REGULATION OF INNATE ANTIVIRAL RIG-I LIKE RECEPTOR (RLR) PATHWAYS VIA PU.1 AND CEBP PROTEINS

THESIS SUBMITTED TO THE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
ANIMAL BIOTECHNOLOGY
BY

JATINDER SINGH CHERA
M.Sc. (Human Genomics)

ANIMAL BIOTECHNOLOGY CENTRE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE
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KARNAL-132001 (HARYANA), INDIA

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This is to certify that the thesis entitled, **"Regulation of Innate Antiviral RIG-I Like Receptor (RLR) Pathways Via PU.1 and CEBP Proteins"** submitted by **Mr. Jatinder Singh Chera** towards the partial fulfillment of the award of the degree of **DOCTOR OF PHILOSOPHY IN ANIMAL BIOTECHNOLOGY** of the **ICAR-National Dairy Research Institute (Deemed University)**, Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

Sachinandan De

Dated: 08.09.2021

(Sachinandan De)
Major Advisor

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Jatinder Singh

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ABSTRACT

Innate immunity is a branch of the immune system that engages in the quick and non-specific defense against many infections, particularly viruses, which have the potential to cause a negative impact on farmers' livelihoods. Pattern recognition receptors (PRRs), which detect conserved pathogen-associated molecular patterns (PAMPs) carried by a wide range of pathogens, are one of the most significant properties that allow the mammalian cells to generate an innate immune response. RIG-I like receptors (RLRs), for example, are involved in the recognition of RNA molecules from viruses and the subsequent development of an immunological signaling cascade to protect the host. RLRs protect animals from viruses such as the Foot and Mouth Disease virus (FMDV), Classical Swine Fever virus, Bluetongue virus, Peste des petits ruminants virus (PPRV), and other Influenza viruses. Polyinosinic:polycytidylic acid or poly(I:C), is a synthetic mimic of viral dsRNA that has been utilized as a vaccine adjuvant and it strongly activates the RLR pathways in animal and cell culture models. Lineage determining transcription factors (LDTFs) including PU.1, CEBPA, and CEBPB play roles in cellular differentiation and immune system control. The regulatory activities of these LDTFs in relation to the RLR pathways have not been well examined.

The goal of this dissertation was to figure out how the LDTFs PU.1, CEBPA, and CEBPB regulate the RLR pathway genes. The effects of the siRNA-mediated silencing of PU.1, CEBPA, and CEBPB in the bovine macrophage cell line (BoMAC) on the RLR pathways were investigated. The impact of LDTF knockdown on the immunological response mediated by poly(I:C) was also studied. Additionally, the ectopic expression of the LDTFs (cloned in a constitutive mammalian expression vector) in non-immune cells for enhancing their immune response against poly(I:C) was also tested. The PU.1 gene was not successfully silenced in BoMAC because the siRNA for PU.1 caused up-regulation rather than down-regulation of its gene expression. CEBPA knockdown was successful resulting in moderate downregulation of the RLR pathways, although CEBPB silencing was unsuccessful. When BoMAC cells were stimulated with poly(I:C), CEBPA silencing inhibited the immunological response via the RLR and DNA sensing pathways. The RLR pathways were expected to be suppressed by the down-regulation of PU.1, but more research is needed to understand the mechanism behind the siRNA-mediated up-regulation of PU.1. Ectopic expression of the LDTFs in non-immune cells were also carried out and it was first tested in the MDBK (Madin Darby Bovine Kidney) cell line, which showed robust expression of the LDTFs. However, these cells were unresponsive to poly(I:C) stimulation and thus, primary buffalo fetal fibroblast and endometrial epithelial cells were selected for the experiments instead. In buffalo fetal fibroblasts and endometrial epithelial cells, PU.1 alone or in combination with CEBPA or CEBPB significantly increased the expression of the RLR and DNA sensing pathway genes. In endometrial epithelial cells, however, PU.1 in conjunction with CEBP proteins resulted in a stronger activation of the RLR pathway genes than PU.1 alone. CEBPA, like PU.1, was able to up-regulate the pathway genes, although to a lesser level, whereas CEBPB had no effect on the genes. PU.1 alone and in conjunction with CEBPA or CEBPB was also able to prime the fibroblasts and epithelial cells to generate powerful immune response against poly(I:C). CEBPA had a dampened priming activity, but CEBPB did not prime the cells to produce an immunological response to poly(I:C). These findings suggest that PU.1 is critical for the control of the RLR and DNA sensing pathways, albeit CEBPA plays a minor role in this regulation and CEBPB does not participate on its own. In the event of RNA virus infections, PU.1 and CEBPA could be exploited as therapeutic targets for either enhancing or inhibiting the immune responses.

सारांश

जन्मजात रोग प्रतिरोधक शक्ति, प्रतिरक्षा प्रणाली की एक शाखा है जो कई संक्रमणों विशेषत: किसानों की आजीविका पर नकारात्मक प्रभाव डालने की क्षमता रखने वाले विषाणुओं के खिलाफ त्वरित और गैर-विशिष्ट रक्षा प्रदान करती है । रोगजनकों की एक विस्तृत श्रृंखला द्वारा जनित संरक्षित रोगजनक से जुड़े आणविक पैटर्न (पीएएमपी) का पता लगाने वाले पैटर्न पहचान रिसेप्टर्स (पीआरआर), स्तनधारी कोशिकाओं को एक सहज प्रतिरक्षा प्रतिक्रिया उत्पन्न करने की अनुमति देने वाले सबसे महत्वपूर्ण गुणों में से एक हैं। उदाहरण के लिए, आरआईजी-I जैसे रिसेप्टर्स (आरएलआर), विषाणु से आरएनए अणुओं की पहचान और मेजबान की रक्षा के लिए एक प्रतिरक्षाविज्ञानी सिग्नलिंग कैस्केड के विकास में शामिल हैं। आरएलआरस जानवरों को फुट एंड माउथ डिजीज वायरस (एफएमडीवी), क्लासिकल स्वाइन फीवर वायरस, ब्लूटॉन्ग वायरस, पेस्ट डेस पेटिट्स रूमिनेन्ट्स वायरस (पीपीआरवी) और अन्य इन्फ्लुएंजा वायरस से बचाते हैं। पॉलीआईनोसीनिक:पॉलीसाईटीडायलिक एसिड या पॉली(आई:सी), वायरल डबल स्ट्रेन्डेड आरएनए की एक कृतिम नक़ल है जिसे वैक्सीन एडजुवेंट के रूप में उपयोग किया गया है। यह पशु और सेल कल्चर मॉडल में आरएलआर मार्ग को दृढ़ता से सक्रिय करता है। पीयू.1, सीईबीपीए और सीईबीपीबी सहित वंश निर्धारण प्रतिलेखन कारक (एलडीटीएफ) कोशिका-विभेदन और प्रतिरक्षा प्रणाली के नियंत्रण में भूमिका निभाते हैं। आरएलआर मार्ग के संबंध में इन एलडीटीएफ की नियामक गतिविधियों की अच्छी तरह से जांच नहीं की गई है। इस शोध का लक्ष्य यह पता लगाना था कि एलडीटीएफस पीयू.1, सीईबीपीए और सीईबीपीबी कैसे आरएलआर मार्ग जीन को नियंत्रित करते हैं। बोवाइन मैक्रोफेज सेल लाइन (बीओएमएसी) में आरएलआर मार्ग पर पीयू.1, सीईबीपीए, और सीईबीपीबी की एसआई-आरएनए की मध्यस्थता वाली साइलेंसिंग के प्रभावों की जांच की गई। पॉली(आई:सी) द्वारा मध्यस्थता वाली प्रतिरक्षा प्रतिक्रिया पर एलडीटीएफ नॉकडाउन के प्रभाव का भी अध्ययन किया गया। इसके अतिरिक्त, पॉली(आई:सी) के विरुद्ध प्रतिरक्षा प्रतिक्रिया को बढ़ाने के लिए गैर-प्रतिरक्षा कोशिकाओं में एलडीटीएफ (एक सारभूत स्तनधारी अभिव्यक्ति वेक्टर में क्लोन) की अस्थानिक अभिव्यक्ति का भी परीक्षण किया गया। पीयू.1 जीन को बीओएमएसी में सफलतापूर्वक साइलेंस नहीं किया जा सका क्योंकि पीयू.1 के लिए एसआई-आरएनए जीन अभिव्यक्ति के घटाव के बजाय बढ़ोतरी का कारण बना। सीईबीपीए नॉकडाउन सफल रहा, जिसके परिणामस्वरूप आरएलआर मार्गों का मध्यम घटाव हुआ, हालांकि सीईबीपीबी साइलेंसिंग असफल रहा। जब बीओएमएसी को पॉली(आई:सी) से उत्तेजित किया गया, तो सीईबीपीए साइलेंसिंग ने आरएलआर और डीएनए संवेदन मार्ग के माध्यम से प्रतिरक्षात्मक प्रतिक्रिया को कम कर दिया। पीयू.1 के डाउन-रेगुलेशन द्वारा आरएलआर मार्गों के दुर्बल होने की आशा थी, लेकिन पीयू.1 के एसआई-आरएनए की मध्यस्थता वाले अप-रेगुलेशन के पीछे के क्रियाविधि को समझने के लिए और अधिक शोध की आवश्यकता है। गैर-प्रतिरक्षा कोशिकाओं में एलडीटीएफस के अस्थानिक अभिव्यक्तियाँ का पहले एमडीबीके (मैडिन डार्बी बोवाइन किडनी) सेल लाइन में परीक्षण किया गया, जिसमें एलडीटीएफस के प्रचंड अभिव्यक्तियाँ पेश हुईं। हालाँकि, ये कोशिकाएँ पॉली(आई:सी) उत्तेजना के प्रति अनुत्तरदायी थीं और इस प्रकार, प्राथमिक भैंस भ्रूण फाइब्रोब्लास्ट और एंडोमेट्रियल उपकला कोशिकाओं को इसके बजाय प्रयोगों के लिए चुना गया। भैंस भ्रूण फाइब्रोब्लास्ट और एंडोमेट्रियल उपकला कोशिकाओं में पीयू.1 ने अकेले एवं सीईबीपीए या सीईबीपीबी के संयोजन में भी, आरएलआर और डीएनए संवेदन मार्गों के जीन की अभिव्यक्ति में काफी वृद्धि करी। हालांकि, एंडोमेट्रियल उपकला कोशिकाओं में अकेले पीयू.1 की तुलना में पीयू.1 एवं सीईबीपी प्रोटीनस के संयोजन ने आरएलआर मार्गों के जीनस को अधिक उत्प्रेरित किया। सीईबीपीए, पीयू.1 की तरह, मार्गों के जीनस को अप-रेगुलेट करने में सक्षम था, हालांकि कम स्तर तक, जबकि सीईबीपीबी का जीनस पर कोई प्रभाव नहीं पड़ा। अकेले पीयू.1 और पीयू.1 सीईबीपीए या सीईबीपीबी के संयोजन में भी, पॉली(आई:सी) के विरुद्ध शक्तिशाली प्रतिरक्षा प्रतिक्रिया उत्पन्न करने के लिए फाइब्रोब्लास्ट्स और उपकला कोशिकाओं को तैयार करने में सक्षम रहा। सीईबीपीए में यह तैयारी करवाने की क्षमता कम पाई गई, परन्तु सीईबीपीबी कोशिकाओं में पॉली(आई:सी) के विरुद्ध प्रतिरक्षात्मक प्रतिक्रिया प्रस्तुत करवाने की तैयारी में विफल रहा। इन निष्कर्षों से पता चलता है कि पीयू.1 आरएलआर और डीएनए संवेदन मार्गों के नियंत्रण के लिए महत्वपूर्ण है, यद्यपि सीईबीपीए इस विनियमन में एक छोटी भूमिका निभाता है और सीईबीपीबी स्वयं कोई भाग नहीं लेता है। आरएनए विषाणु के संक्रमण की स्थिति में, पीयू.1 और सीईबीपीए का उपयोग प्रतिरक्षा प्रतिक्रिया को बढ़ाने या बाधित करने के लिए चिकित्सीय प्रयोग में लाया जा सकता है।

