tissue regeneration, production of extracellular matrix, epithelial cell migration, and differentiation. There are only few ISEMF cell lines available from ruminant origin. Limited number of studies on innate immune response of ISEMF cells have impeded our understanding of the role of ISMEF cells in innate immunology.

In the study, we first established primary ovine ileal epithelial cell (OIEC) cultures and subsequently immortalized primary OIEC with hTERT gene (hTERT-OIEC). Both primary and hTERT immortalized OIEC cells displayed typical cobblestone morphology and expressed cytokeratin protein validating their epithelial origin. Epithelial cells were able to polarize, form tight junction proteins and express different surface glycoconjugates. Furthermore, we developed and characterized ovine ileal sub-epithelial
myofibroblast cells (ISEMF) from the same 3-day old male lamb and immortalized them using SV40 large T-antigen (SV40-ISEMF). We stimulated primary ovine ISEMF with lipopolysaccharide (LPS), peptidoglycan (PGN), flagellin, imiquimod, poly I:C and poly I:C with lyovec for 3 h and 24 h. We analyzed mRNA expressions of various ovine TLRs (1,2,3,4,5,6 and 9) and cytokines. Upon ligand stimulation, primary ovine ISEMF cells were able to modulate their innate immune responses by expressing variable levels of TLRs and cytokines. Hence, both OIEC and ISEMF cell lines can serve as valuable in vitro tools in elucidating enteric disease pathogenesis.
1. Literature review

1.1 Innate immunity at the mucosal site

Over past few decades increase in scientific studies in the field of innate immunology has enabled in better understanding of host-pathogen interactions. Role of mucosal tissues like digestive tract, urogenital tract, respiratory tract in innate immunity has been greatly appreciated by several studies. Digestive tract harbors trillions of microbes and this unique interaction of intestinal microbiome with the host has gained the spotlight in the field of mucosal immunity. Discovery of pattern recognition receptors (PRRs) in late 1990s and, after a decade, development of tools for characterization of culture-independent microbiome were the major two advancements in unravelling the world of innate immunity (1-4).

The major role of digestive system is to digest the food and absorb the nutrients. During the ingestion of food, microorganisms make their way towards the intestine and colonize. Small intestine harbors a large number of microbes and highest number of immune cells in the body. Intestinal epithelial cells (IECs), the outermost cells of the intestine, act as the first line of defense. The intestinal epithelium is the largest mucosal surface of the body and is constantly renewed by intestinal epithelial stem cells residing in the intestinal crypts. IECs can be classified into major two groups: enterocytes and secretory IECs. Enterocytes take part in metabolic activities of the intestine like digestion and absorption of nutrients. Secretory IECs comprise goblet cells, Paneth cells, and enteroendocrine cells. Secretory cells are specialized in creating barrier between host tissue and microbes. Goblet cells secret mucins, heavily glycosylated proteins, which
prevent direct contact between microbes and underlying epithelial cells. Antimicrobial peptides (AMPs) secreted by Paneth cells also create chemical barrier between host and the microbes in the intestine (5-7).

IECs are constantly exposed to the microbes and pose unique challenge to maintain balance between immunity against pathogens and tolerance towards commensal. In the absence of innate immune system this mutualism between host and microbes is lost which validates the idea that sensing of microbes by host cells is highly regulated in a coordinated manner (8, 9). Sensing of microbiota by IECs aids to gather information about surrounding microbial environment (1). Though IECs are considered as nonprofessional immune cells, they are equipped with wide range of innate immune receptors. Innate immune receptors including toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-1 like receptors (RLRs), the C-type lectin receptors, the absent in melanoma 2 (AIM2) like receptors and the OAS-like receptors help in constant surveillance for the presence of pathogenic microorganisms in the host tissue (10). Toll-like receptors present in IECs sense presence of microbial proteins and signal through myeloid differentiation protein MyD88 or TIR-domain-containing adapter-inducing interferon-β (TRIF) to generate inflammatory immune responses. Paneth cells, a subset of IECs, express high level of NOD2 receptors which upon sensing peptidoglycan initiate array of immune responses including autophagy, cytokine secretion, and epithelial cell regeneration. NOD1 expressed in IECs plays role in mediating homeostatic bacterial colonization and generation of lymphoid follicles in the intestine (8, 11-17).

Pattern recognition receptors (PRRs) expressed in IECs also play role in clearance of infected cells. IECs expressing NLRC4 (NLR family CARD-domain-containing
protein 4) recognize flagellin and initiate the expulsion of pathogen. A member of NOD-family protein NLRP6 (NLR Family Pyrin Domain Containing 6) expressed in IEC regulates the interaction between host and microorganisms by producing cytokines like IL-18 and inhibiting expression on antimicrobial peptides. Lower expression of NLRP6 leads to intestinal dysbiosis, increased susceptibility to bacterial infection, and altered microflora in the gut. NLRP6 also regulates viral microbiome interaction in the gut (18-21).
Fig 1: Diagrammatic representation of mucosal barrier in the intestine. In small intestine segregation between microbes and IECs is dictated mainly by AMPs produced by Paneth cells. However, in large intestine mucin produced by goblet cells creates the barrier between host and microbiome. (3)
Fig 2: Diagrammatic overview showing imbalance between mucosal barriers and gut microbes resulting to intestinal inflammation. (3)
1.2 Intestinal epithelial cells role in innate immunity

Intestinal epithelial cells (IECs) create barrier between environmental factors and host immunity. This barrier includes mucus layer, secreted antimicrobial peptides, and several other innate immune receptors to activate immune system. As described by Ryu Okumura and Kiyoshi Takeda (3), IECs play a role in segregation and mediation to maintain the homeostasis. IECs create physical and chemical barrier between host immune cells and gut microbiome referred as segregation. Segregation between host immune compartments and gut microbes prevent unwanted intestinal inflammation. Similarly, IECs also mediate signals between host tissue and gut microbes. IECs upon sensing gut microbes produce immune chemical messengers (cytokines and chemokines) which stimulate adaptive immune responses from T-cells and B-cells. Furthermore, chemokines attract immune cells like macrophages and neutrophils to the site and initiate the immune responses. Activation of IECs has also been linked to increase the activity of antigen-presenting cells (APCs) in lymphoid tissue leading to production of antigen-specific IgA antibody responses in the intestinal mucosal surface. Intestinal disease such as inflammatory bowel disease (IBD) has been associated with reduced production of mucus and AMPs. Cytokines such as IL-17 and IL-22 produced by activated T-cells control overgrowth of opportunistic pathogens in the intestine (3).

1.2.1 Mucosal barriers of the intestine

There are mainly two types of intestinal barriers: physical and chemical barrier. Physical barrier comprises of mucus layer on intestinal surfaces, glycocalyx on absorptive IEC, and tight junction proteins. The main role of these barriers is to limit the microbial invasion into host tissue. Mucus secreted by goblet cells consists of mucin
glycoproteins and forms gel like structure. Goblet cells are more abundant in large intestine than in small intestine. Microbial load is higher in large intestine than in small intestine; hence, the mucus layer is thicker in large intestine (22-25). Mucus layer comprises of two distinct layers, inner and outer layer (26). A gel forming O-glycosylated protein, MUC2 plays an important role in maintaining the mucus barrier on the intestinal surface. The inner mucus layer is mostly free of any microbial invasion and mice model lacking MUC2 have been linked to increase bacterial invasion in colonic epithelium (26).

Intestinal epithelial cells also produce defensin family of proteins which are antimicrobial proteins. Secretion of immunoglobulin IgA is also mediated by IECs. These chemical barriers protect the host tissues against invasion from gut microbiome. Paneth cells, the antimicrobial peptide producing cells, are absent in large intestine. So, large intestine heavily relies on thick mucus layer produced by goblet cells in creating the barrier from the luminal content (27). However, special proteins like glycosylphosphatidylinositol (GPI)-anchored protein known as Ly6/ plaur domain-containing 8 (Lypd8) found in large intestine helps in segregating gut microbes from underlying epithelium of large intestine (28). Similarly, regenerating islet-derived 3 (Reg3) protein produced by Paneth cells in small intestine cells also creates chemical barrier on the intestinal mucosal surface (29, 30).

1.2.2 Mediation role played by intestinal epithelial cells

Intestinal microenvironment is altered by various factors such as viral infections, changes in diet, stress and sometimes even by jet lag (31-36). These changes in intestinal environment can lead to generation of immune responses leading to intestinal dysbiosis. IECs play crucial role in signaling any changes in gut environment to host immune cells.
Commensal bacteria like segmented filamentous bacteria found in ileum induce production of serum amyloid A protein which helps in differentiation of Th17 cells. These bacteria also induce production of IL-22 (37-40). Attachment of certain bacteria like *Citrobacter rodentium* upregulates the production of reactive nitrogen species (37). Production of cytokines upon sensing the microbes by IECs recruits immune cells to the mucosal surface. Similarly, M cells found in follicles of the intestinal epithelium uptake the antigen from the intestinal lumen and present it to APC cells. This function of M cells significantly play role in the production of antigen specific IgA antibody (41).
1.3 History of toll-like receptors

Field of innate immunology started kicking off following the discovery of the toll protein in *Drosophila melanogaster*. Initially, before the identification of toll-like receptors (TLRs), role of innate immunity was considered only as initiator of adaptive immune system. The roles of inflammatory cytokines such as interleukin-1 (IL-1), IL-6, tumor necrosis factor (TNF), and antiviral cytokines interferons (IFN) were not elucidated (42). TLRs are the first recognized pattern-recognition receptors (PRRs) which are present on the cell surface or endosomes. TLRs sense either conserved molecular pattern from the microbes referred as pathogen associated molecular patterns (PAMPs) or proteins from the damaged cells or tissues referred as damage associated molecular pattern (DAMPs). Charles Janeway postulated the theory of PRRs recognizing PAMPs (43). It all started after the identification of IL-1 receptor (IL-1R) in mammals (44). In 1980s, IL-1R was identified and their involvement in the T cell activation, inflammation was studied. In 1988, IL-1R gene was cloned and its structure was predicted (45) and in 1991, IL-1R domain was found to be homologous with cytosolic domain of Toll protein found in *Drosophila melanogaster* (46). By that time, role of toll protein was only considered in the development of dorso-ventral polarity in drosophila. Then later it was established that toll and IL-1R protein were sharing common amino acids required for nuclear factor-Kb (NF-kB) signaling (47). NF-kB is a transcription factor and was first identified in B cells (48, 49). NF-kB controls number of genes responsible for inflammation and generation of immune responses and both IL-1R and toll protein were found to activate NF-kB signaling pathway.
Around 1994, proteins with leucine-rich repeats were identified from mammals and those proteins resembled with toll protein than IL-1R (50). In 1997, a protein homologous to toll was identified from mammals and named as hToll (51). Co-transfection of human monocytes with CD4-hToll chimeric protein led to the expression of genes regulated by NF-Kb including CD80 gene (51). CD80 is a protein that involves in the stimulation of T cell through CD28 (42). This experiment provided an important link between innate and adaptive immunity and the role of hToll in the signaling of antigen presenting cells was considered significantly important. This experiment also provided important clue that hToll might be an ideal PRRs recognizing PAMPs, the same theory that Janeway postulated earlier (43).

By the year 1998, five toll homologues proteins were identified from mammals and they were coined as Toll-like receptors (TLRs) and the initial hToll protein was renamed as TLR4 (52). These discoveries led scientists to speculate role of TLRs in innate immunology to use lipopolysaccharide (LPS), a gram-negative bacterial endotoxin, as PAMP candidate (42). During 1960s at the Jackson laboratory they observed mouse colony resistance to LPS and believed it be under the control of a single gene named \( LPS^d \) (42). Later in 1998 gene \( Lps^d \) was identified as \( Tlr4 \) and this work in \( Lps^d \) was awarded with novel prize (53). In 1999, TLR4 knockout mice failed to respond to LPS and this confirmed TLR4 as a receptor for LPS (54).

After proving the involvement of TLR4 in LPS recognition, other PAMPs were tested as potential ligands for TLRs. TLR2 was found to recognize peptidoglycan, TLR1 to sense triacylated lipopeptides, TLR6 engages with diacylated lipopeptides, TLR5 recognize bacterial flagellin (55-59). Until the discovery of TLR3 in 2001, role of TLRs
in viral infection was not identified (60). TLR3 senses double-stranded RNA and generates both NF-kB and type I IFN responses (60). Till date 10 TLRs in human have been identified. Similarly, in bovine 10, ovine 10 and in mice 13 TLRs have been identified (42, 61, 62). The detail timeline chart about the history of TLRs is shown below:
Fig 3: Detail timeline history of TLRs and different milestones of TLR research (42).
1.4 TLRs signaling pathway

Toll-like receptors (TLRs) are type-I transmembrane protein consisting of leucine-rich ectodomains recognizing PAMPs, transmembrane domains and intracellular Toll-interleukin 1 receptor (TIR) which mediates downstream signaling. TLR1 to TLR9 are conserved in most of the species, however in mice TLR10 is not functional due to retroviral insertion and TLR11 to 13 have not been identified in humans. Based on the cellular location, TLRs are broadly classified into two groups, cell surface TLRs and intracellular TLRs. Cell surface TLRs comprises of TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 and mostly recognize microbial surface components including lipids, lipoprotein, glycolipids. Intracellular TLRs are present only in the vesicles like endosomes, endolysosomes, lysosomes, endoplasmic reticulum and they comprise of TLR3, TLR7, TLR8, TLR9, TLR11, TLR 12 and TLR13. Intracellular TLRs sense nucleic acids derived from microbes and occasionally in autoimmunity can recognize self-nucleic acids as well (63-66).

TLR4 was the first identified member of TLR family and recognizes lipopolysaccharide (LPS), an outer membrane protein of gram-negative bacteria. LPS is widely known for causing sepsis (67). TLR4 forms a complex with MD-2, a cell surface protein and binds with LPS to initiate immune responses (68). Five out of six hydrophobic chains of LPS bind with MD-2 and remaining one engages with TLR4, this TLR4-MD2-LPS complex then becomes activated and initiates downstream signaling (69, 70). Similarly, CD14 and LPS-binding protein (LBP) are also involved in generating TLR4 mediated immune responses (71). LPB and CD14 binds with LPS and presents LPS bound with LPB to TLR4-MD2 complex. Apart from LPS, TLR4 is also involved in
sensing mouse mammary tumor viruses, *streptococcus pneumonia* pneumolysin, respiratory virus fusion proteins, and paclitaxel (67).

TLR2 is another extensively studied member of TLR family. TLR2 is mainly involved in recognizing peptidoglycan, a major component of gram-positive bacterial cell wall (67). TLR2 heterodimerizes with TLR1 or TLR6. TLR1/2 complex recognize triacylated lipopeptides present on gram-negative bacteria and mycoplasma. Similarly, TLR2/6 complex senses diacylated lipopeptides from gram-positive bacteria and mycoplasma (59, 71). TLR2 ligands mainly mediates the production of inflammatory cytokines through NF-Kb signaling. However, in monocytes, upon viral infection TLR2 can mediate production of type I interferons (72).

TLR5 senses engagement with bacterial flagellin (64). Intestinal dendritic cells have high expression of TLR5 on their surfaces (67). TLR3 has horseshoe shaped ectodomain which enables its binding with double-stranded RNA (dsRNA) (73, 74). Most viruses produced dsRNA as their replication intermediate and this is sensed by endosomal TLR3. In humans, lower expression of TLR3 was found be linked with increase in susceptibility to viral infections. TLR7 senses single-stranded RNA (ssRNA) from viruses, small interfering RNAs, and RNA from streptococcus B bacteria in conventional dendritic cells (75). TLR8 is closely related to TLR7 and senses bacterial and viral ssRNA (76). TLR9 responds to unmethylated 2’-deoxyribo cytidine-phosphate-guanosine (CpG) motif DNA commonly found in bacteria and viruses (77). TLR9 drives TH1 responses. Apart from CpG, TLR9 also recognizes hemozoin, a product of hemoglobin digestion in plasmodium (77).
TLRs synthesis takes place in endoplasmic reticulum, then transported to golgi and finally gets trafficked to either cell surface or to their precise intracellular location. TLR7 and TLR9 are found in endoplasmic reticulum in unstimulated cells and gets transported to endolysosomes upon stimulation. This transportation of TLRs from ER to endosomes is mediated by UNC93B1 protein (78-80).
Table 1: Details on TLR’s cellular location and their ligands (66).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Cellular localization</th>
<th>Microbial component(s)</th>
<th>Origin(s)</th>
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<tbody>
<tr>
<td>TLRs</td>
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<tr>
<td>TLR1/TLR2</td>
<td>Cell surface</td>
<td>Triacyl lipopeptides</td>
<td>Bacteria</td>
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<tr>
<td>TLR2/TLR6</td>
<td>Cell surface</td>
<td>Diacyl lipopeptides</td>
<td><em>Mycoplasma</em></td>
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<td>Lipoteichoic acid</td>
<td>Gram-positive bacteria</td>
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<td>TLR2</td>
<td>Cell surface</td>
<td>Lipoproteins</td>
<td>Various pathogens</td>
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<td>Peptidoglycan</td>
<td>Gram-positive and -negative bacteria</td>
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<td>Lipoarabinomannan</td>
<td><em>Mycobacteria</em></td>
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<td>Porins</td>
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<td>Envelope glycoproteins</td>
<td>Viruses (e.g., measles virus, HSV, cytomegalovirus)</td>
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<td>GPI-mucin</td>
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<td>Cell surface/endosomes</td>
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<td>TLR5</td>
<td>Cell surface</td>
<td>Flagellin</td>
<td>Flagellated bacteria</td>
</tr>
<tr>
<td>TLR7/8</td>
<td>Endosome</td>
<td>ssRNA</td>
<td>RNA viruses</td>
</tr>
<tr>
<td>TLR9</td>
<td>Endosome</td>
<td>CpG DNA</td>
<td>Viruses, bacteria, protozoa</td>
</tr>
<tr>
<td>RLRs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIG-I</td>
<td>Cytoplasm</td>
<td>dsRNA (short), 5′-triphosphate RNA</td>
<td>Viruses (e.g., influenza A virus, HCV, RSV)</td>
</tr>
<tr>
<td>MDA5</td>
<td>Cytoplasm</td>
<td>dsRNA (long)</td>
<td>Viruses (picorna- and noroviruses)</td>
</tr>
<tr>
<td>NLRs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOD1</td>
<td>Cytoplasm</td>
<td>Diaminopimelic acid</td>
<td>Gram-negative bacteria</td>
</tr>
<tr>
<td>Receptor</td>
<td>Cellular localization</td>
<td>Microbial component(s)</td>
<td>Origin(s)</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------</td>
<td>------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NOD2</td>
<td>Cytoplasm</td>
<td>MDP</td>
<td>Gram-positive and -negative bacteria</td>
</tr>
<tr>
<td>NALP1</td>
<td>Cytoplasm</td>
<td>MDP</td>
<td>Gram-positive and -negative bacteria</td>
</tr>
<tr>
<td>NALP3</td>
<td>Cytoplasm</td>
<td>ATP, uric acid crystals, RNA, DNA, MDP</td>
<td>Viruses, bacteria, and host</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAI</td>
<td>Cytoplasm</td>
<td>DNA</td>
<td>DNA viruses, intracellular bacteria</td>
</tr>
<tr>
<td>AIM2</td>
<td>Cytoplasm</td>
<td>DNA</td>
<td>DNA viruses</td>
</tr>
<tr>
<td>PKR</td>
<td>Cytoplasm</td>
<td>dsRNA, 5′-triphosphate RNA</td>
<td>Viruses</td>
</tr>
</tbody>
</table>

Cytoplasmic portion of TLRs consist of TIR domain which recruits adapter proteins to initiate downstream signaling cascade (81). There are five main proteins involved in TLR signaling cascade; myeloid differentiation factor 88 (MyD88), TIR
domain containing adaptor protein inducing interferon-β (TRIF, also called TICAM1), MyD88-adaptor-like (MAL or TIRAP), TRIF-related adaptor molecules (TRAM or TICAM2), and sterile α-and armadillo-motif containing protein (SARM) (82, 83).

Among these proteins, MyD88 and TRIF act as major TIR adaptor protein. Similarly, TRAM and MAL act by translocating MyD88 or TRIF to activate TLRs. While SARM acts as negative regulator of TLR signaling (84-87).

1.4.1 MyD88 dependent pathway

Following the engagement of TLR with PAMPs, MyD88 forms a complex known as myddosome by recruiting members of IL-1R associated kinases (IRAK family). Signaling cascade starts after the activation of IRAK4 which then activates IRAK1. Activation of IRAK1 leads to autophosphorylation of IRAK1 at several sites. IRAK1 activation is followed by IRAK2 activation (88-90). Activated IRAK1 engages with TNF receptor associated factor 6 (TRAF6) which is E3 ubiquitin ligase and synthesize polyubiquitin linked to Lys63. TRAF6 promotes the polyubiquitination on itself and TAK1 protein. TAK1, member of mitogen activated protein (MAP) kinase kinase kinase (MAPKKK), form association with TAB1, TAB2, and TAB3 proteins. Members of TAB protein families regulate the activation of TAK1 kinase. Activated TAK1 results into activation of two distinct signaling pathway; NF-kB pathway and MAPK signaling pathway (64, 91, 92).

In mammalian cells NF-Kb, dimeric transcription factor, consists of five members: RelA (P65), RelB, CreI, p105 (precursor of p50), and p100 (precursor of p52). Under normal conditions NF-Kb is inactivated and found in the cytoplasm. During
inactive phase, NF-Kb is in contact with its inhibitory proteins IkB (inhibitor of kB) and these inhibitory proteins constrain the nuclear localization signal of NF-Kb. TAK1 binds to IKK (IkB kinase) and this leads to the phosphorylation of NF-Kb inhibitors leading to unmasking of nuclear localization signal of NF-Kb (93, 94). This results into the nuclear localization of NF-Kb and transcription of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-a. Similarly, activated TAK1 leads to activation of MAPK family of proteins such as ERK1/2, P38 and c-Jun N-terminal Kinase (JNK). This results into activation of activator protein 1 (AP-1) family of transcription factors. Activation of AP-1 also leads to expression of genes responsible in inflammatory immune responses (67, 95).
Fig 4: Schematic flow of TLR signaling along with negative regulators of TLR signaling.
adapted from (64)
1.4.2 TRIF-dependent pathway

This pathway is also often referred as MyD88-independent pathway. In this pathway, TRIF interacts with TRAF6 and TRAF3, then TRAF6 recruits RIP-1 kinase which activates TAK1 kinase. Activated TAK1 kinase results into activation of NF-Kb and MAPK pathway ultimately leading to the production of inflammatory cytokines. However, TRAF3 engages with IKK kinases complex and activates TBK1, IKKe and NF-Kb essential modulator (NEMO). Activated NEMO then phosphorylates interferon regulatory factor (IRF3) which is a transcription factor for the expression of IFN genes. Then, activated IRF3 dimerizes and translocate into the nucleus leading to the mRNA expression of type-I interferon genes (63, 67).

An inositol lipid, PtdIns5P also activates IRF3. PtdIns5P forms a complex by interacting with TBK1 and IRF3, this close interaction between TBK1 and IRF3 results into phosphorylation of IRF3. Phosphoinositide Kinase, FYVE-Type Zinc Finger Containing (PIKfyve) kinase was found be involved in the making of PtdIns5P (96).
Fig 5: Intracellular TLR signaling pathway and graphic illustration of TLR trafficking.
1.4.3 Negative regulators of TLR signaling

Over stimulation of TLRs can be detrimental to the host tissues. To maintain balance between immunity and tolerance negative regulation of TLR signaling is important. Excessive stimulation of TLRs can cause autoimmune disorders or can develop inflammatory conditions. TLR signaling is inhibited at multiple steps by number of molecules. MyD88 signaling is inhibited by ST2825, SOCS1, and Cb1-b (97). Similarly, SARM and TAG blocks the activation of TRIF pathway (98). These molecules block the binding of MyD88 or TRIF to the TLRs or the adaptor molecule. TRAF6 is negatively regulated by A20, USP4, CYLD, TANK, TRIM30a (99, 100). Likewise, TRAF3 activation is suppressed by SOCS3 and DUBA molecule (101). Stability of genes resulting from TLR signaling are also regulated by miRNA like miR-146a, miR199a, and miR148/152 (102). Regnase-1 and TPP negatively regulates the stability of inflammatory cytokines (63).
2. Establishment and Characterization of Ovine Ileal Epithelial Cell lines.

**Abstract**

Development of continuous cell line has been an indispensable tool for studying disease pathogenesis at cellular and molecular level. Intestinal epithelial cells maintain barrier between the luminal content and internal tissue and thus faces unique challenge in maintaining homeostasis. In this study, our main goal was to establish and characterize an intestinal epithelial cell line from a 3-day old sheep. We also immortalized ovine ileal epithelial cells (OIEC) with hTERT and generated hTERT immortalized ovine ileal epithelial cells (hTERT-OIEC). Both primary and hTERT immortalized cells displayed typical cobblestone morphology and expressed cytokeratin validating their epithelial origin. Similarly, cells were able to polarize, form tight junctions and express different surface glycoconjugates. Primary and hTERT immortalized ovine ileal epithelial cell lines developed in this study may serve as an invitro model of studying disease pathogenesis and innate immune responses.

**Keywords:** Ovine intestinal epithelial cell line, immortalization, hTERT, tight junction protein, lectin-binding profile.
2.1 Introduction

The intestinal mucosal surface plays an important role in creating balance between tolerance and immunity to maintain homeostasis (3, 103). Intestinal epithelial cells (IECs), uppermost layer of the intestinal mucosal surfaces, are primarily designed to absorb nutrients and water. In recent years, the role of intestinal epithelial cells have been explored beyond absorption and they play crucial role in shaping innate immune response in the host (6, 104). In addition to the physical barriers IECs maintains chemical barrier to segregate the luminal content from interacting with lamina propria and subsequently entering the systemic circulation. Viscous mucus produced by a subset of IECs, goblet cells, forms a protective layer above the intestinal epithelial creating a physical barrier for the pathogens. Similarly, tight junction proteins attach to the adjacent epithelial cells and form a tight seal to limit the movement of molecules and entry of pathogens into the host body. Furthermore, chemicals such as antimicrobial peptide (AMP), defensin proteins secreted by paneth cells, which are specialized subset of IECs, inhibits the contact between gut microbes and mucosal surface (3). Upon stimulation from microbes, IECs mediate the recruitment of immune cells like macrophages, neutrophils and T-cell through productions of various chemokines and cytokines (105).