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CHAPTER –1

Introduction

INTRODUCTION

The immune system is a constantly evolving system that helps the host to defend itself against wide range of pathogens and hazardous self-associated molecules. The two important arms of immunity are the innate and the adaptive immunity. The innate immunity serves as the first line of defense which a pathogen encounters and it comprises of several barriers; immune cells such as macrophages, neutrophils, NK cells, dendritic cells, etc. along with various non-immune somatic cells (Mehraj et al., 2019). One of the characteristic features of innate immunity is the presence of germline-encoded pattern recognition receptors (PRRs) that recognize different signature molecules from the pathogens called pathogen-associated molecular patterns (PAMPs). The PAMPs can be polysaccharides, peptides, lipoproteins, nucleic acids, etc. that are detected by host cellular PRRs. The different PRRs are TLRs (Toll-like receptors), RLRs (RIG-I like Receptors), NLRs (NOD like Receptors), CLRs (C-type Lectin Receptors) and ALRs (AIM2 like Receptors) (Carty et al., 2021). RLRs, ALRs and some TLRs are involved in the detection of different forms of pathogenic nucleic acids. Endosomal TLR3, TLR7 and TLR8 along with RLRs are involved in the detection of pathogenic RNA (Hung et al., 2018; Streicher and Jouvenet, 2019). RLRs comprise of 3 receptor proteins namely RIG-I (Retinoic acid-Inducible Gene I), MDA5 (Melanoma Differentiation-Associated protein 5) and LGP2 (Laboratory of Genetics and Physiology 2) that are present in the cytoplasm and are involved in the detection of many types of RNA viruses (Rehwinkel and Gack, 2020). Endosomal TLR9, cytosolic receptors like ALRs, DAI (DNA-Dependent Activator of IFN-Regulatory Factors), cGAS (cyclic GMP-AMP synthase), DDX41 (DEAD-Box Helicase 41) and STING (Stimulator of Interferon Genes) are involved in the detection of DNA molecules (Tan et al., 2018). The viruses causing diseases in animals have the potential to become a burden on the livelihoods and the overall economy of nations due to the rapid evolution of their proteins and resistance to host immune responses (especially in case of RNA viruses). Many highly virulent livestock associated viruses like FMDV (Foot and Mouth Disease virus), Bluetongue virus, PPRV (Peste des petits virus), Classical Swine Fever virus, Influenza virus, etc. are detected by the RLR pathways (Chauveau et al., 2012; Dong et al., 2013; K. Li et al., 2021; P. Li et al., 2021; Malik and Zhou, 2020). A viral dsRNA analog polyinosinic:polycytidylic acid or poly(I:C) has been widely used as a vaccine adjuvant

to boost the immune response against many viral infections and it has been shown to get sensed by RIG-I and MDA5 (Qiao et al., 2021). The two forms of poly(I:C) widely used are low molecular weight (LMW) and high molecular weight (HMW) which are of lengths ranging from 0.2-1 kb and 1.5-8 kb respectively (Mueller et al., 2019). MDBK (Madin Darby Bovine Kidney) epithelial cell line has proved useful for various studies related to many bovine viruses (Fay et al., 2020; Kopliku et al., 2015; Zhu et al., 2016, 2015) and thus makes it a suitable non-immune cellular model for RLR pathway studies although, these cells failed to exhibit RLR pathway activation in response to poly(I:C) in this dissertation as discussed later. As a result, primary fetal fibroblasts and endometrial epithelial cells from buffalo were chosen as the cell model of choice. Fibroblasts are mesenchymal cells that play an important role in the detection of pathogens and have the capability to generate immune response when transfected with poly(I:C) (Vats et al., 2020). Bovine endometrial epithelial cells have been reported as a model for RLR pathway studies conducted using poly(I:C) as an immune-stimulant (Carneiro et al., 2017). Bovine macrophage cell line (BoMAC) was developed by SV40 plasmid transformation of bovine peritoneal macrophages (Stabel and Stabel, 1995) in which, several viral and bacterial studies have been conducted (Z. Li et al., 2018; Maina et al., 2019; Rola-Łuszczak et al., 2014). BoMAC is also reported to generate immune response against poly(I:C) (Toka et al., 2019).

Lineage determining transcription factors (LDTFs) are the transcription factors that are responsible for the determination of the lineage to which a cell would terminally differentiate and thus, gain specialized functions. In various combinations, the LDTFs have the capability of promoting terminal differentiation of cells to various lineages like myeloid, lymphoid, neural, pluripotent, etc. (Hosokawa and Rothenberg, 2021; Kurotaki et al., 2020; J.-E. Lee et al., 2019; Oh and Jang, 2019). The LDTFs can be used to manipulate cell fates such as reprogramming of somatic cells into pluripotent stem cells (Takahashi and Yamanaka, 2006; Yu et al., 2007) as well as conversion of terminally differentiated cells of one lineage into the cells of other lineages (Hybiak et al., 2020; Kadkhodaeian, 2021; Niu et al., 2013; Qin et al., 2020; Wang et al., 2021). PU.1 is one such LDTF which belongs to the ETS (Erythroblast Transformation Specific) family of transcription factors that is encoded by the *Spi1* gene. It is responsible for the differentiation of various cell types, predominantly myeloid and lymphoid cells (Guanglan et al., 2020). Due to the ability of PU.1 to cause commitment of cells to

myeloid lineages, researchers have successfully demonstrated the conversion of non-myeloid cells into myeloid-like cells and up-regulation of the immune response genes by ectopic expression of PU.1 alone or along with other associated transcription factors like CEBPA and to a lesser extent, CEBPB (Feng et al., 2008; Laiosa et al., 2006; McClellan et al., 2015; Orsini et al., 2019; Rosa et al., 2018). Ectopic expression of PU.1 in HeLa cells leads to the establishment of monocyte like enhancers which further causes the expression of various monocyte-specific genes that are otherwise repressed in the HeLa cells. The enhancer selection and the associated monocyte gene expression (including inflammatory response involving NF-κB) became stronger when PU.1 was co-expressed with CEBPA (Jin et al., 2011). PU.1 also plays an important role in the modulation of several innate immunity PRR genes such as TLR2, TLR4 and Dectin-1 (Haehnel et al., 2002; C. Liu et al., 2017; Roger et al., 2005) while there haven't been much studies conducted to directly correlate CEBP transcription factors with innate immunity. CEBPA is known to up-regulate the expression of anti-microbial peptide cathlecidin (Dhawan et al., 2015). Various innate immune signaling also cause the induction of CEBP transcription factors which further induce the genes that play role in the downstream events of immune signaling. CEBPs have also been reported to play role in LPS mediated inflammatory response (Hu et al., 1998). CEBPA also plays an active role in the TLR3 mediated pathway against HIV (Human Immunodeficiency Virus) infection (Bhargavan et al., 2016). The roles of LDTFs in the regulation of the RLR pathways is still not well established and therefore, to unravel the underlying mechanisms, following objectives were taken up for this dissertation:

1. To analyze the RLR pathway and effector genes in bovine macrophage cell line (BoMAC) subjected to knock-down of PU.1 and C/EBP proteins
2. To express PU.1 and C/EBP proteins in non-immune cells to enhance the activity of RLR pathway and effector genes

CHAPTER –2

Review of Literature

2.1. The innate immunity

The first and the foremost system in the body that rapidly acts against invading pathogens is the innate immunity. Innate immune response acts against wide variety of pathogens in a non-specific manner in contrast to adaptive immunity which is specific for individual pathogens. The innate immune system comprises of physical barriers like skin, epithelial and mucus layers, phagocytic cells such as myeloid cells, serum proteins like complement and acute phase proteins, antimicrobial molecules like lysozyme, lectins and anti-microbial-peptides as well as pattern recognition receptors (PRRs) that are able to detect signature conserved molecular patterns on pathogens called pathogen associated molecular patterns (PAMPs) (Kaur and Secord, 2019; Shimizu et al., 2021). After the detection of PAMPs by PRRs, signaling cascades are triggered that lead to the release of cytokines including interferons in order to get rid of the pathogen (Brubaker et al., 2015; Carty et al., 2021). There are several classes of PRRs which include TLRs, RLRs, NLRs, CLRs and ALRs (Carty et al., 2021). RLRs, ALRs and some TLRs are involved in the detection of different forms of pathogenic nucleic acids. RIG-I, MDA5 and LGP2 come under the RLR category which are present in the cytoplasm and detect wide range of RNA viruses (Rehwinkel and Gack, 2020). RIG-I is capable of detecting 5' triphosphate/diphosphate containing ssRNA or dsRNA which are the characteristics of many RNA viruses while MDA5 detects longer dsRNA viral molecules (Rehwinkel and Gack, 2020). The role of LGP2 has been proposed in the regulation of RIG-I and MDA5 but it remains to be clearly established (Sanchez David et al., 2019). DAI or ZBP-1 (Z-DNA binding protein) elicits immune response after it detects Z-form of RNA or DNA in the cells infected with viruses such as Influenza virus and Murine Cytomegalovirus (Koehler et al., 2020). Detection of PAMPs by PRRs is the hallmark of innate immune responses that is responsible for generation of pro-inflammatory cytokines and interferons which further lead to the expression of Interferon-stimulated genes (ISGs) that help in the clearance of viral load (Carty et al., 2021; Schoggins, 2019).

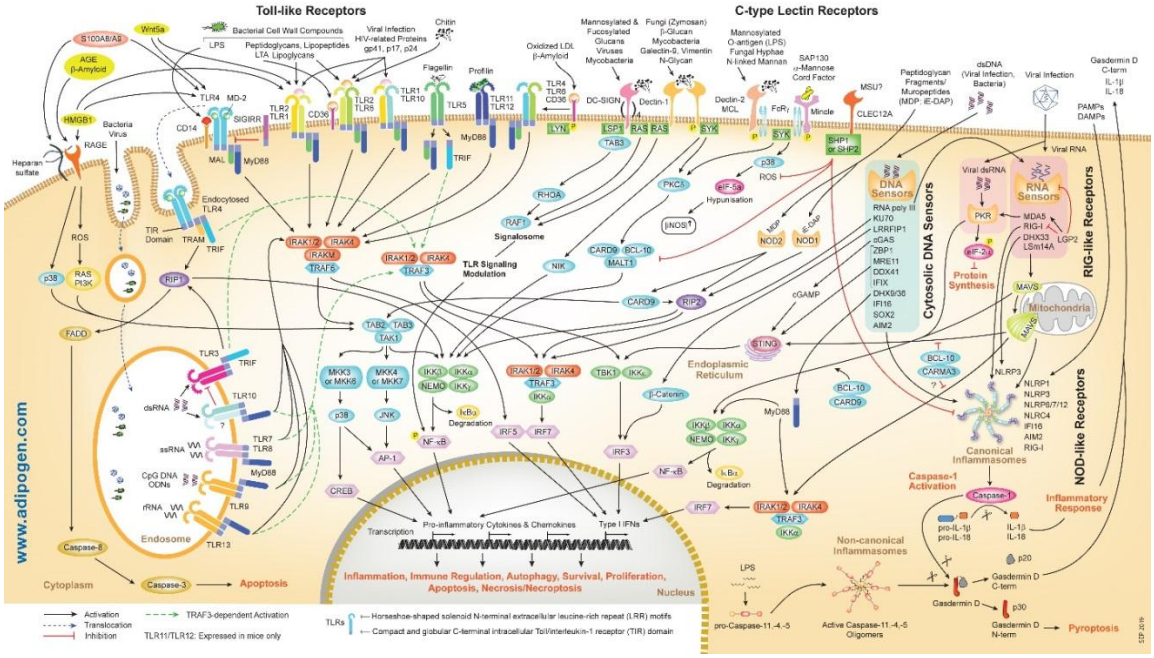


Figure 2.1: PRR signaling pathways. All of the PRR signaling pathways converge to the level of transcription factors comprising of CREB, AP1, NF-κB, IRF3 IRF5 and IRF7 which are responsible for transcriptional up-regulation of pro-inflammatory cytokines, apoptotic genes and interferons. The nucleic acid sensing PRR signaling including endosomal nucleic acid sensors converge at the level of mediator TRAF3 (TNF Receptor Associated Factor 3), TRAF6 (TNF Receptor Associated Factor 6) and IKKs (IκB kinases) that lead to the activation of transcription factors NF-κB, IRF3 and IRF7 for generating antiviral immune responses. (Image downloaded from www.adipogen.com)