Presence of various glycans on the surface of IECs helps in interacting with microbes by functioning as receptor for viruses or serving as site of attachment for bacteria (106). The adherence of C. jejuni reduced significantly when the glycosylation pattern of Caco2 was alerted (107). So, the presence or absence of specific sugar moieties on the intestinal surface makes the species susceptible or resistant to the pathogen. Recognition of mannose, galactose, N-acetylneuraminic acid, N-acetylgalactosamine, N-
acetylgucosamine, sialic acid and fucose group of glycans by microbes facilitates their attachment on the host cell surface (107-109). Following adherence, pathogen attempts to invade the epithelial cells barrier by disrupting the tight junction proteins. Tight junction proteins are considered as the first line of defense against penetration of epithelial cells. They are either transmembrane proteins like claudin, occludin or formed by cytoplasmic plaque like Zonula occludens (ZO-1). Presence of tight junction proteins limits the movement of ions and molecules through the epithelium (110). It helps in creating apical and basal polarity which can be measured in terms of resistance and recorded as trans-epithelial electric resistance (TEER) (111).

Species-specific IECs model provides close insight of host pathogen interaction and disease development process in the species. Recently, Campylobacter jejuni, chiefly colonize in small intestine, is associated globally with one of the major infectious cause of abortions in sheep (112, 113). Although attempts have been made using ferrets and murine in studying infection of Campylobacter jejuni however, lack of appropriate model has hindered in understanding the different outcomes in different species infected with the pathogen (114). Sheep is a natural host of Campylobacter Jejuni, and small intestine is the colonizing site for the bacteria. So, mechanistic detail of the disease can be studied in invitro using appropriate cell line model developed from the primary site of infection. Similarly, presence of the antibodies against emerging influence D virus in the serum of sheep and goats have gained widespread interest in studying the pathogenesis of influenza D virus in small ruminants (115). Salmonella sps., Escherichia coli, Mycobacterium avium subspecies paratuberculosis and rotavirus have been associated with diarrhea and other gastrointestinal upsets in sheep (116-118). Thus, intestinal
epithelial cell line model from sheep provides close resemblance of in vivo infection and help in developing strategies in dealing with various mucosal pathogens.

Multiple cell lines have been developed from sheep’s mammary gland, rumen, omasum, uterus, trophoblast, and kidney (119-123). Similarly, intestinal epithelial cell line models are available from bovine, porcine, and avian origin (124-127). However, there is no epithelial cell line available from the intestine of sheep. Thus, we have established and characterized ovine ileal epithelial cell line (OIEC) from 3-day old lamb. Often cells are transformed using immortalizing agents including human telomerase reverse transcriptase gene, simian virus 40 large T antigen, and human papilloma virus to generate continuous cell line (124, 128, 129). Due to their enhanced proliferation ability without entering the senescence, transformed continuous cell line provide a stable model for invitro study. In this study, we established primary ovine intestinal cells (OIECs) and immortalized primary OIEC with human telomerase reverse transcriptase (hTERT) gene. We also compared hTERT immortalized ovine ileal epithelial cells (hTERT-OIEC) with primary cells for different biochemical and physiological characteristics.
2.2 Materials and methods

2.2.1 Isolation and establishment of ovine ileal epithelial cell (OIEC) culture

Institutional Animal Care and Use Committee (IUCAC) of South Dakota State University, USA approved all the protocols of the animal experiment. Ileal intestinal tissue from 3-day old male lamb was collected in ice-cold DMEM/F-12 media containing 1% antibiotics. Both ends of the tissues were ligated and the luminal content was washed three times with DMEM/F-12 media with antibiotics. Then, tissues were cut open longitudinally and submerged in per-warmed dispase solution (1.2 units/ml, Roche Pharmaceutical) at 37°C for 90 minutes. After the incubation, suspension containing large number of digested cells were collected and centrifuged at 1200rpm for 5 minutes. Supernatant was discarded and isolated primary epithelial cell culture was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% Non-essential amino acids and 1% antibiotics (Penicillin-streptomycin). Cells were cultured at 37°C and 5% CO₂ in humidified incubator. Upon ~80% confluency, cells were passaged using 0.25% trypsin-EDTA (Ethylenediaminetetraacetic acid). Established epithelial cells morphology was observed under phase-contrast microscope.

2.2.2 Immunocytochemical staining of primary and hTERT immortalized epithelial cells

Primary and immortalized cells were stained against epithelial, fibroblast and muscle cell specific markers following previously described protocol (130). Briefly, cell suspension of 1*10^5 per ml was prepared from either primary-OIEC or hTERT-OIEC. Cytospins were prepared by centrifuging 100ul of cell suspension at 800 rpm for 5
minutes using cytofuge (Cytospin 3, Thermo Shandon Ltd., UK). Slides were air dried for 2 hours and fixed with absolute acetone for 7 minutes. After rehydrating slides with 1X PBS, endogenous peroxidase activity of cells was blocked with 0.3% hydrogen peroxide and 0.1% sodium azide in 1X PBS for 7 minutes. For blocking non-specific protein binding, cells were incubated with 1% goat serum for 20 minutes. Endogenous biotin was blocked using avidin/biotin kit (Vector laboratories) as per manufacture’s recommendation. Cells were treated with either primary antibody or isotype control antibody. Cell were incubated with 100ul of 1ug/ml primary mouse monoclonal antibodies (Sigma Aldrich) specific against cytokeratin (mAb C6909; IgG2a isotype), vimentin (mAb V5255; IgM isotype), alpha-smooth muscle actin (mAb A2457; IgG2a isotype), or desmin (mAb D1033; IgG1 isotype) for 1 hour at room temperature. For isotype control M9144 (IgG2a isotype), M5170 (IgM isotype), and M9269 (IgG1 isotype) antibodies (Sigma-Aldrich) were used. After an hour of incubation, slides were washed three times with PBS. Then, cells were incubated with isotype specific biotinylated goat-anti mouse IgG2a, IgM or IgG1 (Caltag laboratories) antibodies at 1:2000 dilution for 30 minutes. Following secondary antibody incubation, cells were incubated with streptavidin-horseradish peroxidase (Vector laboratories) for 30 minutes. After that, ready-to-use DAB (Diaminobenzidine) substrate (Vector laboratories) was added to develop the brown color for positive reaction. Finally, cells were counterstained with hematoxylin (Vector laboratories) to visualize the nucleus. Slides were observed and images were taken using BX53 upright microscope.
2.2.3 Human telomerase reverse transcriptase (hTERT)-mediated immortalization of cells

For hTERT immortalization of established primary-OIEC, earlier described protocol was adapted (124). Briefly, half-million primary-OIEC of passage 25 were seeded onto 3 wells of 6 well-plate. After 18 hours of seeding, cells were washed with 1X PBS. Then, 2ml serum free OPTI-MEM media (Gibco) was added onto cells. Similarly, 15ul of lipofectamine (Invitrogen) was mixed with 485ul of OPTI-MEM media and the mixture was incubated for 10 minutes at room temperature. After the incubation, in a separate tube, 45ul of 100ng/ul pGRN-145 plasmid (ATCC) containing hTERT gene was mixed with 205ul of the lipofectamine-OPTIMEM mixture and the whole mixture was incubated further for 30 minutes at room temperature. Then, in the first and second well, 125 ul of lipofectamine-OPTIMEM mixture without the plasmid was added. In the third well, 125ul of the lipofectamine and OPTIMEM mixture containing the plasmid was added. After 12 hours, cells were washed with 1X PBS and fresh DMEM media was added to replace OPTIMEM media. Cells in the first well were left as such after the addition of fresh media. However, in the second and the third wells selection antibiotic (hygromycin B) was added at 100ug/ml concentration. Thus, first well served as positive control for the cell growth, second well served as negative control of transfection and also as positive control for the cell death by selection antibiotic, and third well was used for generating hTERT-immortalized continuous OIEC cell line. Colonies resistant to selection antibiotics from the transfected well were propagated continuously in the presence of selection antibiotic using earlier described DMEM/high-glucose media.
2.2.4 Confirmation of hTERT-mediated immortalization of ovine ileal epithelial cells

Conventional PCR and immunocytochemistry were performed to confirm the development of hTERT immortalized OIEC. Genomic DNA was isolated from the primary-OIEC and hTERT-OIEC using DNeasy Blood & Tissue Kit (Qiagen). Nanodrop2000 spectrophotometer was used to quantity the extracted DNA. Equal amount (100ng) of isolated DNA was taken from primary OIEC, hTERT-OIEC and pGRN plasmid with h-TERT gene to amplify the hTERT gene using gene specific primers (Forward 5’ CGGAAGAGTGCTGAGCA; Reverse 5’ GGATGAAGCGGAGTGGA). PCR was performed using Taq PCR kit following the manufacturer’s protocol (New England Biolabs, Catalog number E5000S). The following PCR conditions was used; initial denaturation at 95°C for 10 min, 40 cycles of denaturation, annealing and extension at 95°C for 30 sec, 60°C for 30 sec, 72°C for 60 sec and final extension at 72°C for 60 sec. The PCR products were resolved in agarose gel and visualized using ODYSSEY-FC gel imaging system.

Immortalization was further confirmed at protein level by immunocytochemistry. Slides were prepared either from primary-OIEC or hTERT-OIEC. Non-specific staining was blocked by treating cells with 5% goat serum for 20 minutes. For the detection of hTERT protein, cells were incubated with 100ul (1.25ug/ml) of either rabbit anti-TERT polyclonal IgG (Santa Cruz Biotechnology Inc; SC7212) or normal rabbit IgG (SC3888) as isotype matched control. Slides were incubated overnight at 4°C. After washing slides three times with PBS, 100 ul of (1:1000 diluted) biotinylated goat-anti rabbit IgG (southern Biotech, cat 4050-08) was added. After incubating for 30 minutes cells were
washed and incubated with streptavidin-HPR (Vector laboratories) solution for 30 minutes. For colorimetric detection of the protein DAB (Vector laboratories) substrate was added. Nuclear counterstaining was performed by using hematoxylin (Vector laboratories) for 2 mins. Images were taken using Olympus BX53 upright microscope.

2.2.5 Study of cell growth kinetics

Twenty thousand primary-OIEC (Passages 23, 36, and 56) or hTERT-OIEC (passages 15, 26, and 60 after immortalization) cells were seeded in duplicates in 6-well plates. Total of five 6 well plates were setup. All the 6-well plates consisted of two wells of each primary OIEC and hTERT-OIEC. After 48 hours of seeding, cells in one of the 6-well plate were trypsinized and counted using hemocytometer and applying trypan blue exclusion method. The process was repeated every 24 hours for 5 days. The experiment was performed in triplicates using early, mid and late passage cells. Population doubling time was calculated using the formula DT= T \ln 2/\ln(X2/X1), where T denotes incubation time in hours, X1 and X2 refers to number of cells at the beginning and end of the incubation time (131).

2.2.6 Barrier integrity

TEER was measured using Evom voltmeter in ohms. Approximately 5*10^5 primary-OIEC or hTERT-OIEC cells were seeded onto 24mm diameter, 0.4um pore size polycarbonate filter inserts (Corning, Catalog number 3412). Two milliliters media was added on upper and basal chamber of the filter. In one well, filter insert was left unseeded for measuring background resistance. After 24 hours of seeding, resistance was measured
every 24 hours for a period of 10 days. The media was also changed in all the chambers after measuring the resistance. The experiment was performed in triplicates using early, mid and late passage cells.

After 10 days, indirect immunofluorescence assay was performed on polarized cells for staining tight junction proteins. Cells in the filter inserts were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton-X in PBS. Cells were blocked with 5% goat serum and incubated with rabbit polyclonal antibodies against occludin, ZO-1, claudin-1 and claudin-3 for one hour at room temperature. Then cells were incubated with Alexa-488 conjugated secondary goat anti rabbit IgG antibodies for 30 min at room temperature followed by nuclear counterstaining with propidium iodide. Images were taken using BX53 upright fluorescence microscope.
Table 2: Lectins and inhibitors used for studying surface glycans of Primary-OIEC and hTERT-OIEC (130).

<table>
<thead>
<tr>
<th>Lectins</th>
<th>Binding specificity of lectins</th>
<th>Inhibitors</th>
<th>Inhibitor concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Glucose/mannose group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Canavalia ensiformis</em> agglutinin (Con-A)</td>
<td>α-Man, α-Glc</td>
<td>α-Methyl mannoside, α-methyl glucoside</td>
<td>200 mM each</td>
</tr>
<tr>
<td><em>Pisum sativum</em> agglutinin (PSA)</td>
<td>α-Man, α-Glc</td>
<td>α-Methyl mannoside, α-methyl glucoside</td>
<td>200 mM each</td>
</tr>
<tr>
<td><em>Lens culinaris</em> agglutinin (LCA)</td>
<td>α-Man, α-Glc</td>
<td>α-Methyl mannoside, α-methyl glucoside</td>
<td>200 mM each</td>
</tr>
<tr>
<td><strong>2. N-Acetylgalactosamine group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Griffonia simplicifolia</em> lectin I (GSL-1)</td>
<td>α-GalNAc, α-Gal</td>
<td>Galactose</td>
<td>200 mM</td>
</tr>
<tr>
<td>Soybean (<em>Glycine max</em>) agglutinin (SBA)</td>
<td>α-GalNAc,</td>
<td>N-Acetylgalactosamine</td>
<td>100 mM</td>
</tr>
<tr>
<td><em>Dolichos biflorus</em> agglutinin (DBA)</td>
<td>α-GalNAc,</td>
<td>N-Acetylgalactosamine</td>
<td>100 mM</td>
</tr>
<tr>
<td><em>Ricinus communis</em> agglutinin (RCA-120)</td>
<td>β-GalNAc, β-Gal</td>
<td>Galactose</td>
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</tr>
<tr>
<td><em>Sophora japonica</em> agglutinin (SJA)</td>
<td>β-GalNAc,</td>
<td>N-Acetylgalactosamine</td>
<td>100 mM</td>
</tr>
<tr>
<td><em>Vicia villosa</em> agglutinin (VVA)</td>
<td>β-GalNAc,</td>
<td>N-Acetylgalactosamine</td>
<td>100 mM</td>
</tr>
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<td><strong>3. N-Acetylglucosamine group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lycopersicon esculentum</em> (tomato) lectin (LEL)</td>
<td>β-GlcNAc</td>
<td>Chitin hydrolysate</td>
<td>200 mM</td>
</tr>
<tr>
<td>Lectins</td>
<td>Binding specificity of lectins</td>
<td>Inhibitors</td>
<td>Inhibitor concentration (mM)</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>--------------------------------</td>
<td>--------------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td><em>Solanum tuberosum</em> (potato) lectin (STL)</td>
<td>β-GlcNAc</td>
<td>Chitin hydrolysate</td>
<td>200 mM</td>
</tr>
<tr>
<td><em>Wheat</em> (<em>Triticum vulgaris</em>) germ agglutinin (WGA)</td>
<td>β-GlcNAc</td>
<td>Chitin hydrolysate</td>
<td>200 mM</td>
</tr>
<tr>
<td><em>Datura stramonium</em> lectin (DSL)</td>
<td>β-GlcNAc</td>
<td>Chitin hydrolysate</td>
<td>200 mM</td>
</tr>
<tr>
<td><em>Griffonia simplicifolia</em> lectin II (GSL-2)</td>
<td>α, β-GlcNAc</td>
<td>Chitin hydrolysate</td>
<td>200 mM</td>
</tr>
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<td>Succinylated WGA (SWGA)</td>
<td>β-GlcNAc</td>
<td>Chitin hydrolysate</td>
<td>200 mM</td>
</tr>
<tr>
<td><em>Peanut</em> (<em>Arachis hypogaea</em>) agglutinin (PNA)</td>
<td>β-Gal</td>
<td>Chitin hydrolysate</td>
<td>200 mM</td>
</tr>
<tr>
<td><em>Erythrina cristagalli</em> lectin (ECL)</td>
<td>β-Gal, β-GalNAc</td>
<td>Lactose</td>
<td>200 mM</td>
</tr>
<tr>
<td><em>Artocarpus integrifolia</em> (Jacalin)</td>
<td>β-Gal</td>
<td>Galactose</td>
<td>400 mM</td>
</tr>
</tbody>
</table>

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
2.2.7 Lectin binding assay

Staining percentage of 23-different types of lectins on primary-OIEC and hTERT-OIEC was determined using flow-cytometry. Briefly, half million cells were added onto 96-well U-button plate and 100ul of 10ug/ml biotinylated lectin was added onto cells. Similarly, to determine the specificity of lectin binding, same concentration of lectin and its specific inhibitors was added onto next well (Table 2) and incubated for an hour at 4°C. After washing, cells were incubated with 50ul of 5ug/ml of streptavidin-FITC (Fluorescein isothiocyanate) for 30 min. Finally, cells were fixed with 1% paraformaldehyde. Background staining was determined by incubating cells with only streptavidin-FITC and autofluorescence of cells were checked without adding any lectins and streptavidin-FITC. Staining percentage of cells were determined using FACS caliber cytometer (BD, USA) by creating histogram plot of forward scattering. Background staining was subtracted from all the wells and true staining percentage was calculated. No inhibitors were used for UEA-1, PHA-E and PHA-L lectins. Primary cells between passage 30 to 40 and immortalized cells between passage 35 to 45 (10 to 20 after immortalization) were used for the assay and mean staining percentage of three experiments were used for the statistical analysis.
2.2.8 Testing for mycoplasma contamination

Primary and hTERT immortalized OIEC grown in chamber slides were fixed in 4% paraformaldehyde, permeabilized by 0.2% Triton-X in PBS and stained with DAPI (Invitrogen) for the presence of mycoplasma contamination in the cell culture (132). Slides were mounted with slow fading reagent (Invitrogen, ref s36937) and observed under BX35 upright fluorescence microscope.

2.2.9 Statistical analysis

For comparing the lectin staining percentage between primary-OIEC and hTERT-OIEC, two-tailed two sample unequal variance $t$ test was applied. Population doubling time, TEER values between the two cell types were compared using student $t$ test. A p-value of less than 0.05 was considered as statistically significant.
Fig 6: Ovine ileal epithelial cells (OIEC) demonstrate normal epithelial cell morphology under phase-contrast microscope. A and B, Primary-OIEC and hTERT-OIEC culture after 24 hours of seeding. C (Primary-OIEC) and D (hTERT-OIEC) after 48 hours of seeding. Scale bars represents 50 µm.
Fig 7: Immunocytochemical (ICC) staining of primary-OIEC confirms the epithelial origin of the cells. Left column represents staining against protein of interest (POI) and right represents isotype control. Brown color (due to DAB substrate breakdown) indicates positive staining and was visualized strongly against cytokeratin (top left image). All other staining against vimentin, a-SMA, desmin and isotype control were negative as shown by the absence of clear brown staining. Displayed images are representative of three independent ICC experiments. Scale bar represents 50 µm.
Fig 8: Immunocytochemical (ICC) staining of hTERT-OIEC confirms the epithelial origin of the cells. Left column represents staining against protein of interest (POI) and right represents isotype control. Brown color (due to DAB substrate breakdown) indicates positive staining and was visualized strongly against cytokeratin (top left image). All other staining against vimentin, α-SMA, desmin and isotype control were negative as shown by the absence of clear brown staining. Displayed images are representative of three independent ICC experiments. Scale bar represents 50 µm.
2.3 Results

2.3.1 Primary OIEC and hTERT immortalized OIEC display epithelial phenotype

Both the cell types displayed normal epithelial cell morphology when cultured on normal tissue culture flask. Cells were polygonal, cuboidal and showed typical cobblestone morphology when observed under phase contrast microscope (Fig 6). Initially primary cells were heterogenous with higher proportion of epithelial cells and few fibroblast-like cells. To obtain homogenous population, cells were treated with 0.25% trypsin EDTA for 3 min then, detached cells were discarded, and fresh media was added on remaining cells. The procedure was repeated until homogenous population of epithelial cells were obtained. Once homogeneity was obtained, cells grew faster, and the state of confluency was achieved in every 3-4 days. We were able to take primary-OIEC for more than 60 passages and hTERT-OIEC were frozen after 105 passages. Primary-OIEC and hTERT-OIEC maintain their normal morphology during late passages as well.

Immunocytochemistry validated the epithelial origin of both the cell types. Cytokeratin is considered as universal epithelial cell marker and both primary OIEC and hTERT OIEC stained strongly against cytokeratin (Fig 7,8). Cells stayed negative against vimentin, alpha-smooth muscle actin, and desmin (Fig 7,8). Isotype controls were also negative showing the specificity of the staining (Fig 7,8). ICC was performed routinely on both the cell types to check the phenotypic stability and cytokeratin was consistently positive in both the cell types while other markers were negative.
Fig 9: Gel image reveals presence of hTERT gene in hTERT-OIEC. Lane 1 shows 2 log ladder, lane 2 represents no template control, lane 3 represents primary-OIEC, lane 4 represents pGRN-145 plasmid with hTERT gene (positive control) and lane 5 represents hTERT-OIEC. Product size is 125 base pair.
Fig 10: Immunocytochemical (ICC) staining shows presence of hTERT protein in hTERT-OIEC. A and B, Primary-OIEC staining against hTERT specific antibody and isotype control respectively. C and D, hTERT-OIEC staining against hTERT specific antibody and isotype control respectively. Brown color indicates positive staining. In figure 5C, presence of hTERT protein in hTERT-OIEC was expressed by strong positive brown staining. Primary-OIEC (Fig 5A) stained very faintly for hTERT protein. Scale bar represents 50 µm.
2.3.2 Immortalization was confirmed by conventional PCR and immunocytochemistry

Genomic DNA was extracted from hygromycin resistant hTERT-OIEC and primary-OIEC, then conventional PCR was performed using hTERT gene specific primers. Agarose gel image revealed the presence of hTERT gene in hTERT immortalized OIEC (Fig 9). As expected, product size of pGRN 145 plasmid containing hTERT gene and hTERT immortalized OIEC were same (125bp). In contrast, no product was observed from primary OIEC (Fig 9).

Immortalization was further confirmed at protein level by immunocytochemical staining of both primary-OIEC cells and hTERT-OIEC using hTERT specific antibody. Immortalized OIEC cells were strongly positive for hTERT protein whereas primary OIEC cells stained very weakly or were negative against hTERT protein (Fig 10). Isotypes controls stayed negative for both the cell types showing the specificity of the staining.
Fig 11: Growth curve of primary-OIEC and hTERT-OIEC. Cells were counted in hemocytometer using trypan blue dye exclusion method. Initially 20000 cells were seeded, and cells were counted for 5 days. Values are averages of three independent experiments. Cell count wasn’t significantly different (p>0.05) between the cell types for all 5 days. Error bar represents standard error of mean.
Fig 12: Mean population doubling time (PDT) of primary-OIEC (16.30 hours) and hTERT-OIEC (16.40 hours). PDT was calculated from mean of three independent experiments. PDT difference between the cell types was statistically non-significant (p>0.05). Error bar represents standard error of mean.

2.3.3 Primary and hTERT OIEC exhibits stable proliferation ability

Growth curve of primary OIEC and hTERT-OIEC cells were similar. Both cell types had similar growth pattern with hTERT OIEC cells reaching peak at day 4 and slowing down afterwards whereas, primary OIEC cell count continued to rise till day 5 (Fig 11). Similarly, population doubling time of primary-OIEC and hTERT-OIEC was 16.30 hours and 16.40 hours respectively (Fig 12). There was no significant difference between population doubling time and cell growth of both the cell types.
Fig 13: TEER reading of primary-OIEC and hTERT-OIEC over a period of 10 days.

Cells were seeded on polycarbonate filter inserts. Cells polarize within 4 days of seeding as shown by peak on the 4th day. Green line denotes background resistance from unseeded filter with media. TEER reading between the cell types were non-significant (p>0.05) for all 10 days. Values are mean of three independent experiments. Error bar represents standard error of mean.
Fig 14: Immunofluorescence revealed expression of tight junction protein in primary-OIEC. Left column represents tight junction protein staining (green) visualized by alexa-488 conjugated secondary antibody. Centre column represents nuclear counter staining (red) performed by propidium iodide. Right column contains merged image of respective row. Occludin, ZO-I and Claudin-III were mainly expressed on cell membrane and cell-cell junction. Images are representative of three independent experiments. Scale bar represents 20 µm.
Fig 15: Immunofluorescence revealed expression of tight junction protein in hTERT-OIEC. Left column represents tight junction protein staining (green) visualized by alexa-488 conjugated secondary antibody. Centre column represents nuclear counter staining (red) performed by propidium iodide. Right column contains merged image of respective row. Occludin, ZO-I and Claudin-III were mainly expressed on cell membrane and cell-cell junction. Images are representative of three independent experiments. Scale bar represents 20 µm.
2.3.4 Cells were able to exhibit barrier integrity and express functional tight junction protein.

Barrier integrity was recorded quantitatively by measuring trans-epithelial electric resistance (TEER). Primary-OIEC and hTERT-OIEC were able to polarize within 4 days of seeding (Fig 13). Primary OIEC cells showed overall higher TEER readings than hTERT-OIEC and the values were highest on 4th day for both the cells types. Primary-OIEC measured up to 737 Ω on 4th day and hTERT-OIEC measured highest of 436 Ω on the same day (Fig 13). After 4th day, TEER readings declined slowly in primary OIEC however, in hTERT-OIEC the TEER readings remained stable from 5th day to 8th day and then decreased.

Similarly, we accessed barrier integrity in polarized cells on 10th day of seeding by performing indirect immunofluorescence for tight junction protein. Cells were stained against various tight junction proteins; occludin, ZO-I, claudin-I, Claudin-III. Occludin, ZO-I, and Claudin-III was detected in both primary OIEC and hTERT-OIEC (Fig 14,15). In contrast, claudin-I was not detected in both the cell types (data not shown). Tight junction proteins were mainly present on the cell membrane and cell-cell junction. However, along with membrane and junctional staining, ZO-I and Claudin-II had some nuclear localization in both the cell types (Fig 14,15). Isotypes controls were used to confirm the specificity of the staining and were negative in both the cell types (Fig 14,15).
Fig 16: Lectin binding profile of primary-OIEC. Cells were stained with biotinylated lectins followed by streptavidin-FITC binding. Staining percentage was determined using FACS caliber. Black bar represents staining percentage of the lectin and gray bar represents staining percentage of the lectin after incubation with the inhibitor. For UEA-I, PHA-E, and PHA-L lectins no inhibitors were used. Values are mean of three independent experiments. Error bar represents standard error of mean.
Fig 17: Lectin binding profile of hTERT-OIEC. Cells were stained with biotinylated lectins followed by streptavidin-FITC. Staining percentage was determined using FACS caliber. Black bar represents staining percentage of the lectin and gray bar represents staining percentage of the lectin after incubation with inhibitor. For UEA-I, PHA-E, and PHA-L lectins no inhibitors were used. Values are mean of three independent experiments. Error bar represents standard error of mean.
Fig 18: Comparison of lectin binding profile between primary-OIEC and hTERT-OIEC. Black bar and gray bar represent staining percentage of primary-OIEC and hTERT-OIEC respectively. Values represents average of three independent experiments. Asterisks (*) indicate significantly different staining percentage, one asterisk (*) represents p <0.05, two asterisks (**) represents p<0.01, and three asterisks represents (***) p<0.001. Error bar represents standard error of mean.
Fig 1: Primary-OIEC and hTERT-OIEC were free of mycoplasma contamination. A and B show DAPI nuclear staining of Primary-OIEC and hTERT-OIEC respectively. Scale bar represents 10 µm.