2.2. Innate immunity against RNA and DNA viruses

RNA and DNA from many viruses have the potential to initiate innate immune signaling cascades. The RNA of viruses in mammalian cells is mainly detected by cytosolic RLRs as well as endosomal TLR3, TLR7 and TLR8 (Uehata and Takeuchi, 2020). Viral DNA can be sensed by ALRs, endosomal TLR9, cGAS, STING, DAI and DEAD box helicases such as DHX9, DHX36, DDX41 (Briard et al., 2020; Koehler et al., 2020). The RLR RIG-I is capable of detecting 5' triphosphate/diphosphate containing short single stranded RNA (ssRNA) or double stranded RNA (dsRNA) which are the characteristics of RNA viruses such as those from *Flaviviridae*, *Rhabdoviridae, Orthomyxoviridae, Filoviridae* and *Paramyxoviridae* families while MDA5 detects longer dsRNA molecules from the members of *Picornaviridae* (Gitlin et al., 2006; Goubau et al., 2014; Hornung et al., 2006; Kato et al., 2006; Pichlmair et al., 2006; Rehwinkel and Gack, 2020; Thompson et al., 2011). The role of LGP2 has been proposed in the regulation of RIG-I and MDA5 but it remains to be clearly established (Sanchez David et al., 2019). All

RLRs possess DEAD box helicase domain and a C-terminal domain (CTD), while RIG-I and MDA5, but not LGP2, additionally have a N-terminal caspase recruitment domain (CARD), which interacts with CARD of adaptor molecule called MAVS/ IPS-1 (mitochondrial anti-viral signaling protein). The helicase domain is involved in the detection of viral RNA which causes an ATP-dependent change in conformation that allows CARD-CARD interaction between the RLR and MAVS and thus, K63-linked ubiquitin conjugation via tripartite motif protein 25 (TRIM25) (Okamoto et al., 2018). The interaction of many CARDs from RLRs and MAVS cause their aggregation recruits further signaling adaptors and kinases like IκB kinase (IKK) family of kinases, IKKa/b/g, TBK1 and IKKe. These kinases lead to the activation of NF-κB and IRFs which translocate into the nucleus to induce interferons and pro-inflammatory cytokines. Interferons bind to the interferon receptors on the cell membrane which follow the JAK-STAT pathway to induce the expression of ISGs (IFN-stimulated genes) (Owen et al., 2019). The dsRNA analog poly(I:C) has been shown to get sensed by RIG-I and MDA5 (Chen et al., 2017). The widely used two forms of poly(I:C) are low molecular weight (LMW) and high molecular weight (HMW) which are of lengths 0.2-1 kb and 1.5-8 kb respectively (Mueller et al., 2019). MDA5 has been shown to get activated by HMW poly(I:C) while RIG-I is activated by LMW poly(I:C) (Kasumba and Grandvaux, 2019; Kato et al., 2011). Poly(I:C) stimulation of ovarian fibroblasts has been reported to generate anti-HIV immunity by secretion of antimicrobials and activation of CD4+ T-cells (Patel et al., 2018). Intranasal administration of poly(I:C) along with inactivated Influenza vaccine also leads to generation of high amounts of IgA and serum IgG antibodies against both homologous and heterologous viral infections (Moriyama et al., 2017; Thomas et al., 2015). Poly(I:C) has also been used to improve immunogenicity against malarial subunit vaccine rPfMSP-142 by production of Th1 antibodies (Mehrizi et al., 2018). Poly(IC:LC) which is poly(I:C) stabilized with poly-L-lysine and carboxy-methyl cellulose has also been shown to be effective against SARS-Coronavirus and Ebola-Zaire virus infection by generating broad spectrum innate antiviral immunity and also, long term protective immunity (Kende et al., 2019; Kumaki et al., 2017).

The DNA sensor cGAS, detects DNA from various retroviruses such as HIV, SIV (Simian Immunodeficiency Virus) and MLV (Murine Leukemia Virus). When DNA binds to cGAS, a molecule called 2'3'-cGAMP is formed from ATP and GTP which acts as a secondary messenger for activating STING; the protein responsible for downstream

activation of interferon pathways (Motwani et al., 2019). ALRs consist mainly of two proteins, AIM2 and IFI16 (Kumar et al., 2019). Both AIM2 and IFI16 sense dsDNA in the cytoplasm (and in the nucleus by IFI16) from various pathogens like HSV (Herpes Simplex Virus) and Vaccinia virus. The activation of these receptors occurs in sequence-independent manner which leads to the production of type I interferons through the STING pathway along with the formation of inflammasome via signaling through a protein called ASC (Apoptosis-associated Speck-like protein containing a Carboxy-terminal CARD) (Chang et al., 2019; Jin et al., 2012; Kerur et al., 2011; Rathinam et al., 2010; Sapre and Nair, 2020; Unterholzner et al., 2010). IFI16, cGAS and STING have also been shown to play roles in the Interferon-β induction against *Listeria monocytogenes* infection (Hansen et al., 2014). DAI or ZBP-1 (Z-DNA binding protein) has been shown to elicit immune response after it detects Z-form of DNA (or RNA) in the cells infected with viruses such as Influenza virus and Murine CMV (Cytomegalovirus) (Kuriakose and Kanneganti, 2018). DDX41 acts as a sensor for viral DNA and bacterial secondary messengers like cyclic-di-GMP (Y. Jiang et al., 2017). The RNA and DNA sensors also have the potential to detect RNA and DNA from the host itself under distressful conditions such as cell death, cancer, metabolic stress and autoimmune disorders (Briard et al., 2020; Khodarev, 2019).

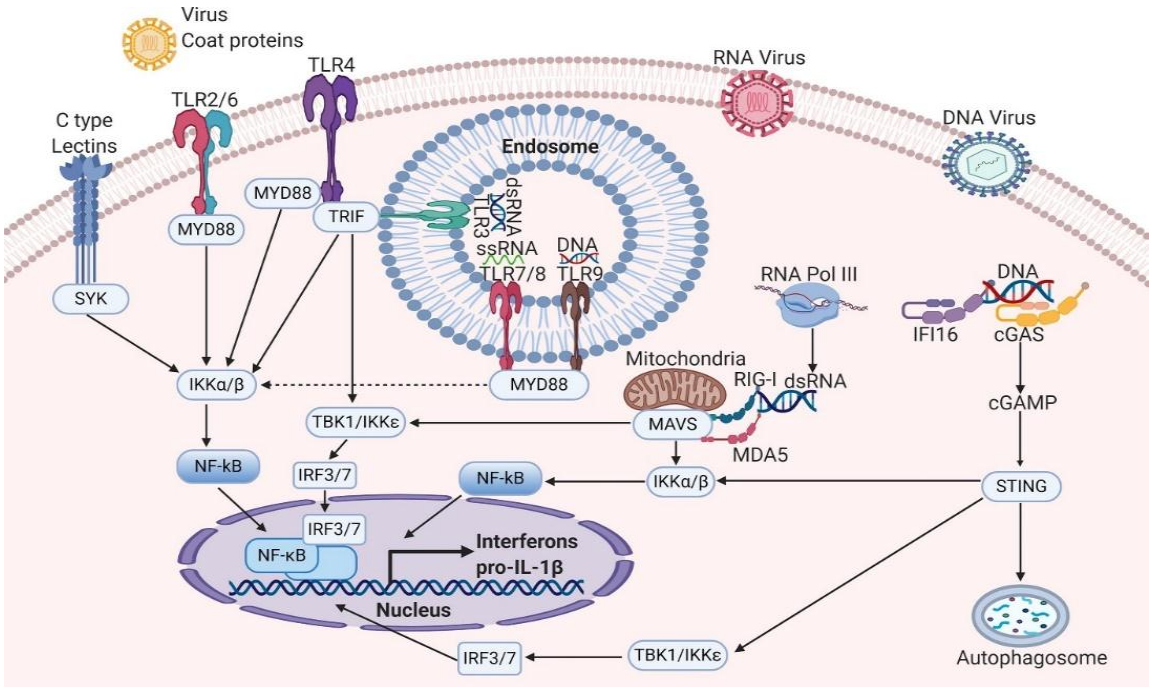


Figure 2.2: Conventional RNA and DNA sensing pathways that act against viruses in mammalian cells. The cytosolic receptors and endosomal TLRs recognize different types of RNA and DNA viruses in order to generate a signaling cascade that leads to the induction of inflammatory cytokines and antiviral interferons. (*Source: Carty et. al., 2020)*

The nucleic acid sensing pathways play major roles against many deadly viruses in livestock animals that cause huge losses to economy and livelihoods. Peste des petits virus (PPRV) causes huge economic losses to the goat and sheep industry each year (Bardhan et al., 2017) and it is detected by the RLR pathways (P. Li et al., 2021). Foot and Mouth Disease virus (FMDV) is a serious threat to livestock industry that can also be sensed through RLR pathways (K. Li et al., 2021). Zoonotic Influenza A viruses (IAVs) occur in wide variety of animals and pose a serious threat to both, the economy and human lives (Borkenhagen et al., 2019). RIG-I is the main RLR involved in the detection of such IAV infections (Weis and Te Velthuis, 2021). Another RNA virus, Bluetongue virus is also capable of causing economic losses to the livestock industry (Gethmann et al., 2020) which is detected by RIG-I and MDA5 (Chauveau et al., 2012). Recently, RIG-I has also been reported to act against gastrointestinal parasite *Haemonchus contortus* in sheep that is capable to cause huge losses in the sheep industry (Banerjee et al., 2021). The DNA sensors IFI16 and cGAS are able to sense DNA from wide variety of viruses (H. C. Lee et al., 2019). These sensors are also known to detect the DNA of intracellular bacteria (Patrick et al., 2016). However, in livestock DNA viral diseases, not much study has been carried out in deciphering the roles of DNA sensors. In one report, cGAS-STING pathway has been shown to act against *Mycobacterium bovis* infection that potentially causes economic loss to cattle farming (Li et al., 2019).

2.3 Evasion of immune response by RNA and DNA viruses

The pathogenic viruses have acquired many strategies to obliterate the PRRs along with associated pathway proteins in order to suppress the inflammatory responses of the host. There is a constant evolutionary arms race between viruses and the host involving incorporation of changes in their virulence and immunity related gene sequences respectively in order to gain competitive advantage over one another (Kikkert, 2020). Due to these mechanisms, delayed immune responses are generated that lead to establishment of peak viral titers prior to detection by the host (Menachery et al., 2014). Many RNA viruses including highly pathogenic coronaviruses, flaviviruses, arteriviruses, noroviruses and picornaviruses shield their dsRNA replicative intermediates from innate immune sensors in replication compartments (García-Nicolás et al., 2018; Wolff et al., 2020). Influenza A virus and recently discovered bunyaviruses engage in ‘cap-snatching’ which is stealing of 5’-cap structures from host mRNA in order to prevent detection by innate RNA sensors (De Vlugt et al., 2018; Olschewski et

al., 2020). Coronaviruses also possess viral RNA capping enzymes which helps them to evade host immunity (Hartenian et al., 2020). Crimean–Congo hemorrhagic fever virus, Borna disease virus (BDV), and hantavirus (HTNV) remove the 5′-triphosphate group of their RNA in order to evade RIG-I detection (Y. Liu et al., 2017). The N protein of Rabies virus also helps in preventing the viral RNA detection by RIG-I (Katz et al., 2017). Coronavirus nsp15 endoribonuclease sequesters dsRNA intermediates away from the RNA sensing PRRs (Deng et al., 2017). Influenza A viruses possess NS1 protein that has been reported to promote host immunity evasion by several ways; one of which is sequestering of viral RNA away from RNA sensors (Nogales et al., 2018). A very deadly Zaire Ebolavirus (a filovirus) encodes VP35 protein that binds dsRNA to prevent RIG-I detection and signaling (Woolsey et al., 2019). Additionally, several viruses encode proteins that cause disruption or degradation of innate sensors to evade immune responses. RIG-I, MDA5 and LGP2 have been reported to get degraded/inhibited by several picornaviruses including FMDV, EMCV (Encephalomyocarditis Virus), poliovirus and rhinovirus (Zhang et al., 2020). The classical swine fever virus N^{pro} protein antagonizes RLR mediated immune response by interacting with IRF3 to prevent IFN-I induction and apoptosis (Hardy et al., 2021). The DNA virus HSV-1 encodes US11 and UL37 proteins that are involved in the inhibition of MDA5 and RIG-I (Zhu and Zheng, 2020). Similar to sequestration of RNA, several viruses sequester viral DNA from the immune receptors. ORF52 protein of gammaherpesviruses is able to bind cytosolic DNA as well as cGAS to prevent immune responses (Wu et al., 2015). The capsid protein of HIV-1 interacts with host proteins that plays a vital role in shielding the viral DNA in the host (Sultana et al., 2019). The DNA sensor cGAS is cleaved/inhibited by different sets of proteins in Dengue virus, Zika virus, Vaccinia virus, HSV-1, KSHV (Kaposi's Sarcoma-associated Herpesvirus) and HCMV (Human Cytomegalovirus) (Eaglesham and Kranzusch, 2020). HSV-1 encoded VP22 inhibits cGAS by binding to it and the tegument protein UL41 of the virus leads to the degradation of cGAS and IFI16 mRNAs (Zhu and Zheng, 2020). IFI16 is also reported to be targeted by pUL83 and ICP0 proteins encoded by herpesviruses (Christensen and Paludan, 2017).

2.4. Role of epigenetics and lineage determining transcription factors (LDTFs) in cellular functions

Epigenetics is a vast term that comprises of the molecular mechanisms which act together in harmony to bring changes in the gene expression without any influence on

the gene sequence (Li, 2021). It consists of many components like covalent histone modifications, RNA interference, DNA methylation, ATP-dependent remodelers of chromatin and 3D genome landscape (Kim and Kaang, 2017; Morrison, 2020). Covalent modifications occur on the tail of histones proteins that form the octameric structure called nucleosomes which help in wrapping up the DNA. Histones H2A, H2B, H3 and H4 are part of the nucleosomes and post translational modifications on the amino acid residues of the histone tails lead to different outcomes such as gene expression or repression (Rothbart and Strahl, 2014). These modifications are initiated by different enzymes like HAT (histone acetyl transferase), HDAC (histone deacetylase), HMT (histone methyl-transferase), etc. which deliver different chemical groups on the amino acid residues of histone tails.

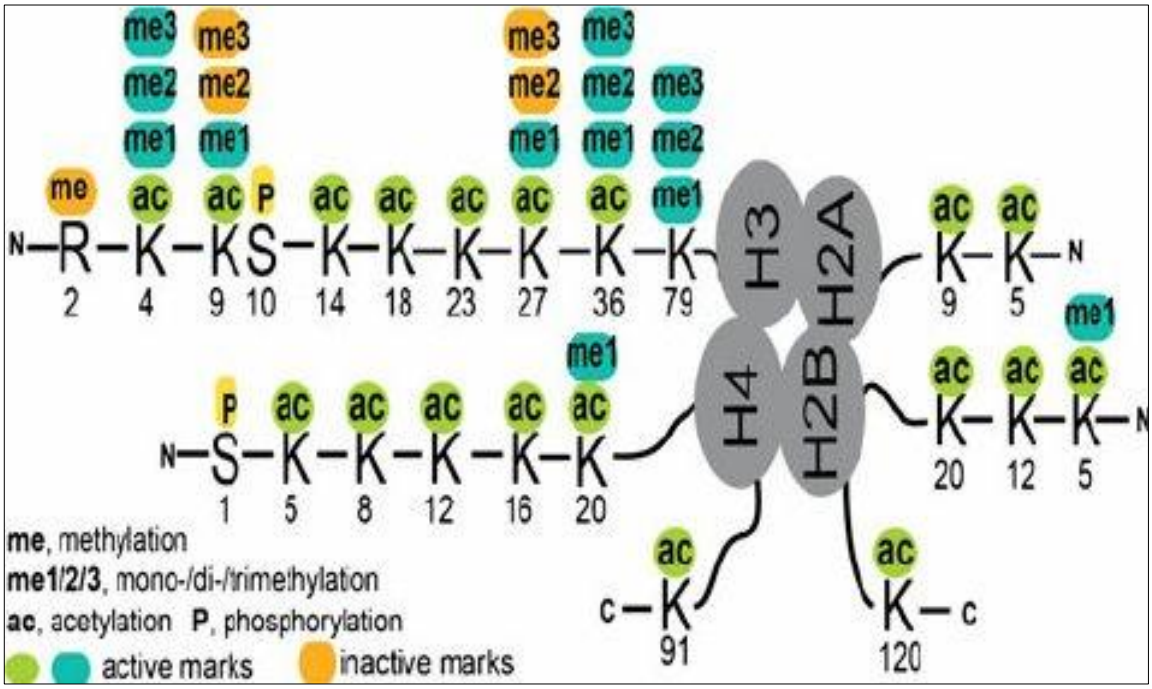


Figure 2.3: Covalent modifications of amino acids on histone tails. The widely studied modifications on histone tail amino acids have been shown in the figure. Most of the modifications occur on lysine residues. The numeric values denote the lysine position of the respective histone tail (*Source: Dai, H. and Wang, Z.; 2014)*

The overall genome architecture of the cells is shaped by intricate organization of DNA in 3D space because of which certain regions of genome come in contact with other regions depending on the cell type. Non-coding enhancer sequences play important role in gene regulation as these sequences recruit accessory proteins that increase the transcription of the genes with whose promoters they come in contact with (Andersson and Sandelin, 2020). The presence of H3K4me1 and H3K27me3 (methylation) is a

characteristic of poised enhancer (which gets activated under certain conditions such as differentiation) while H3K27ac (acetylation) is a marker for active enhancer that increases the transcription of a gene. The enhancers only possessing H3K27me3 are repressed enhancers (Nguyen et al., 2015).

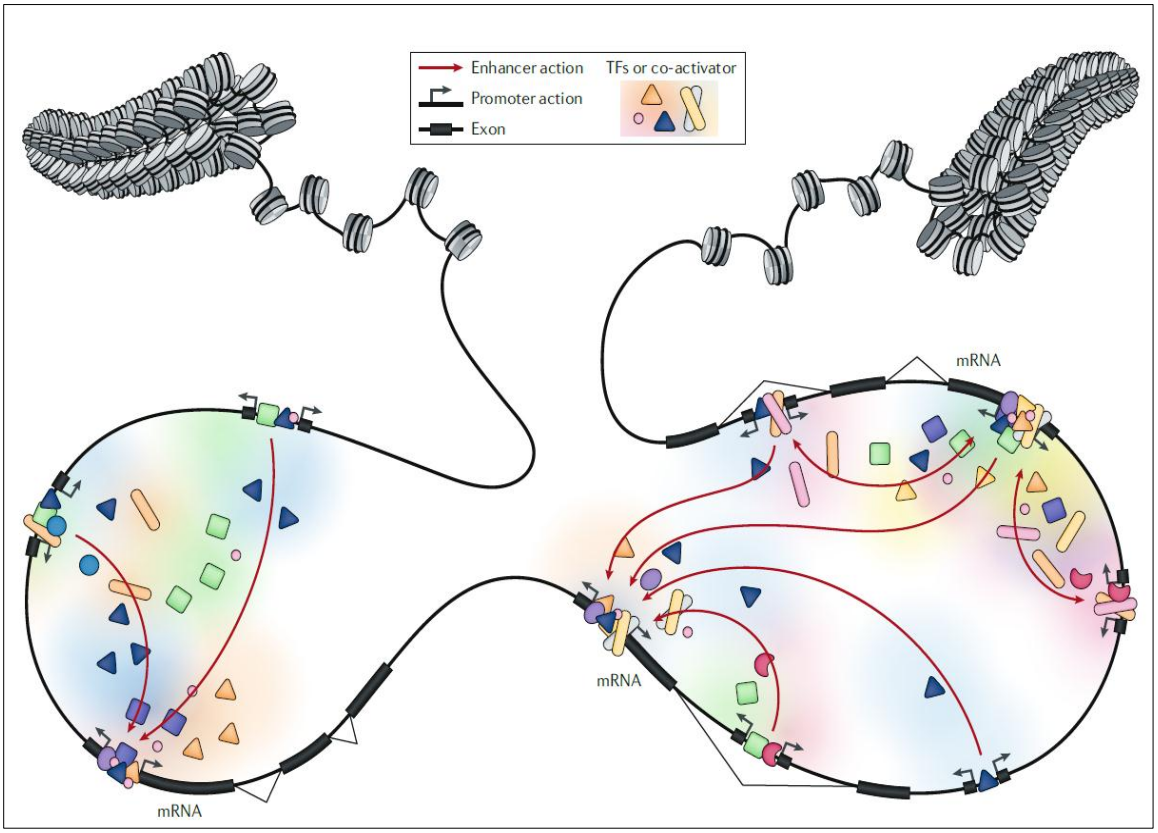


Figure 2.4: Schematic representation of transcriptional regulation via networks of enhancers and promoters established by transcription factors and RNA polymerases. *In the figure, a complex promoter-enhancer relationship has been depicted in which enhancers are increasing the mRNA levels of the genes by interacting with their promoters via transcription factors and associated cofactors/activators that ultimately recruit RNA polymerases.* (*Source: Andersson and Sandelin, 2020).*

The poised enhancers are bound by special transcription factors called lineage determining transcription factors (LDTFs) and signal dependent transcription factors (SDTFs) that are involved in the differentiation and other cell specific functions like immune response only when required. The LDTFs are responsible for shaping the genome by selecting pre-existing enhancers for specific cell types while in cooperation with SDTFs, they can select *de novo* enhancers in response to stimulus (Hagman, 2015; Ostuni et al., 2013; Pouzolles et al., 2016).