2.4 Glycosylation profile of Primary OIEC and hTERT-OIEC

Binding profile of 23 different lectins was investigated using flow cytometry. Jacalin, STL, WGA, DSL, SWGA SNA, PSA, RCA-120, and PHA-L stained strongly (>80%) on both the cell types (Fig 16,17,18). Conversely, staining of GSL-II, DBA, SJA, VVA were either negative or extremely low (<10%) on both the cell types. Similarly, only on hTERT-OIECs lectins LEL, MAL-I and PHA-E stained extremely high (>80%) and conversely, lectins PNA, ECL, and SBA stained low (<10%) on the immortalized cells. On statistical analysis, staining profile of LEL, STL, PNA, ECL, MAL-I, MAL-II, DBA, UEA-I, PHA-E and PHA-L were found to be significantly different (p <0.05) between primary-OIEC and hTERT-OIEC. Binding specificity of lectins onto cells were determined by using specific inhibitors for each lectin and most of the inhibitors reduced the staining percentage on both the cell types. Staining percentage of SNA and MAL-I was inhibited only partially on both the cell types when treated with
inhibitors. Non-specific background staining was determined by treating cells with only secondary antibody and was less than 5 percentage.

2.4.1 Cells were free from mycoplasma contamination

Cells were fixed, permeabilized and stained with DAPI to confirm absence of any mycoplasma contamination in the cell cultures. Cells were observed under fluorescence microscope and were found to be free from mycoplasma contamination (Fig 19).

2.5 Discussion

In the study, we have established continuous primary sheep ileal epithelial cell line and then immortalized the primary cells with human telomerase gene. Development of continuous cell line have been indispensable tool for studying disease pathogenesis at cellular and molecular level. Epithelial cell plays pivotal role in pathogen recognition, binding, entry of the pathogen, disease prognosis and outcome of the disease (104). Immortalization procedure using hTERT gene is more desired due to its safe nature than other viral transfection methods and the procedure is considered to retain most of the phenotypic and biochemical properties of the original primary cells (133, 134).

Intestinal epithelial cells constantly remain in contact with both commensal and pathogenic microbes and play distinctive role in maintaining homeostasis by creating a balance between tolerance and immunity. Slight imbalance can lead towards autoimmunity or the infection (105). So, to explore different properties of intestinal epithelial cells, in this study we have developed an intestinal epithelial cell line model from 3-day old male lamb. Initially, epithelial cell cultures were more heterogenous with
some-fibroblast like cells, and this inevitable problem of epithelial cell culture was avoided by 3 minutes trypsin treatment. Unlike epithelial cells, fibroblast cells are loosely adhered to the surface and do not form tight junction proteins and this difference in their property was explored to obtain homogenous population of epithelial cells. This technique of 3 minutes trypsin treatment has been adapted in previous studies and found to be effective in removal of fibroblast-like cells (135, 136).

During invitro culture, epithelial cells display unique polygonal cobblestone morphology but several studies have reported age related changes in morphology, loss of physiological properties and alteration in phenotypic character of the cells (136). Primary OIEC and hTERT-OIEC were able to grow up to 60 and more than 100 passages respectively. The morphological characteristics, proliferation capacity, expression of cytokeratin, lectin binding profile of the cells were examined before and after the hTERT immortalization and cells were able to retain their normal epithelial cell properties. These characteristics were routinely inspected over time on both the cell types to examine the phenotypic stability. Cells were able to maintain their normal morphological characters, express cytokeratin and were able to polarize even in late passages.

Cell senescence is one of the major constraints of cell culture as somatic cells can divide for a finite number of times before they enter senescence, often referred as hayflick limit (137). Often cell cultures are immortalized to escape the senescence however, several studies have demonstrated cells can spontaneously immortalized and proliferate continuously for long time (138, 139). Spontaneous immortalization of the primary-OIEC could be the reason as in this study primary-OIEC cells grew beyond 60 passages. Primary bovine ileal epithelial cell line developed in an earlier study was able
to proliferate beyond 100 passages without losing its physical characteristics (124).

hTERT gene prevents telomere shortening, initiates telomerase activity which ultimately prolongs the life span of cells (133). Numerous cell lines have been developed using hTERT transfection including porcine intestinal epithelial cell, bovine intestinal epithelial cells, and several more (124, 140, 141). Growth curve analysis revealed strong proliferation capacity of both primary-OIEC and hTERT-OIEC with a doubling time of 16.30 and 16.40 hours respectively. Katwal et;al., observed slightly lower mean population doubling time in primary (14.16 hours) and hTERT immortalized (13.33 hours) bovine ileal epithelial cells. In another study bovine small intestinal epithelial cells had higher population doubling time than primary-OIEC and hTERT-OIEC cell lines established in the present study (131). This discrepancy in population doubling time (PDT) might be the result of differences in the age groups of the donor animal, animal species and many other unknown factors (124, 132, 135)

Intestinal epithelial cells line maintains a barrier between external luminal content and underlying lamina propria. Intercellular junctions anchored by tight junctions, adherent junctions and desmosomes play an important role in maintaining this barrier integrity (142, 143). Any disarrangement in the epithelial cell barrier due to stress, injury, or pathogens can seriously affect the host and develop conditions such as colitis and inflammatory bowel disease (144). Leakage in the epithelial barrier can result into increase in permeability and entry of unwanted foreign materials into the circulation via paracellular route (7). Once, the microbes, potential allergens or any other luminal contents enter the body’s internal tissue, series of innate and adaptive immune responses are triggered (145). Various enteric pathogens such as *Salmonella, Entamoeba*
*histolytica*, E.coli gain access to underlying lamina propria by disrupting the tight junctions through the engagement of proteases, adhesins and other proteins (110). Various studies have appreciated the role of tight junctions in protecting the host body from potential infection sources (145, 146). Furthermore, this barrier property of epithelial cells also helps body in selective absorption of nutrients, water, ions, and macromolecules. Several studies have suggested tight junction proteins as potential drug target site in combating enteric infections (7, 147). In our present study, both primary-OIEC and hTERT-OIEC were able to polarize and express tight junction proteins. Occludin, ZO-1 and claudin-3 were mainly expressed in the cell membrane; however, some nuclear localization of ZO-1 and claudin-3 was also observed in both the cell types. A similar pattern of nuclear and peripheral expression of claudin-3 and ZO-1 was observed in porcine tracheal and nasal turbinate epithelial cells (136). Occludins are major transmembrane tight junction proteins and play a crucial role in sealing the paracellular movement of molecules which helps in creating apical and basal polarity on the epithelial lining (148). This polarity can be measured in terms of trans epithelial electrical resistance (TEER). Both the primary-OIEC and hTERT-OIEC cells in this study exhibited high TEER readings (436-737Ω) confirming their ability to polarize on trans well filter membranes. Other intestinal epithelial cell line models, depending on the conditions and equipment used, measure a wide range of TEER. Human epithelial colorectal adenocarcinoma cells (Caco-2) cells measure from 250 to 4000 Ω.cm² (149, 150), human immortalized colon cell line (HCEC) 200 Ω.cm² (151), porcine jejunal epithelial cells from 384 to 2830 Ω.cm² (152, 153), canine intestinal epithelial measured more than 900 Ω.cm² (154).
Mucosal surface serves as major site of pathogen entry and studies have consistently established the fact that interactions between microbes and intestinal surface carbohydrates play key role in disease development (106, 155). The uppermost layer of mucosal surface is rich in glycolipids, glycoproteins, proteoglycans and mucin secreted by epithelial cells. In this study, we investigated the lectin-binding profile of primary OIECs and whether hTERT immortalization modified the glycosylation pattern of the primary-OIECs. Glycosylation profile of primary-OIEC and hTERT-OIEC was studied using 23 different lectins. After analyzing the results, 9 out of 23 lectins staining percentages were significantly different between the two cell types. Previous studies have reported change in expressions of glycoconjugates after immortalization (156-158). These changes could be the result of altered gene expression in immortalized cells. Several studies have shown the linkage between glycosylation pattern and gene regulation (159). Gene transcription has been shown to play major impact on formation and expression of surface glycoconjugates and any modifications in cell may lead to altered expression of glycoconjugates. Due to such potential differences in gene expression, synthesis of enzymes like glycosyltransferase and glycosidases responsible for majority of the glycosylation might be different between the cell types causing some changes in the staining percentage after immortalization (160).

Primary-OIEC and hTERT-OIEC express Sia2-3 gal (SNA) and Sia2-6gal (Mal-I and Mal-II) receptor specific for influenza virus attachment (136). Mannose group of lectins facilitate the adherence of enteric bacteria onto epithelial layer and both primary-OIEC and hTERT-OIEC cells display mannose glycan (106). Enteric protozoans like E. histolytica use N-acetyl-D-galactosamine lectin to interact with intestinal epithelium and
encroach mucus barrier (110). Thus, both the cell lines may be used as an invitro model for studying mucosal bacterial, protozoan, and viral diseases pathogenesis.
2.6 Conclusion

We successfully isolated, established and characterized ovine intestinal ileal epithelial cells. To our best knowledge, this is the first ever ovine intestinal epithelial cell line developed from ileum of 3-day-old lamb. To overcome the replication senescence, we immortalized the primary-OIEC with hTERT gene and then we compared the immortalized cell line with primary cell line for the expression of different phenotypic and biochemical markers. Immortalized cells were able to retain the properties of primary-OIEC cells. Our results suggest these cell lines may be used as an *in vitro* model for studying viral and bacterial pathogenesis. Both primary-OIEC and hTERT-OIEC can be polarized and express various tight junction proteins and thus they can be used for studying drug delivery, host-pathogen interactions, and antigen uptake. We further plan to use these cell lines in studying their role in innate immunity against enteric diseases.
3. Development and characterization of ovine ileal sub-epithelial myofibroblast (ISEMF) cells and studying its responses to various bacterial and viral ligands

**Abstract**

The intestinal mucosa is constantly in contact with the luminal content, and any form of disruptions in the uppermost intestinal epithelium exposes underlying sub-epithelial myofibroblasts to the luminal content, which results in the generation of immune responses from these cells. Intestinal sub-epithelial myofibroblasts (ISEMFs) play significantly important role in wound healing, tissue regeneration, production of extracellular matrix, epithelial cell migration, and differentiation. There are only few ISEMF cell lines available from ruminants and limited number of studies on innate immune response of ISEMF cells have impeded our understanding of role of ISMEF cells in innate immunity. Here, we have developed and characterized ovine ileal sub-epithelial myofibroblast cells (ISEMFs) from 3-day old male lamb and immortalized them using SV40 large T-antigen. We stimulated primary ovine ISEMF with lipopolysaccharide (LPS), peptidoglycan (PGN), flagellin, imiquimod, poly I:C and poly I:C with lyovec for 3 h and 24 h. We analyzed mRNA expressions of various ovine TLRs (1,2,3,4,5,6 and 9) and cytokines. Upon ligand stimulation, primary ovine ISEMF cells were able to modulate their innate immune responses by expression of various TLRs and cytokines. Hence, these ovine ISEMF cell lines can serve as a valuable tool in elucidating innate immune responses of intestinal subepithelial myofibroblasts to various bacterial/viral ligands and enteric pathogens.

**Keywords:** ovine myofibroblast cells, TLRs, ligand stimulation.
3.1 Introduction

Intestinal subepithelial myofibroblast (ISEMF) cells are the mesenchymal cells lying underneath the intestinal epithelium (161). Originally described by Pascal, Kaye, and Lane (162) in 1968, ISEMFs have gained widespread interest in immunological studies (156, 163-166). Two types of myofibroblasts are found in the intestine: ISEMFs and interstitial cells of Cajal (ICC) (167). ICC are mainly found in the submucosa and muscularis propria of the intestine and help in smooth peristaltic movement of the intestine (168). ISEMFs typically characterized by stellate morphology are found throughout the gut and are more dense in crypts than in colon or villi of small intestine (167).

The intestinal mucosa is constantly in contact with the luminal content and any form of disruptions in the uppermost intestinal epithelium exposes underlying ISEMF to the luminal content resulting into generation of immune response from ISEMF cells (169). ISEMFs express pattern recognition receptors including toll like receptors (TLRs) for recognizing conserved pathogen associated molecular patterns (PAMPs) from pathogens (156). Hence, ISEMFs are very important in elucidating pathophysiology of diseases such as inflammatory bowel disease (IBD) which mainly results from aggravated immune responses from intestinal mucosal and submucosal compartment (170-172). Similarly, ISEMFs play significantly important role in wound healing, tissue regeneration and the production of extracellular matrix (ECM) (173). Studies have demonstrated the role of underlying intestinal myofibroblast cells in epithelial cell migration, differentiation and regeneration. Supernatant collected from human colonic
myofibroblast cells (CCD-18co cell line) drives the maturation of Caco2 cell line through Wnt-5a signaling pathway (174).