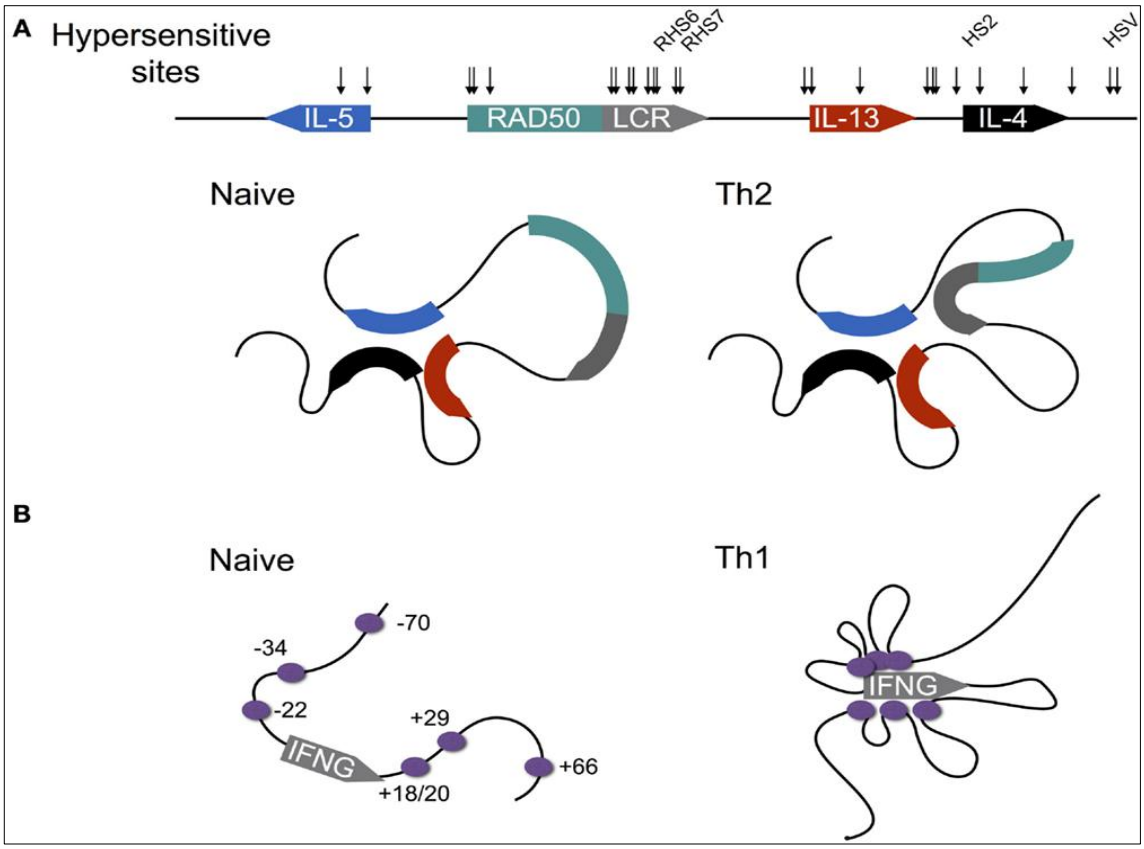


Figure 2.5: Chromatin looping of enhancer and promoter regions for Interferon gamma gene in different cell types. ***A)*** *The promoters of the genes IL-5, IL-13 and IL-4 are clustered together in naïve CD4+ T-cells. The 3' intron of RAD50 gene contains an LCR (locus control region) for the genes mentioned above that aids in their transcriptional up-regulation during differentiation. This LCR loops onto the IL genes when naïve cells differentiate into Th2 cells and thus, causing their transcriptional up-regulation.* ***B)*** *IFN-gamma in mouse undergoes similar mechanism but when differentiating into Th1 cells (purple dots are enhancer regions that loop on the IFN-gamma gene when differentiated into Th1 cell type). (Source: Nguyen et al., 2015)*

2.5. Role of PU.1 and CEBP transcription factors in epigenetics and immunity

One important LDTF for determining myeloid and lymphoid immune cell lineages is PU.1 of the ETS (E- twenty six) family that is encoded by Spi1 gene which binds to purine rich signature sequences on the genome (typically AGGAAGTG motifs) (Pang et al., 2018; Rothenberg et al., 2019). PU.1 is also critical for regulation of immunity related genes (Ghisletti et al., 2010; Jin et al., 2011). Various reports suggest the active role played by PU.1 in expression of PRRs such as Dectin-1, TLR2 and TLR4. In THP-1 cells treated with *Aspergillus fumigatus* conidia, Dectin-1, TLR2 and TLR4 were significantly up-regulated when PU.1 was overexpressed while they were down-regulated when PU.1 was silenced (C. Liu et al., 2017). PU.1 overexpression also led to

increase in release of pro-inflammatory cytokines and phagocytosis. In another report, Dectin-1 was found to be up-regulated in HEK293T cells in presence of PU.1 as it was found to bind the promoter of Dectin-1 (Wang et al., 2016). Opportunistic pulmonary pathogenic fungus *Pneumocystis* has been shown to down-regulate PU.1 in alveolar macrophages which leads to Dectin-1 down-regulation and knocking down of PU.1 also reduced Dectin-1 expression (Zhang et al., 2010). TLR4 is involved in the detection of gram negative bacteria and PU.1 expression has been shown to increase TLR4 activation by binding to its promoter (Roger et al., 2005; Tsatsanis et al., 2006). In synovial macrophages of rheumatoid arthritis patients, down-regulation of LPS stimulated PU.1-TLR4 expression through drugs inhibited proinflammatory cytokine release which reduced disease severity (Park et al., 2013). PU.1 is also involved in the regulation of TLR2 (PRR involved in detection of cell wall components of bacteria, fungi and parasites) expression by binding to its promoter with the help of additional transcription factors like Sp3 (Haehnel et al., 2002). C/EBP alpha and beta are CCAAT binding transcription factors that cooperate with PU.1 in selection of macrophage specific enhancers (including *de novo* enhancers) and subsequently, in their differentiation (Heinz et al., 2010; Kaikkonen et al., 2013). These are also involved in processes like immune response and adipogenesis (C. Li et al., 2018). C/EBP alpha has also been shown to promote PU.1 expression by binding to its promoter (Cai et al., 2008). CEBPA is known to regulate the expression of anti-microbial peptide cathlecidin in cooperation with vitamin D and chromatin remodeler Brahma protein (Dhawan et al., 2015). Although, various reports have suggested the other way round i.e., the innate immune signaling causes the up-regulation of CEBP transcription factors to cause the transcription of downstream genes. Roles of CEBPs (including CEBPA and CEBPB) have been suggested in LPS induced inflammation (Hu et al., 1998) and CEBPA is known to get activated in the TLR3 mediated pathway in HIV infection (Bhargavan et al., 2016).Therefore, the complex interactions between the DNA, histones and transcription factors decide the fate of a cell and gives it special abilities to tackle a situation in a manner which is superior to other cells.

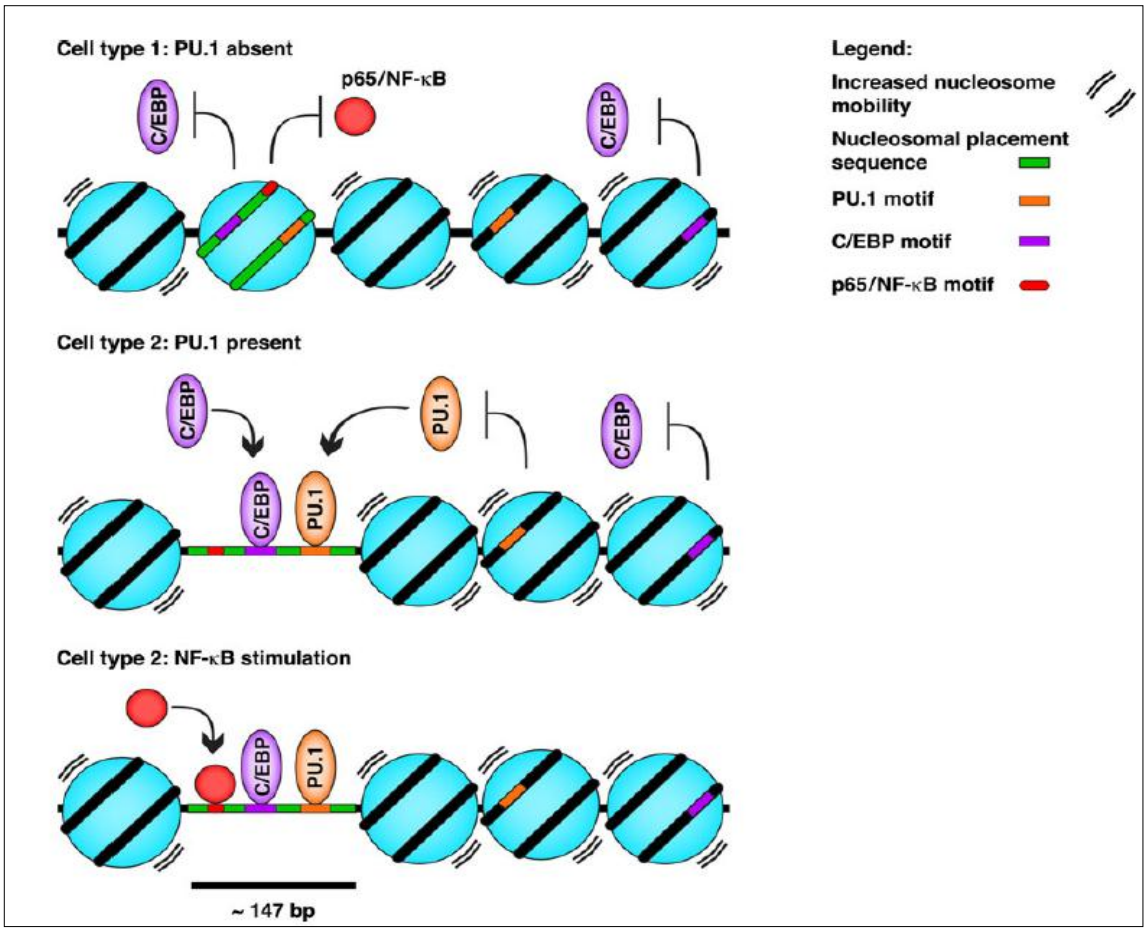


Figure 2.6: Schematic representation of LDTF mediated cell-specific gene expression. In cell type 1 where PU.1 is absent, the nucleosome containing the PU.1 and C/EBP motifs remains intact and thus, the gene regulatory regions harbored by it are silenced. In cell type 2 where PU.1 is present (such as myeloid cells), PU.1 and C/EBP bind to their motifs causing eviction of the nucleosome and thus, exposing the DNA region. Some lineage specific genes are transcribed in this manner while some may require additional signaling TFs like NF-κB to complete the protein interaction scaffold and cause the recruitment of RNA polymerase for transcription of the gene (as in the case of stimulation of PRRs with PAMPs). (*Source: Gosselin and Glass, 2014)*

CHAPTER –3

Materials & Methods

MATERIALS AND METHODS

3.1. Ethics statement

The study was approved by the Institutional Animal Ethics Committee (IAEC) of National Dairy Research Institute, Karnal, Haryana, India. No animal was specifically slaughtered for this study. The fetus and the uterus for fibroblast and endometrial epithelial cell culture respectively were obtained from a Municipal slaughterhouse (Ghazipur, New Delhi).

3.2. Primer designing for full length LDTF genes

Full length mRNA sequences of PU.1, CEBPA and CEBPB genes from bovine were retrieved from NCBI. The ORFs for the genes were determined using Expasy Translate tool (https://web.expasy.org/translate/) and the melting temperature (Tm) of each primer was kept ~60^{o}C using MBCF Oligo Calculator (http://mbcf149.dfci.harvard.edu/docs/oligocalc.html). Both forward and reverse primers for each gene included restriction enzyme sites of NheI site at 5' end of forward primer and XhoI site at 3' end of reverse primer according to MCS of pIRES-EGFP-Puro (a gift from Michael McVoy- Addgene plasmid #45567). Initially, PU.1 was not amplified with the given primer pair, so another reverse primer from 3' UTR was designed and used for PU.1 amplification. For joining of PU.1 with CEBPA or CEBPB, primers for overlap extension PCR were also designed in a way that the reverse primer of PU.1 gene was incorporated with the upstream sequence of IRES element at its 3' end while the end of the IRES element was incorporated at the 5' end of the forward primer of CEBPA and CEBPB (Figure 3.1). Individual sequence for each primer is given in the Table 1 and Table 2 of Annexure I.

Figure 3.1: Representation of the primer regions used for overlap extension PCR of PU.1 and CEBP genes with inclusion of IRES element. *The upstream gene (PU.1) has its reverse primer joined with the upstream sequence of IRES element (shown in red). The upstream region was not exactly taken from IRES start site due to very high GC content (shown in dark blue). The 5' end of the forward primer of downstream gene (CEBPA or CEBPB) was joined with the downstream end of IRES sequence (shown in light blue).*

3.3. PCR amplification of the LDTF genes, their cloning and sequencing

Buffy coat from the whole bovine blood was isolated by carrying out repeated RBC lysis till a white coat remained as the pellet. The buffy coat was directly resuspended in TRI reagent (Sigma) and RNA isolation was carried out (discussed later in section 3.6). PU.1

gene was initially amplified using forward and reverse primer but however, the product generated was not PU.1 and therefore, the gene was amplified again with forward and 3' UTR reverse primer. Thus, the product obtained was used as template for 2nd reaction using primers with restriction sites. CEBPA and CEBPB genes being intron-less, were amplified directly from bovine genomic DNA. PCRs for amplification of the genes were performed using Q5 polymerase (NEB). The reaction components for each PCR and Thermal Cycler conditions are given in the Table 4 and Table 5 respectively of Annexure II. For overlap extension PCR, the initial products were amplified in the same manner as discussed above and the products obtained were gel purified. The purified products were used as primers along with forward primer of PU.1 with NheI site and reverse primer of CEBPA or CEBPB with XhoI site in a single reaction to obtain the product PU.1-IRES-CEBPA and PU.1-IRES-CEBPB. The protocol for the reaction was taken u pfrom a previous report (Horton et al., 2013) with modifications which cannot be currently disclosed here as it is still under refinement. The PCR products were gel purified and restriction digested along with the vector pIRES-EGFP-Puro. These were double digested with NheI (NEB) and XhoI (NEB) using CutSmart buffer. The cut vector was dephosphorylated using FastAP (Thermo Scientific) and ligated with individual PCR products using T4 DNA ligase (ThermoFisher Scientific) according to manufacturer's instructions. The ligated plasmids and inserts were transformed into XL-1 Blue strain of *E.coli* (made competent using Hanahan method) and spread on LB agar plates supplemented with 50 µg/ml ampicillin. The plates were incubated at 37^{o}C overnight and the obtained colonies were inoculated in 5 ml LB broth containing 50 µg/ml of ampicillin for overnight at 37^{o}C in shaker incubator. Plasmids were isolated using alkaline hydrolysis method which were then screened for the positives and then sequenced. The sequences of LDTF genes are given in the Table 6 of Annexure III. The positive plasmids containing bacteria were preserved as glycerol stocks in 50% glycerol (in autoclaved dH_2O) and frozen at -80^{o}C. A little amount of glycerol stock was scraped and inoculated in 20 ml of LB broth containing 50 µg/ml of ampicillin for overnight at 37^{o}C in shaker incubator. The bacterial suspensions were then subjected to endotoxin-free plasmid isolation using PureLink HiPure Plasmid DNA Purification Kit (Invitrogen) and all the steps were performed according to the manufacturer's instructions. The extracted plasmids were resuspended in autoclaved 1X TE buffer (pH-8.0) and stored at -20^{o}C till further use.

3.4. Cell culture

Primary buffalo fetal fibroblasts were obtained from the ear pinna of buffalo fetus. Small pieces of ear pinna were cut and placed in a T-25 flask (Nunc) with complete Dulbecco's Modified Eagle's medium (DMEM) (D2906, Sigma Aldrich) supplemented with 10 % Fetal Bovine Serum (FBS) (16000044, Invitrogen), 25mM HEPES, L-Glutamine (2 mM), 1X antibiotic-antimycotic solution (Gibco, Thermo Scientific) in a humidified incubator at 37°C with 5% CO_2. The fibroblast cells migrated from the pieces and adhered to the flasks. The pieces were removed from the flask after which the cells were allowed to grow and passaged for further experiments. At 80% confluency, the cells were trypsinized using 0.25% Trypsin.EDTA (Sigma) prepared in 1X DPBS (Dulbecco's Phosphate Buffered Saline) at 37°C followed by inactivation of trypsin using complete DMEM and then pelleting the cells at 1500 RPM for 5 minutes in pyrogen and nuclease-free 15 ml conical tubes (Invitrogen). The pellet was resuspended in complete DMEM and then cultured in the flasks as mentioned before. Buffalo endometrial epithelial cells were obtained from the uterus and was provided as a kind gift by Ankit Pal (Ph.D. student, ABTC). The lumen of uterus was washed 3 times using 1X DPBS without calcium and magnesium supplemented with 1X antibiotic-antimycotic solution and 0.1% BSA. The ends of the uterine horn were tied so that trypsin to be added later was retained within it. 15-20 ml of sterile 1X DPBS containing 0.25% trypsin was added to the tied lumen. Epithelial cells were allowed to get trypsinized from the lumen and suspend in the solution at 37^{o}C for 1 hour. The cell suspension was filtered through a 70 μm plastic strainer (Corning) to remove the debris. The filtrate was washed 3 times with 1X DPBS + 1X antibiotic-antimycotic + 0.1 % BSA by centrifugation at 600g for 10 minutes. The pellet was resuspended in complete DMEM and allowed to grow in T-25 flask. The further processing of the cells was exactly the same as mentioned for fetal fibroblasts. Bovine Macrophage Cell Line (BoMAC) was provided as a kind gift by Dr. Judith Stabel (Research Microbiologist, Infectious Bacterial Diseases Research, USDA). BoMAC were also cultured in complete DMEM medium (with supplements as described for fetal fibroblasts) at 37^{o}C with 5% CO_2. BoMAC was also passaged just like fibroblasts and epithelial cells for further experiments. MDBK (Madin Darby Bovine Kidney) cell line was provided as a kind gift by Dr. R.S Kataria (Principal Scientist, NBAGR) and the culture of these cells were performed in the same manner as described above for other cell types. All the cell types were also cryopreserved using 10% DMSO (Sigma) in

culture medium containing 20% FBS. Initially, the cell aliquots were kept overnight at -80^{o}C in Mr. Frosty freezing container (Thermo Scientific) containing isopropanol followed by transfer to liquid nitrogen canister. Revival of the cells were performed by thawing the cells at room temperature, addition of 10X fresh complete DMEM with 10% FBS (9 ml media in 1 ml cryopreserved cells) followed by centrifugation at 1500 RPM for 5 minutes and subsequent culture in T-25 flasks.

3.5. Transfection protocols

3.5.1 Transfection of vector pIRES-EGFP-Puro with inserted LDTF genes

The transfections for MDBK cell line, fetal fibroblasts and endometrial epithelial cells were performed in 24-well plates by seeding 2.5x10^{5} cells in 500µl DMEM + 10% FBS + 1X antibiotic-antimycotic solution per well. At 80% confluency, media was replaced with OptiMEM reduced serum media (Gibco, Thermo Scientific) and transfection was performed using Lipofectamine 3000 (Invitrogen). 0.75µl of lipofectamine was complexed with 500 ng of empty vector and vector + inserts along with P3000 (volume in µl equal to 2X the µg of DNA) according to the manufacturer's instructions. MDBK cell line was transfected for 16 hours while fetal fibroblasts and endometrial epithelial cells were transfected for 36 hours (as these time points exhibited highest expression of the LDTFs).

3.5.2 Transfection of esiRNAs against LDTFs

The esiRNAs (endoribonuclease prepared siRNAs) for bovine PU.1, CEBPA and CEBPB genes along with GFP esiRNA (used as control) were obtained (Sigma). A stretch of sequence from the coding region of the gene (Table 7 of Annexure III) was selected and dsRNA was transcribed from it. The dsRNA was then cleaved using endoribonuclease which resulted in the generation of heterogenous overlapping siRNAs that targeted the whole stretch of sequence. From the stock, 50 nM and 100 nM concentrations of siRNAs were complexed with Lipofectamine 3000 in the same manner as described in previous section with only exception that P3000 was not added to the RNA. The transfection was carried out for 48h and 72h time periods in the BoMAC cell line.

3.5.3 Transfection of poly(I:C)

For LMW and HMW poly I:C (Invivogen) transfection, complex of Lipofectamine 3000 and poly(I:C) was performed in exactly the same manner as discussed in 3.5.1 section.

For MDBK cell line without LDTFs expression, concentration ranges of 1, 2, 2.5 and 5 µg/ml of poly(I:C) were used to transfect the cells for 6 hours to determine the optimum concentration. For MDBK expressing the LDTFs, poly(I:C) at final concentration of 1 µg/ml was used to stimulate the RLR pathways. In case of fetal fibroblasts and endometrial epithelial cells expressing the LDTFs, a final concentration of 25 ng/ml poly(I:C) was transfected for 6 hours as higher concentrations exhibited toxicity against the cells. For esiRNA transfected cells, 1 µg/ml poly(I:C) was transfected for 6 hours.

Note: After the transfection protocols, all the cell types were harvested in 400 µl TRI Reagent (Sigma) and stored at -80^{o}C until RNA extraction was performed.

3.6. RNA extraction and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The cells harvested in TRI reagent were thawed at room temperature and 100 µl chloroform was added followed by vigorous vortexing and incubation at room temperature for 5 minutes. The mixture was then centrifuged at 12,000 RPM for 10 minutes at 4^{o}C. The upper aqueous phase was taken in a separate tube and equal volume of isopropanol was added, mixed and incubated in ice for 10 minutes. The solution was centrifuged at 12,500 RPM for 15 minutes at 4^{o}C to obtain a pellet. The pellet was then washed 2 times with 80% chilled ethanol and finally dissolved in 15 µl nuclease free water (Thermo Scientific). The quality and quantity of RNA were checked by agarose gel electrophoresis and NanoDrop 2000 (Thermo Scientific) respectively. A total of 500 ng of RNA was used for cDNA preparation for each sample. For RT-PCR, 10 µl reactions were prepared using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific) according to the manufacturer's instructions. The prepared cDNAs were used immediately for further experiments.

3.7. Semi-quantitative PCR experiments

The cDNA prepared was diluted 3 times i.e., upto 30 µl with nuclease free water to get an effective concentration of 16.67 ng/µl. 10 µl reactions were prepared in 96 well plates (Brand) with each well containing 5 µl KAPA SYBR® FAST qPCR Master Mix (2X) (Kapa Biosystems), 0.2 µl of each forward and reverse primers (10 µM stock), 2 µl diluted cDNA and 2.6 µl nuclease-free water. The PCR was performed in Light Cycler 480 II (Roche) according to the kit protocol with annealing temperature set at 61^{o}C. Melting curves for all the genes were also prepared to check the PCR accuracy. The

obtained datasets were analyzed using delta-delta-Ct (ΔΔCt) method (Livak and Schmittgen, 2001) using RPS18 as housekeeping gene (Axtner and Sommer, 2009). The primer sequences for the genes analyzed are given in the Table 3 of Annexure I.

3.8. Statistical analyses

The datasets have been presented as the mean values ± standard deviation (SD) for 3 biological replicates (n=3). One-Way ANOVA (Analysis of Variance) followed by Dunnett's post hoc multiple comparison test for finding significant differences between the control and the transfected groups for each gene were performed for the qPCR experiments.

CHAPTER –4

Results and Discussion

RESULTS AND DISCUSSION

The LDTFs (Lineage Determining Transcription Factors) play a very important role in cellular differentiation and cell-specific gene regulation. PU.1 is a myeloid specific LDTF which is encoded by the *Spi1* gene that binds to purine rich GAGGAA sequences (predominantly present in enhancer and promoter regions). PU.1 is involved in the regulation of several differentiation and immunity related genes. CEBPA and CEBPB are CCAAT enhancer binding proteins that cooperate with PU.1 to further enhance its gene regulatory activity. In previous studies, PU.1 and CEBP LDTFs have been over-expressed to change the lineages of terminally differentiated cells such as epithelial cells and fibroblasts to macrophage-like cells. PU.1 also regulates the expression of pattern recognition receptors like Dectin-1, TLR2 and TLR4. So, it was expected that PU.1 might be a key transcription factor in the regulation of other innate immune pathways as well. Therefore, the ability of PU.1 along with CEBPA and CEBPB to regulate the genes of innate RLR (RIG-I like receptor) pathways were tested on bovine non-immune and immune cells. The RLR pathways play a very important role in defense against highly pathogenic RNA viruses such as FMDV, PPRV, Bluetongue virus, Influenza and bacteria like *Mycobacterium bovis*. The viruses have developed mechanisms to hijack the immune pathways by destroying or inactivating the PRRs or the associated downstream molecules. Therefore, enhancing the expression of the immune receptors and downstream molecules at early stages or prior to viral infection may help in improving the health status of animals prone to such viruses. The knock-down of these LDTFs PU.1, CEBPA and CEBPB in immune cells (macrophage cell line BoMAC) along with the ectopic expression of these LDTFs (cloned in mammalian expression vector pIRES-EGFP-Puro) in non-immune cells (fibroblasts and epithelial cells) were performed and their effects on the RLR pathways were assessed.