There are very few ISEMF cell lines available from ruminants. There are two reported bovine ISEMF cell lines (156, 175) and others are from humans (176), rats (177, 178) or mice (179-181). There are limited number of studies on innate immune responses of ISEMF cells to various pathogen associated molecular patterns (PAMPs). In this study, we have developed and characterized primary ovine ileal ISEMF cells and immortalized primary ISEMFs using SV40 large T antigen. Further, we stimulated primary ovine ISEMFs with various bacterial and viral ligands for 3hrs and 24hrs time points and observed the expression profiles of various TLRs and cytokines.

3.2 Materials and Methods

3.2.1 Isolation and establishment of primary sheep ileal sub-epithelial myofibroblast cell culture

The animal experiment protocol was approved by Institutional Animal Care and Use Committee (IUCAC) of South Dakota State University, USA. Ileal intestinal tissue from 3-day old male lamb was collected in ice-cold DMEM/F-12 media containing 1% antibiotics. Both ends of the tissues were ligated and the luminal content was washed three times with DMEM/F-12 media with antibiotics. Then, tissues were cut open longitudinally and submerged in per-warmed dispase solution (1.2 units/ml, Roche) at 37°C for 90 minutes. After the incubation, suspension containing large number of digested cells were collected and centrifuged at 1200rpm for 5 minutes. Supernatant was discarded and primary fibroblast cell culture was maintained in Dulbecco’s modified
Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% Non-essential amino acids and 1% antibiotics (Penicillin-streptomycin). Cells were cultured at 37°C and 5% CO₂ in humidified incubator. Upon ~80% confluency, cells were passaged using 0.25% trypsin-EDTA (Ethylenediaminetetraacetic acid). Established myofibroblast cells’ morphology was observed under phase-contrast microscope.

3.2.2 Immortalization of ISEMF cells

For SV40 immortalization of primary ISEMF cells earlier described protocol was adapted (124). Half million ovine ISEMF cells of passage 9 were seeded in 3 wells of 6-well plate. After 18 hours of seeding, cells were washed with 1x PBS and cultured in serum free OPTI-MEM media (Gibco). First well was left as such with no interventions. Second well was used as negative control and cells in third well were transfected with mixture of lipofectamine-Psv3-neo plasmid (ATCC ® 37150) vector containing SV40 large T antigen gene. Transfection of the plasmid was performed by following manufacturer’s protocol (Lipofectamine 2000). After 6 hours of transfection, cells were washed and cultured in earlier described DMEM/high glucose media. Selection antibiotics genetin (G418, Thermo Fisher, cat 10131-035) at 1000µg/ml concentration was added in second (negative control) and third (transfected) well. Upon confluency cells from transfected well were transferred into T75 flask as labeled as passage 10-1. Where 10 represents total number of times culture had been passaged and 1 represents passage number after immortalization.
3.2.3 Confirmation of SV40 immortalization

Conventional PCR was performed to confirm the SV40 immortalization of ovine ISEMF cells. Genomic DNA was extracted from primary ovine ISEMFs and SV40 immortalized ovine ISEMFs using DNeasy Blood & Tissue Kit (Qiagen) and quantified using nanodrop2000 spectrophotometry. Equal amount (100ng) of extracted DNA from both cell types, and Psv3-neo plasmid with SV40 gene was amplified using SV40 gene specific primers (Table 3). PCR was performed following the manufacture’s protocol (New England Biolabs, Catalog number E5000S). Following PCR conditions were used: initial denaturation at 95\(^0\) C for 5 min followed by 40 cycles of denaturation, annealing and extension at 95\(^0\) C for 30 sec, 55\(^0\) C for 60 sec, 72\(^0\) C for 60 sec and final extension at 72\(^0\) C for 10 minutes. PCR products were resolved in agarose gel and visualized using ODYSSEY-FC gel imaging system.

Table 3: Primers used to amplify SV40 large T antigen gene in SV40 immortalized ovine ISEMF cells.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Product size</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward: 5’-AGCAGACACTCTATGCCTGTGTGGAGTAAG-3’</td>
<td>751 bp</td>
<td>(182)</td>
</tr>
<tr>
<td>Reverse: 5’-GACCTTTTGAGGCTTCTGGATGCAACTGAG-3’</td>
<td></td>
<td></td>
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3.2.4 Immunocytochemistry (ICC) of primary ISEMFs.

Immunocytochemical staining of primary ovine ISEMF was performed using monoclonal antibodies against cytokeratin, vimentin, alpha-smooth muscle actin (ASM) and desmin. Cytospins were prepared from 100ul of \(10^6\) cells/ml suspension (Cytospin 3, Thermo Shandon Ltd., UK). Slides were air dried for an hour and fixed in acetone for 7 mins. Slides were then immersed in 1X PBS with 0.3% hydrogen peroxide and 0.1% sodium azide for blocking peroxidase activity. Non-specific protein binding was blocked by incubating cells in 1% goat serum for 20 mins. Avidin/biotin kit was used to block endogenous biotin as per manufacture’s protocol (Vector laboratories). Cells were then treated with either primary antibody or isotype control antibody. Cells were incubated with 100ul of 1ug/ml primary mouse monoclonal antibodies specific against cytokeratin (mAb C6909; IgG2a isotype), vimentin (mAb V5255; IgM isotype), alpha-smooth muscle actin (mAb A2457; IgG2a isotype), or desmin (mAb D1033; IgG1 isotype) for 1 hour at room temperature. For isotype control M9144 (IgG2a isotype), M5170 (IgM isotype), and M9269 (IgG1 isotype) antibodies were used. Cells were washed with 1X PBS and incubated with isotype specific biotinylated goat-anti mouse IgG2a, IgM or IgG1 (Caltag laboratories) antibodies at 1:2000 dilutions for 30 minutes. Following secondary antibody incubation, cells were incubated with streptavidin-horseradish peroxidase (Vector laboratories) for 30 minutes. Ready-to-use DAB (Diaminobenzidine) substrate (Vector laboratories) was added to develop the brown color for positive reaction. Hematoxylin was used for the nuclear counter staining. Slides were observed and images were taken using BX53 upright microscope.
3.2.5 Stimulation of Primary ISEMF cells with PRR ligands

PRRs of primary ovine ISEMF cells were stimulated with various bacterial and viral ligands. For the stimulation of surface TLRs bacterial ligands; lipopolysaccharide (LPS, catalog: LG529) from *Escherichia coli O55:B55* at 5µg/ml concentration, peptidoglycan (PGN, catalog: tlr1-pgn) from *Staphylococcus aureus* at 10µg/ml concentration and flagellin (FLA, catalog: tlr1-stfla) from *Salmonella typhimurium* at 100ng/ml were used (Table 2). Viral nucleoside analogs; imiquimod (catalog: tlr1-imqs) at 5µg/ml concentration, polyinosinic:polycytidylic acid (Poly I:C, catalog: tlr1-pic) at 5µg/ml, and poly I:C with lyovec (poly I:C/lyovec, catalog: tlr1-piclv) at 1µg/ml were used to stimulate endoplasmic and cytosolic PRRs (Table 4). Cells were stimulated for 3hrs and 24hrs and RNA samples were collected using end-time alignment method. Primary ISEMF cells between passage 8 to 12 were used in the stimulation assay. Half million cells were seeded into 6 well plate and after 48 hours of incubation cells were stimulated with the ligands. Each stimulation was performed in triplicates.
Table 4: Details of ligands used in the stimulation of primary ovine ISEMF cells.

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Nature of ligands</th>
<th>Source</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipopolysaccharide</td>
<td>Surface</td>
<td><em>Escherichia coli</em> O55:B55</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td>Peptidoglycan</td>
<td>Surface</td>
<td><em>Staphylococcus aureus</em></td>
<td>10 µg/ml</td>
</tr>
<tr>
<td>Flagellin</td>
<td>Surface</td>
<td><em>Salmonella typhimurium</em></td>
<td>100 ng/ml</td>
</tr>
<tr>
<td>Imiquimod</td>
<td>Endosomes</td>
<td>Synthetic nucleoside analogue</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>Endosomes</td>
<td>Synthetic analogue of double stranded RNA</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td>Poly I:C with lyovec</td>
<td>Cytoplasm</td>
<td>Poly I:C with transfecting reagent lyovec</td>
<td>1 µg/ml</td>
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</table>
3.2.6 RNA extraction and reverse transcription

After 3hrs and 24hrs of ligand stimulation, cells were washed twice with 1X PBS followed by 0.05% trypsin EDTA (corning) treatment. After pelleting the harvested cells, RNA was extracted using RNeasy Mini Kit (Qiagen, catalog: 74101) following manufacturer’s protocol. NanoDrop 2000 was used to quantify the extracted RNA samples. For cDNA preparation, 1µg RNA was reverse transcribed using TaqMan reverse transcription kit (Applied Biosystems, catalog: N8080234) following manufacturer’s protocol. Prepared cDNA was diluted in 1:5 ratio for downstream application.

3.2.7 Real time quantitative PCR for studying changes in gene expression

For RT-qPCR, 1 µl of diluted cDNA, 0.5 µl each of forward and reverse primers (10µM), 5µl of SYBR green master mix ( RT² SYBR® Green ROX, Catalog: 330523, Qiagen sciences, Maryland, USA) and 3µl of nuclease free water was added to bring the reaction volume to 10µl. Changes in the gene expression of TLRs 1-6, MyD88, NF-Kb, IL-1beta, TGF-beta, IFN-alpha, IFN-beta, IL-6 and IL-8 were studied using gene specific primers (Table 5). Beside these genes, changes in expression of TLR 9 was studied with intracellular ligands only. Thermal cycle for amplification TLR 1-6, TLR 9, MyD88, NF-Kb, IL-1b, TGF-b was; initial denaturation 95°C for 15 min followed by 40 cycles of denaturation, annealing and extension at 94°C for 10 sec, 57°C for 30 sec, 72°C for 40 sec. Similarly, for IFN-a, IFN-b, IL-6 and IL-8 different temperature profile was used: initial denaturation 95°C for 10 min followed by 40 cycles of denaturation, annealing/extension at 95°C for 10 sec and 60°C for 45 sec. Hypoxanthine-guanine
phosphoribosyltransferase (HPRT) gene was used as an internal control. Melt curve was obtained to confirm the specificity of the products. The RT-qPCR was performed using Quantstudio™ 6 Flex Real-Time PCR System (Applied Biosystems, NJ, USA). Other than primers for IL-6 and IL-8 all other primers used in the RT-qPCR were designed in previous studies (183-185).
Table 5: Details of primers used in studying TLRs and cytokine expressions in primary ovine ISEMF cells.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’- 3’)</th>
<th>Accession number</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR 1</td>
<td>F TTGCACATCAGCAAGGTTTT R CACTGTGGTGCTGACTGACA</td>
<td>AM981299</td>
<td>159</td>
<td>(183)</td>
</tr>
<tr>
<td>TLR 2</td>
<td>F GGCTGTAATCAGCGTGTTCA R GATCTCGTTGTCGGACAGGT</td>
<td>AM981300</td>
<td>160</td>
<td></td>
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<tr>
<td>TLR 3</td>
<td>F TCAGCTCCAACCTGGAGAACC R CACCCAGGAGAAGACTCTTTGA</td>
<td>AM981301</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>TLR 4</td>
<td>F TGGATTTATCCAGATGCGAA</td>
<td>R GGcACCAGCTTCTGTAAAC</td>
<td>AM981302</td>
<td>152</td>
</tr>
<tr>
<td>TLR 5</td>
<td>F CGACAACCTCCAAGTTCTCAA R TTTCCcAGGAATTGAATG</td>
<td>AM981303</td>
<td>151</td>
<td></td>
</tr>
<tr>
<td>TLR 6</td>
<td>F TTTGTcCTCAGGAACCAAGC</td>
<td>R TcATATTCCAAGAATTCCAGCTA</td>
<td>AM981304</td>
<td>214</td>
</tr>
<tr>
<td>TLR 9</td>
<td>F CCCTGGAGAAGCTGGGACT</td>
<td>R GACAGGTCCACGAAGAGCAG</td>
<td>AM981305</td>
<td>175</td>
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<tr>
<td>My- D88</td>
<td>F AGGTGCGCGTGATGGTGTTGTT</td>
<td>R TGGTGCGAGGGTATTGTAGTCA</td>
<td>GQ221044.1</td>
<td>203</td>
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<tr>
<td>NF-kB</td>
<td>F CACCTTCTCCAGCCCTTTG</td>
<td>R TGCCACCTCCTCTCCAG</td>
<td>XM_012119</td>
<td>95</td>
</tr>
<tr>
<td>IL-1b</td>
<td>F GAAGCTGAGGAGCCGTGCCCTACGAACA</td>
<td>R CCAGCACCAGGGATTGCTCTCTGTCC</td>
<td>NM_001009</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td>Primer Name</td>
<td>Forward</td>
<td>Reverse</td>
<td>Accession</td>
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<td>---------</td>
<td>---------</td>
<td>-------------</td>
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<tr>
<td>TGF-b</td>
<td>F GGGCTTTTCGCTCAGTGCCCACCTGTC</td>
<td>R CAGAGGGGGTGCCCATGAGGAGCAGG</td>
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<td>IFN-a</td>
<td>F ACCTCCAGCTTTCAAGCACA</td>
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<td>IFN-b</td>
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<tr>
<td>IL-8</td>
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<td>R GGGTGGAAAGGTGGAATG</td>
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<td>100</td>
</tr>
<tr>
<td>HPRT</td>
<td>F GGATTACATCAAAGGCGACTGAACA</td>
<td>R CATTGTCTTTCCAGTGCAATT</td>
<td>NM_001034_035</td>
<td>193</td>
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3.2.8 Statistical analysis of RT-qPCR data