Objective 1:

To analyze the RLR pathway and effector genes in bovine macrophage cell line (BoMAC) subjected to knock-down of PU.1 and C/EBP proteins

4.1. The esiRNA mediated knock-down of PU.1 resulted in its over-expression rather than down-regulation

The control GFP esiRNA along with esiRNAs for PU.1, CEBPA, CEBPB were transfected in BoMAC. PU.1 is involved in the regulation of many immunity related genes while CEBPA and CEBPB establish cooperative interactions with PU.1 in order to enhance the expression of several PU.1-regulated genes. It was expected that predominantly, PU.1 suppression would lead to the down-regulation of the RLR pathways. The siRNAs for all the genes matched 100% with the cow gene sequences (Table 7 of Annexure III). For PU.1, the siRNA spanned the exons 1-3 at the position 1-253 starting from the transcription start site (TSS). CEBPA and CEBPB are intron-less genes and the siRNA span positions from the TSS were 126-442 and 53-369 respectively. PU.1 esiRNA transfected at 50 nM and 100 nM concentrations caused tremendous up-regulation by ~1492 fold rather than down-regulation of its gene at both 48h and 72h time points which was totally in contrast to the function of the siRNAs (Figure 4.1A and C). The knock-down of CEBPA was successful at an esiRNA concentration of atleast 100 nM for 48 hours (Figure 4.1B) which reduced the gene expression level by 55% (Figure 4.1C) while no silencing was observed for CEBPB gene at any esiRNA concentration (Figure 4.1B and C). The reason behind the siRNA-mediated up-regulation of PU.1 maybe because PU.1 is an auto-regulatory transcription factor (Chen et al., 1995; Okuno et al., 2005) and additionally, there are other transcription factors that regulate PU.1 expression which may engage in some positive feedback mechanism. Although, earlier reports have suggested successful silencing of PU.1 in other mammalian models (Atar and Levi, 2005; Pimenova et al., 2021; Xu et al., 2013) but till date, no up-regulation of PU.1 under the influence of siRNA has been reported. The mechanism behind this up-regulation is not clear and further experiments like transcription factor binding analysis through ChIP-Seq along with monitoring of PU.1 promoter and enhancer activity will shed light on the underlying mechanism.

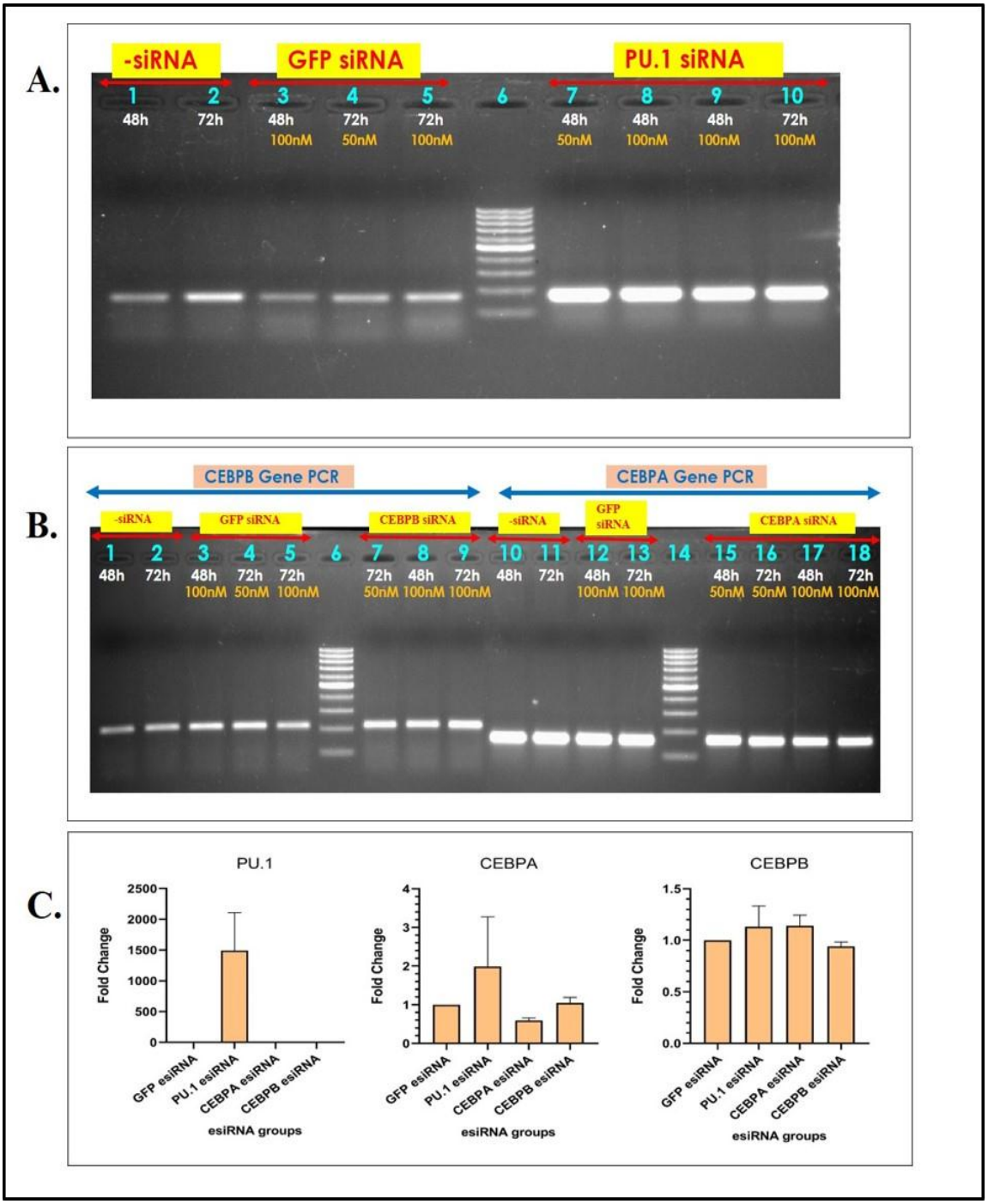


Figure 4.1: Assessment of knock-down of LDTF genes in BoMAC mediated by esiRNAs at varying concentrations and time periods. A. *PCR for PU.1 gene in esiRNA transfected BoMAC cells using qPCR primers and cDNA as the template. The text in yellow boxes above the lanes indicate the type of esiRNA transfected and below the lane numbers, transfection time and concentrations of esiRNAs have been mentioned. Lane 6 contained GeneRuler 100bp ladder.* **B.** *PCR for CEBPB and CEBPA genes using qPCR primers and cDNA prepared from esiRNA transfected BoMAC cells. The text in yellow boxes above the lanes indicate the type of esiRNA transfected while the text boxes above the yellow boxes mention the gene for which the PCR was carried out. Below the lane numbers, transfection time and concentrations of esiRNAs have been mentioned.* **C.** *Semi-quantitative real time PCR for the LDTF genes PU.1, CEBPA and CEBPB in BoMAC cells transfected with each type of esiRNA at 100 nM for 48 h.*

4.2. Knock-down of CEBPA caused the down-regulation of RLR pathway genes and also suppressed the immune response against poly(I:C)

As the knock-down of only CEBPA was successful, the effect of its silencing on the RLR pathway genes and nucleic acid sensors was monitored. It was observed that the expression of most of the RLR pathway genes were suppressed when CEBPA was knocked down (Figure 4.2A). Down-regulation was observed for RIG-I (~37%), MDA5 (~28%), cGAS (~41%), IFI16-l (~36%), DAI (~41%), IRF7 (~34%), ISG56 (~45%) and ISG54 (~40%) while no down-regulation was observed for IFN-β. Although, the knock-down in case of PU.1 and CEBPB was not successful, their data have also been incorporated for reference (Figure 4.2A). When the CEBPA knocked-down cells were stimulated with poly(I:C) for 6 hours, there was suppression of immune response as discussed hereafter in which the nucleic acid sensors RIG-I, MDA5, LGP2, DAI, cGAS, and IFI16-l were down-regulated by 58%, 64%, 63%, 61% and 40% respectively while the transcription factors IRF3 and IRF7 were down-regulated by 33% and 49%. IFN-β expression was also suppressed by 54% which also resulted in the suppression of ISG54, ISG56 and PKR by 54%, 51% and 54% respectively. The data of PU.1 and CEBPB knock-down have also been shown but their results cannot be interpreted with any confidence. The results obtained in these experiments indicate that CEBPA can be targeted for suppression of immune responses against poly(I:C) or dsRNA viruses.

Till date there has been no drug or chemical agent that affects the expression of CEBPA gene. However, various natural plant extracts that have been reported to suppress CEBPA expression also exhibit anti-inflammatory properties. The methanolic extract from strawberries has been shown to suppress LPS-induced inflammation (Gasparrini et al., 2017) and in a recent report, it has been shown to down-regulate CEBPA expression (Forbes-Hernández et al., 2020). Extracts from mulberry fruits have also been reported to reduce inflammatory response and down-regulate CEBPA mRNA (Jung et al., 2019; Lee and Kim, 2020). *Allium hookeri* root extract has also been linked to CEBPA down-regulation as well as inflammation suppression (Kim et al., 2019). Further studies are still required to establish direct correlation between inflammation suppression and reduction of CEBPA expression. Therefore, CEBPA might serve as a target for inflammatory pathways (including RLR pathways). PU.1 down-regulation was expected to cause the major suppression of these pathways, but due to failure of its knock-down, the associated observations could not be drawn and further studies would be required to achieve more clarity behind the siRNA mediated up-regulation of PU.1 expression.