Obtained cycle threshold values (Ct) for various TLRs and cytokines genes were normalized using HPRT gene as housekeeping gene and fold changes in mRNA expression was calculated using earlier described delta-delta Ct ($2^{\Delta\Delta Ct}$) method (190). Equation applied for the calculation was:

$$\Delta\Delta Ct = \Delta Ct \text{ treatment (Ct of target gene in treatment – Ct of reference gene in treatment) – } \Delta Ct \text{ control (Ct of target gene in control – Ct of reference gene in control)}$$

Two-tailed student’s $t$ test was applied to compare the average fold change of three experiments. p-Value less than 0.05 was considered as statistically significant. GraphPad Prism software version 8.4.2 was used to plot the graphs. Fold change data represents mean of three independent experiments with standard error of mean (SEM).
3.3 Results

Fig 20: Phase contrast image of primary and SV40 immortalized ovine ISEMF cells. A) Primary ovine ileal ISEMF cells, B) SV-40 immortalized ovine ileal ISEMF cells. Scale bar on the right bottom corner measures 50 µm.
Fig 2: Immunocytochemical staining of primary ovine ileal ISEMF cells confirms the mesenchymal origin of the cells. Left column represents staining against protein of interest (POI) and right represents isotype control. Brown color (due to DAB substrate breakdown) indicates positive staining and was visualized strongly against a-SMA and vimentin. Isotype controls were negative as shown by the absence of clear brown staining. Displayed images are representative of three independent ICC experiments. Scale bar represents 100 µm.
Fig 22: Gel image reveals presence of SV40 gene in SV40 immortalized ovine ISEMF cells. Lane 1 shows 2 log ladder, lane 2 represents no template control, lane 3 represents primary-ISEMF, lane 4 empty, lane 5 was loaded with Psv3-neo plasmid with SV40 gene (positive plasmid), and lane 6 represents SV40 immortalized.ovine ISEMF and lane 7 represents SV40 immortalized bovine ISEMF cells (positive control), Product size is 706 base pair.
3.3.1 Phenotypic characterization of the primary and SV40 immortalized ovine ISEMFs.

When cultured in uncoated tissue culture flasks, the primary and SV40 immortalized ovine ISEMF displayed normal myofibroblast like characteristics. Cells showed spindled, discoid and stellated shaped morphology typical of myofibroblast like cells (Fig 20). In immunocytochemistry, cells were strongly positive for alpha-smooth muscle actin, a well-known marker for myofibroblast cells. There was also medium grade staining for vimentin, marker for mesenchymal origin and low staining for desmin, a smooth muscle marker. Surprisingly, <10 % also stained for cytokeratin a typical marker of epithelial cells, cytokeratin positive cells were selectively separated by 3 min. trypsin treatment as described earlier (135). We were able to passage primary ISEMF cells up-to passage 17 and SV40 immortalized cells to passage 35 passages without any morphological changes. Cells were routinely screened for the presence of a-SMA and vimentin and were constantly positive throughout the passages.

SV40 immortalized ovine ISEMFs were tested for the presence of SV40 gene. Conventional PCR products when resolved on the agarose gel showed the presence of SV40 gene in the immortalized cells (fig 22).
3.3.2 Innate immune responses of primary ovine ISEMF cells after stimulating with bacterial ligands

After 3 hrs of LPS stimulation, LPS significantly decreased the expression of TLR 4 and TLR5. In contrast, after 24 hours of stimulation, LPS significantly increased the expression of TLR2 and TLR5 while other TLRs expression (TLR1,3,4, and 6) also increased during this period but were not found to be statistically significant. In terms of cytokines and signaling molecule expression, LPS significantly increased the expression of IL-6 and decreased MyD88 expression after 3 hrs of stimulation. Similarly, expression of IL-1b significantly increased after 24 hours of LPS stimulation.

After 3hrs and 24 hrs PGN stimulation, there were no significant alterations in TLRs expression. At 3hrs time point, PGN stimulation significantly decreased the expression of IFN-a. No significant changes were observed in the expression of cytokines and signaling molecules upon PGN stimulation.

After FLA stimulation except decreased expression of TLR6 at 3 hrs time point, there were no significant changes in the expression of TLRs. In terms of cytokines and signaling molecule expression, FLA significantly decreased the expression of MyD88 and TGF-b after 3 hrs of stimulation. FLA stimulation did not change the expression of cytokines and signaling molecules after 24 hrs of stimulation.
3.3.3 Innate immune responses of primary ISEMF cells after stimulation with cytosolic and endosomal viral ligands.

Three hours stimulation of ISEMFs with imiquimod (IMQ) resulted into decrease in gene expression of TLR6. IMQ also significantly downregulated the expression of IFN-β after 24 hrs of stimulation. Poly I:C did not induce any changes in the gene expression after 3hrs and 24 hrs of stimulation. Poly I:C with lyovec upregulated the expression of TLR3 at 3 hrs time point; however, no significant changes were observed in cytokine and signaling molecules gene expression after 3hrs and 24 hrs of stimulation.
Fig. 23: Change in gene expression profile of different TLRs in ovine ISEM cells after LPS stimulation. Each bar represents mean of three independent experiments. Error bar represents standard error of mean (SEM). Fold change was calculated using delta-delta Ct method ($2^{\Delta\Delta Ct}$). Asterisk (*) indicates statistically significant difference, p< 0.05.
Fig 24: Change in gene expression profile of MyD-88, NF-Kb, and different cytokines in ovine ISEMF cells after LPS stimulation. Each bar represents mean of three independent experiments. Error bar represents standard error of mean (SEM). Fold change was calculated using delta-delta Ct method (2^-ΔΔCt). Asterisk (*) indicates statistically significant difference, p< 0.05.
Fig 25: Change in gene expression profile of different TLRs in ovine ISEMF cells after PGN stimulation. Each bar represents mean of three independent experiments. Error bar represents standard error of mean (SEM). Fold change was calculated using delta-delta Ct method ($2^{\Delta\Delta Ct}$). Asterisk (*) indicates statistically significant difference, p< 0.05.
Fig 26: Change in gene expression profile of MyD-88, NF-Kb, and different cytokines in ovine ISEMF cells after PGN stimulation. Each bar represents mean of three independent experiments. Error bar represents standard error of mean (SEM). Fold change was calculated using delta-delta Ct method \((2^{\Delta\Delta Ct})\). Asterisk (*) indicates statistically significant difference, \(p< 0.05\).
Fig 27: Change in gene expression profile of different TLRs in ovine ISEMF cells after flagellin stimulation. Each bar represents mean of three independent experiments. Error bar represents standard error of mean (SEM). Fold change was calculated using delta-delta Ct method ($2^{\Delta \Delta Ct}$). Asterisk (*) indicates statistically significant difference, $p<0.05$. 

Flagellin Stimulation

- **Control**
- **TLR1**
- **TLR2**
- **TLR3**
- **TLR4**
- **TLR5**
- **TLR6**

<table>
<thead>
<tr>
<th></th>
<th>3 hrs</th>
<th>24 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>TLR1</td>
<td>2.50</td>
<td>3.00</td>
</tr>
<tr>
<td>TLR2</td>
<td>1.50</td>
<td>2.00</td>
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<tr>
<td>TLR3</td>
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<td>1.50</td>
</tr>
<tr>
<td>TLR4</td>
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<td>1.00</td>
</tr>
<tr>
<td>TLR5</td>
<td>0.90</td>
<td>0.80</td>
</tr>
<tr>
<td>TLR6</td>
<td>1.10</td>
<td>1.20</td>
</tr>
</tbody>
</table>
Fig 28: Change in gene expression profile of MyD-88, NF-Kb, and different cytokines in ovine ISEMF cells after flagellin stimulation. Each bar represents mean of three independent experiments. Error bar represents standard error of mean (SEM). Fold change was calculated using delta-delta Ct method ($2^{\Delta\Delta Ct}$). Asterisk (*) indicates statistically significant difference, p< 0.05.
Fig 29: Change in gene expression profile of different TLRs in ovine ISEMF cells after imiquimod stimulation. Each bar represents mean of three independent experiments. Error bar represents standard error of mean (SEM). Fold change was calculated using delta-delta Ct method \(2^{\Delta\Delta Ct}\). Asterisk (*) indicates statistically significant difference, \(p< 0.05\).
Fig 30: Change in gene expression profile of MyD-88, NF-Kb, and different cytokines in ovine ISEMF cells after imiquimod stimulation. Each bar represents mean of three independent experiments. Error bar represents standard error of mean (SEM). Fold change was calculated using delta-delta Ct method ($2^{\Delta\Delta Ct}$). Asterisk (*) indicates statistically significant difference, $p< 0.05$. 
Fig 31: Change in gene expression profile of different TLRs in ovine ISEMF cells after poly I:C stimulation. Each bar represents mean of three independent experiments. Error bar represents standard error of mean (SEM). Fold change was calculated using delta-delta Ct method ($2^{\Delta\Delta Ct}$). Asterisk (*) indicates statistically significant difference, $p<0.05$. 
Fig 32: Change in gene expression profile of MyD-88, NF-Kb, and different cytokines in ovine ISEMF cells after poly I:C stimulation. Each bar represents mean of three independent experiments. Error bar represents standard error of mean (SEM). Fold change was calculated using delta-delta Ct method ($2^{\Delta\Delta Ct}$). Asterisk (*) indicates statistically significant difference, p< 0.05.
Fig 33: Change in gene expression profile of different TLRs in ovine ISEMF cells after poly I:C with lyovec stimulation. Each bar represents mean of three independent experiments. Error bar represents standard error of mean (SEM). Fold change was calculated using delta-delta Ct method ($2^{\Delta\Delta Ct}$). Asterisk (*) indicates statistically significant difference, $p< 0.05$. 
Fig 34: Change in gene expression profile of MyD-88, NF-Kb, and different cytokines in ovine ISEMF cells after poly I:C with lyovec stimulation. Each bar represents mean of three independent experiments. Error bar represents standard error of mean (SEM). Fold change was calculated using delta-delta Ct method ($2^{\Delta \Delta Ct}$). Asterisk (*) indicates statistically significant difference, $p< 0.05$. 

Poly I:C with lyovec stimulation

- Control
- MyD-88
- NF-kb
- IL-1b
- TGF-b
- IFN-a
- IFN-b
- IL-6
- IL-8

Fold Change

- 3 hrs
- 24 hrs
3.4 Discussion

In this study, we developed primary and SV40 immortalized ovine ileal subepithelial myofibroblast (ISEMF) cells from ileum of three-day old male lamb. Earlier we used the ileal tissue from the same animal to develop an ileal epithelial cell line (chapter 2). We successfully cultured primary ovine ISEMFs up to 17 passages. These cells showed the typical fibroblast-like morphology and expressed vimentin and alpha-smooth muscle actin markers indicating their mesenchymal origin. We immortalized the primary ISEMF cells with SV40 gene and were able to grow immortalized cells up to passage 35 without any major morphological changes.

LPS is the major outer surface protein present in gram-negative bacteria and plays role in stimulating innate immunity. LPS is primarily recognized by TLR4/MD-2 complex which in turn activates downstream signaling through MyD88 or TRIF pathways and ultimately produce inflammatory cytokines (191). In this study, LPS downregulated the expression of TLR4 and TLR5 genes in primary ovine ISEMFs which coincided with decreased expression of MyD88 in 3 hrs stimulation. Conversely, LPS upregulated the expression of TLR2 and TLR5 after 24 hrs stimulation and IL-6 after 3 hrs time point stimulation.

PGN is a component of bacterial cell wall most abundantly present in Gram-positive bacteria and plays role in determining the structure of the bacterial cell wall. However, in Gram negative bacteria PGN is present as thin layer which is covered by a thick layer of LPS. TLR-2 deficient mice demonstrated and characterized the role of TLR2 in recognition of PGN (192). Similarly, it is well known that PGN promotes inflammatory immune responses in animal model and cell culture studies. PGN
stimulates the production of inflammatory cytokines like IL-1a/b, TNF-a and IL-6 (193). PGN activates NF-kB pathway which contributes to the expression of pro-inflammatory cytokines genes. Similarly, PGN also plays role in activation of mitogen-activated protein kinase (MAPK) signaling pathway which stimulates the expression of inflammatory cytokines via activation of JNK, Erk-1 and p38 kinases (194). However, in the current study PGN did not change the mRNA expressions of tested TLRs and inflammatory cytokines. PGN only downregulated the expression of IFN-a at 3hrs time point. Often purified PGN fails to stimulate the immune responses as demonstrated in a previous study (195). Highly purified PGN from eight different bacterial species were not sensed by TLR2; however, purified lipoprotein, a covalently linked compound with PGN, alone was sensed by TLR2 (195). Our commercially purchased PGN preparation from Staphylococcus aureus could be a purified form of PGN and hence did not to stimulate the expression of inflammatory cytokines and TLRs in ovine ISEMF cells. Furthermore, specific dose of PGN used in this study may have affected the effective stimulation of ovine ISEMFs and, thus, use of different doses of PGN in further studies may clarify the dose effects.

FLA is the major subunit of the protein flagellum which makes up bacterial flagella. Flagellin helps in bacterial motility and adherence to the host cells. FLA is sensed by TLR5 which then can activate either MyD88-dependent or MyD88-independent signaling pathway to generate effective immune responses (196, 197). Recognition of FLA by TLR 5 stimulates the expression of proinflammatory cytokines, chemokines, nitric oxide, hydrogen peroxide and other various host-defense proteins (198, 199). In MyD88-dependent pathway FLA activates TLR5 that recruits MyD-88, an
adapter protein, which then induces MAPK or IκB pathway leading to activation of transcriptional factor AP-1 or NF-κB, ultimately producing proinflammatory cytokines. However, in MyD88-independent pathway FLA is sensed by TLR5/4 heterodimers that recruits TRIF which then activates transcriptional factor IRF3 leading to production of interferons (197, 200). In our study, FLA downregulated the expression of MyD88 at 3 hrs time point which coincided with the downregulation of TGF-b and TLR6 at 3 hrs time point. Since FLA can activate both MyD88-dependent or MyD88-independent pathway, in ISEMF cells TRIF pathway might have been activated which also explains the downregulation of cytokine TGF-b at the same time point. After 24 hrs of FLA stimulation there was no change in expression of any TLRs and cytokines. Though, after stimulating with FLA for 24 hrs, the expression of IL-1b increased by more than 6 folds (6.29±2.91) but was not found be statistically significant.

Imiquimod is a synthetic nucleoside analogue originally developed as potential antiviral agent (201). Over the years of studies have shown that imiquimod plays an important role in generating tumor-related cellular immune responses by activating TLR7 (202, 203). Imiquimod has been approved by Food and Drug Administration (FDA) for treatment of basal cell carcinoma and genital warts (204). Imiquimod induces the signaling cascade by the activation of transcriptional factor nuclear factor-kappa B (NF-Kb). NF-kB family of proteins includes p65, p50/p105, RelB, c-Rel and p52/p100. Under normal conditions NF-kB is in inactivated form located in the cytoplasm; however, following the activation of TLR signaling pathway, inhibitory kB (IκB) protein is phosphorylated releasing activated NF-Kb. This activated NF-kB is translocated into the nucleus leading to the transcription of pro-inflammatory genes. Imiquimod induces the
expression of cytokines such as IFN-a, TNF-a, IL-2, IL-6, IL-8, IL-12 and many others (201). These cytokines induced by imiquimod prefers induction of Th1 immune responses (201, 203). Imiquimod is also found to suppress the negative feedback mechanism that impairs the expression of pro-inflammatory cytokines ultimately favoring the production of inflammatory cytokines. Imiquimod binds to androgen receptor (AR) which suppress the transcription of proinflammatory cytokines. In addition to this, inhibition of adenylyl cyclase activity by imiquimod also helps in the expression of pro-inflammatory genes (201, 205, 206). At higher concentration (25-50ug/ml) imiquimod has been found to induce apoptosis by activating mitochondrial pathways (201). Ratio of pro-to anti apoptotic shifts towards pro apoptotic preferably Bcl-2 associated X (Bax) protein leading to apoptosis of tumor cells (201). In our study, after stimulating with imiquimod the gene expression of TLR6 was downregulated at 3hrs time point and INF-b expression was also downregulated after 24 hours of stimulation. Downregulation of IFN-b at 24 hours-time point was coupled with 3.3-fold increase in IL-1b and 3.8-fold increase in IL-8 expression at 24-hrs time point. However, the upregulation of IL-6 and IL-8 with imiquimod was not found to be statistically significant. Though, we did not study the expression profile of TLR7, receptor for imiquimod, in ISEMF cells, study in HaCat cell line which lacks expression of TLR-7 and TLR-8 showed increased expression of IL-6, IL-8, TNF-a and IL-b after stimulating with imiquimod. Mice lacking My-D88 also showed elevated expression of IL-1b upon imiquimod stimulation (206). Thus, imiquimod can induce the expression of pro-inflammatory cytokines in TLR7 and My-D88 independent manner.
Poly I:C, synthetic dsRNA analogue, is a potent TLR3 ligand and has been used often to generate antiviral INF-I/III responses (207, 208). TLR3 is chiefly found in endosomes and senses dsRNA (209). Many viruses produce dsRNA as a replication intermediate; hence, engagement of dsRNA by TLR3 can greatly influence the outcomes and clearance of such viral infections (207). Experimental inoculation of Poly I:C in rats induced fever in IL-1α dependent manner (210). Similarly, several studies have shown poly I:C to induce apoptosis in cancer cells and its potential use in cancer therapy (209).

Upon engagement with dsRNA, TLR3 activates series of signaling cascade by recruiting TRIF directly to the TIR domain. TRIF then activates TBK-1 which then phosphorylates IRF3 (211). Phosphorylated IRF3 translocate into the nucleus leading to production of type-I interferons (211). However, TLR3 has also been shown to activate NF-kB through TRIF signaling pathway as well (212, 213). TRIF signaling engages TRAF-6 which activates TAK1 which then activates IKK complex leading to phosphorylation of NFkB inhibitor (IkB). This results into translocation of nuclear translocation of NF-kB and production of pro-inflammatory cytokines (214). A study by Zhou et al., found out that high molecular weight poly I:C was more efficient than low molecular weight poly I:C in generating TLR3 responses (207). In our study, poly I:C stimulation did not change the expression profiles of any TLRs and cytokines of ISEMF cells. Though after 3hrs of poly I:C stimulation, the expression of IL-1b increase by 8.3 folds (8.3 ± 7.4), INF-b increased by 9.9 folds (9.9 ± 6.1), and IL-8 increased by 20.3 folds (20.3 ± 18.5) but were not found to be statistically significant. Often TLR3, the receptor for poly I:C, is also located on the cells surfaces of fibroblast, epithelial cells and macrophages (209). Cell membrane localized TLR3 have been found to trigger production of proinflammatory cytokines
whereas, endosomal TLR3 activation results in type I interferon responses (215). Since, TLR3 can activate both IRF3 and NF-kB, the 20-folds increase of IL-8, 9 folds increase of IFN-b and 8 folds increase of IL-1b in ISEMF cells could have been result of activation of both surface and endosomal TLR3. A study in human corneal epithelial cells found out increase in the production of IL-6, and IL-8 and increase in mRNA expression of INF-b after poly I:C stimulation (216).

We also stimulated ISEMF cells with poly I:C/lyovec complex. Lyovec is a lipid based cationic transfecting reagent (217). Since, poly I:C did not change the TLRs and cytokines expression in ISEMF cells, we then used poly I:C with lipid based complex (lyovec) to effectively deliver poly I:C in the cytoplasm. This method of using poly I:C along with lipid based transfecting reagent has been used to stimulate cells in several studies including stimulating human airway epithelial cells (218), mouse macrophages (219), primary human monocyte derived cells (217) and bovine ileal myofibroblast cells (unpublished data from our lab). Intracellular PRRs such as RIG-I and MDA-5 recognize dsRNA. Both RIG-I and MDA-5 are RNA helicases with caspase recruitment domain and helicase domain. Both RIG-I and MDA-5 upon interacting with dsRNA engages with mitochondrial antiviral signaling proteins (MAVS) and activates MAVS pathway leading to activation of NF-kB, IRF3 and IRF7. Activated IRF7 and IRF3 translocate into the nucleus and lead to the transcription of type-I interferon genes (IFN-a and INF-b) (220-224). In our study, poly I:C with lyovec upregulated the expression of TLR3 at 3 hrs stimulation. Expression of IFN-a at 24 hrs was upregulated by 49 folds (49.2 ± 33.2), IFN-b expression at 24 hrs was increased by 649 folds (649.4 ± 321.9) and IL-8 at 3 hrs increased by 10 folds (10 ± 8.5), however, these increase in fold changes of IFN-a, IFN-b
and IL-8 were not found to be statistically significant. Expression of IFN-β at 24 hrs increased at least by 150 folds. Thus, ISEM cells generated robust antiviral immune responses when stimulated by poly I:C with lyovec.

Here, we report first ever ileal sub-epithelial myofibroblast cell line derived from ileum of 3-day old sheep. To our best knowledge, there has not been any study carried out which reports the role of ovine ileal fibroblast cells in innate immunity. Our study was limited to accessing the innate immune responses of primary ovine ISEM cells at mRNA level only. We did not study whether these changes in mRNA levels translated to protein level. We also did not analyze the expression of cytoplasmic PRRs. Despite all these limitations, we studied constitutive expression of TLR 1,2,3,4,5,6 and 9 in primary ISEM cells and their responses to various bacterial and viral ligands.

3.5 Conclusion

In this study, we generated first ever primary intestinal sub-epithelial myofibroblast cell line from ileum of 3-day old male lamb. We also immortalized primary cells using SV40 large T-antigen. We were able to demonstrate constitutive expression of various TLRs and cytokines genes in primary ovine ISEM cells. Similarly, we found that the primary ovine ISEM cells responded to various bacterial and viral ligands and were able to generate innate immune responses. Thus, this cell line can be a good invitro model in investigating innate immune responses at sub-epithelial compartment.
4. Conclusions and future directions

In this study, we successfully established the primary ovine ileal epithelial cell (OIEC) line and immortalized the primary OIEC cell line with hTERT gene. Both the primary and immortalized cells were able to express typical epithelial like cobblestone and cuboidal morphology. Positive staining for cytokeratin in immunocytochemistry (ICC) confirmed epithelial origin of the cells. Similarly, primary and hTERT immortalized OIEC cells retained their normal physiological characteristics even during the late passages. Primary and hTERT immortalized OIEC cells were able to polarize when cultured in trans-well inserts. Indirect immunofluorescence assay (IFA) staining revealed presence of tight junction proteins mainly in the cell membrane of the cells. The expression profile of various glycans on the surface of primary and hTERT immortalized cells was performed using lectin binding assay. Twenty-three different lectins were used and their staining percentage was determined using flow-cytometry. Some differences in the staining percentage between the primary and immortalized OIEC cells were observed. These differences in the staining percentage between primary and immortalized cells could be due to differences in the gene expression profile between the cell types.

Furthermore, we also established primary ileal sub-epithelial myofibroblast cell line (ISEMF) and immortalized it with SV40 large T antigen gene. Primary ISEMF cells stained strongly against alpha-smooth muscle actin and stayed negative against cytokeratin confirming their mesenchymal origin. Primary ISEMF cells were stimulated using bacterial and viral ligands for 3h and 24 h. Gene expression profile of different TLRs and cytokines was assessed using qPCR. Primary ovine ISEMF cells were able to modulate their innate immune responses upon stimulation with the bacterial and viral
ligands. These, OIEC and ovine ISEMF cell lines can serve as good in vitro model of studying enteric disease pathogenesis and developing therapeutic strategies.

To our best knowledge, primary OIEC and ovine ISEMF cells are the first cell lines developed from ileum of sheep. In primary and hTERT OIEC cells we also studied the expression of different TLRs at gene level. Surprisingly, we were unable to detect the expression of the TLRs in both the cell types. This may be due to sensitivity of the qPCR assay or OIEC cells might require proper stimulus for the expression of TLRs. We also examined the expression of TLRs in the ovine ileal tissue and in contrast tissue samples expressed all the TLRs. OIEC cells can be co-cultured in trans well filters with ovine ISEMF cells to mimic in vivo conditions. This co-culture approach can provide additional growth and differentiation factors from fibroblast cells which can induce epithelial cells to express innate immune receptors. Similarly, supernatants from the fibroblast cell cultures can be collected and added onto epithelial cell cultures to stimulate the epithelial cells for expressing TLRs. Rare polymorphism in TLR gene can also be the reason for lack of TLR expression in OIEC cells. However, as in this study both OIEC and ISEMF cell lines were obtained from the same animal but ISEMF cells expressed various TLRs, this possible reasoning is ruled out.

In ovine ISEMF cells, expression of various TLRs and cytokines was only accessed at mRNA level. Whether the changes in mRNA level translated into protein level were not examined. Changes in cytokine protein level can be accomplished by performing ELISA from cell culture supernatant. Similarly, we only used one concentration for stimulating cells at 3hrs and 24hrs time points. Ideal approach for cell stimulation assay would be trying different concentrations of ligands at different time
point intervals and finding the optimum conditions for stimulating the cells. However, despite all these limitations, we were able to show constitutive expression of TLRs in ovine ISEMF cells and these cells responded to bacterial and viral ligands. One interesting observation was upregulation of IFN-beta at 24 hrs time point after poly I:C with lyovec stimulation. Since poly I:C with lyovec stimulates the cytoplasmic PRRs, further studies can be performed in studying the expression profiles of cytoplasmic PRRs like RIG-I and MDA-5 on ovine ISEMF cells.

Intestinal epithelial cells are constantly exposed to gut microbes and pathogens so studying the interaction between OIEC cells and different bacterial pathogens can provide interesting findings on how pathogens gain access into the body by modulating tight junction proteins. Similarly, presence of sialic acid receptors on cell surface of OIEC cells can be explored in studying influenza virus pathogenesis in small ruminants. ISEMF cells helps in wound healing, epithelial cell differentiation and maturation. Recent evidences have shown its role in pathogenesis of inflammatory bowel disease. So, detail understanding of innate immune mechanisms at sub-epithelial compartment using proper ISEMF cell lines can help in better understanding of the diseases like IBD. Hence, both OIEC and ISEMF cell lines can serve as valuable in vitro tools in elucidating enteric disease pathogenesis.
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107


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