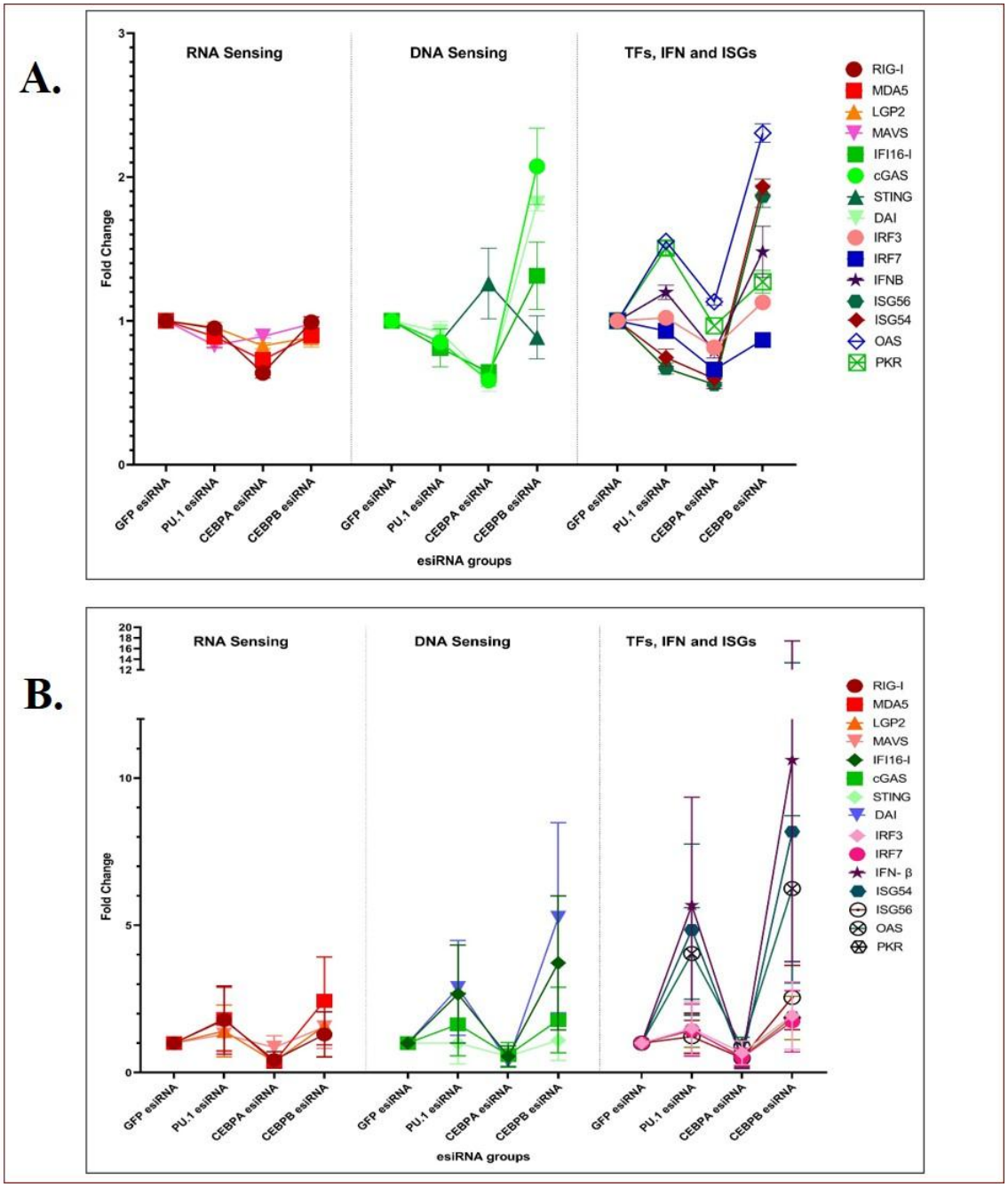


Figure 4.2: Effect of CEBPA knock-down on RLR pathway genes in BoMAC at resting state and when stimulated with poly(I:C) for 6 hours. A. *Semi-quantitative real time PCR data for RLR pathway and DNA sensor genes in the LDTFs knocked-down BoMAC cells (only CEBPA knock-down was successful while PU.1 and CEBPB siRNA data have been added just for reference purpose) where GFP esiRNA transfected cells were taken as the control group. The p-values obtained by One-way ANOVA when GFP esiRNA group was compared with CEBPA esiRNA group have been mentioned in the Table 8 of Annexure-IV.* **B.** *Semi-quantitative real time PCR data for RLR pathway and DNA sensor genes in the LDTFs knocked down BoMAC cells (only CEBPA knock-down was successful while PU.1 and CEBPB siRNA data have been added just for reference purpose) subjected to poly(I:C) stimulation for 6 hours (GFP esiRNA transfected cells were taken as the control group). The p-values obtained by One-way ANOVA when GFP esiRNA group was compared with CEBPA esiRNA group have been mentioned in the Table 9 of Annexure-IV.*

Summary of Objective 1

In this objective, the effects of suppression of the LDTFs PU.1, CEBPA and CEBPB on the regulation of innate antiviral RLR pathways were analyzed and the following observations were deduced:

- In the BoMAC cells, transfection of esiRNA for PU.1 gene caused the up-regulation of PU.1 rather than down-regulation and further studies need to be carried out for better understanding of this phenomenon.
- Knock-down of CEBPA caused the suppression of the RNA and DNA sensing pathway genes expression. CEBPB down-regulation also failed when BoMAC were transfected with CEBPB esiRNA.

CEBPA esiRNA transfection also caused the suppression of the poly(I:C) mediated immune response in BoMAC via the RNA and DNA sensing pathways. CEBPA knock-down caused the suppression of Interferon pathways

Objective 2:

To express PU.1 and C/EBP proteins in non-immune cells to enhance the activity of RLR pathway and effector genes

4.3. Amplification and cloning of the LDTFs PU.1, CEBPA and CEBPB in the pIRES-EGFP-Puro vector

The gene for the LDTF PU.1 was amplified from the bovine blood buffy coat mRNA because PU.1 is abundant in white blood cells. As PU.1 gene was required to be cloned in a mammalian expression vector and transfected into the non-immune cells, primers for full length coding region (from the start codon to stop codon) were used for the PCR amplification. The product size of ~800 bp was generated through nested PCR of PU.1 gene (Figure 4.3).

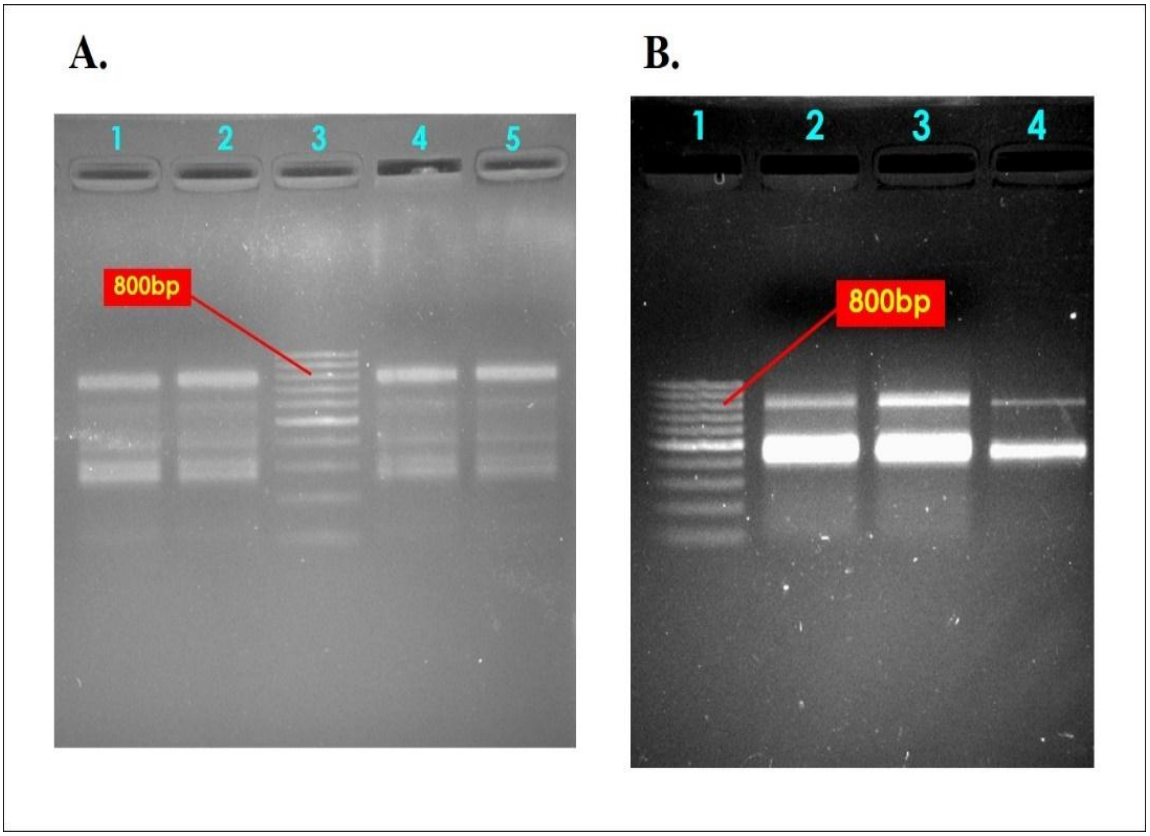


Figure 4.3: Nested PCR for the amplification of PU.1 gene. A. *Lane 1,2,4,5- PU.1 PCR product using forward primer from start codon and reverse primer at 3'UTR, Lane 3- GeneRuler 100bp ladder.* **B.** *Lane 1- GeneRuler 100bp ladder, Lane 2,3,4- PU.1 PCR product using forward primer from start codon and reverse primer till the stop codon. The band near 800 bp represents PU.1 full length CDS (The products in both the figures were electrophoresed in 1.5% agarose gel)*

CEBPA and CEBPB are involved in the cooperative interactions with PU.1 in order to enhance the expression of several PU.1-regulated genes. As both these genes were intron-less, they were amplified directly from the bovine genomic DNA and because of >70% GC content in both the genes, a GC enhancer (included with Q5 polymerase) was added to the PCR for both the genes which led to their amplification (Figure 4.4). The amplification failed when GC enhancer was not added to the reactions. Both, the CEBPA and CEBPB products which were generated from the PCR were found to be ~1000 bp.

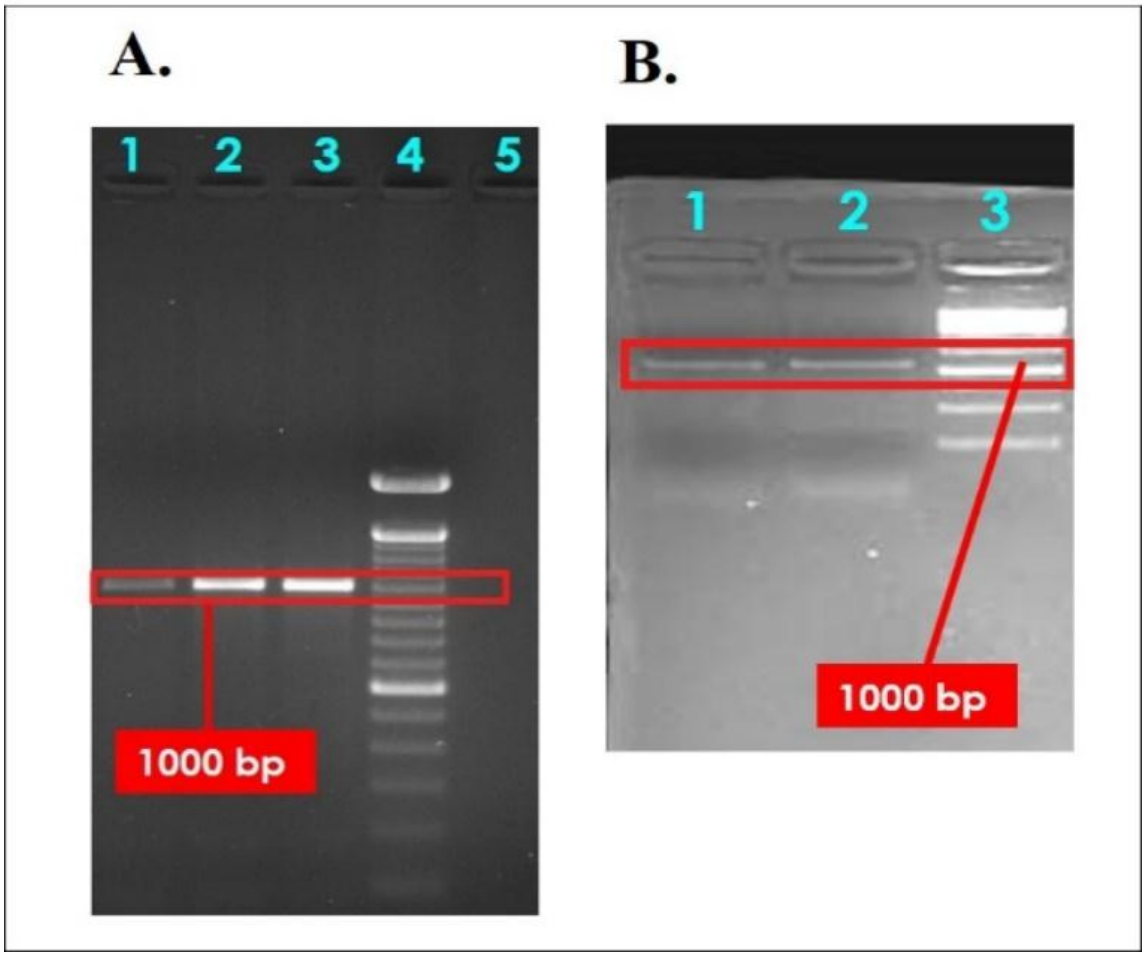


Figure 4.4: PCR amplification of CEBPA and CEBPB genes. A. *Lane 1,2,3- CEBPA PCR products, Lane 4- TrackIt 100bp ladder.* **B.** *Lane 1,2- CEBPB PCR products, Lane 3- GeneRuler 1kb ladder*

PU.1 gene was also joined to the CEBPA and CEBPB genes with an IRES (internal ribosome entry site) element spanning between them through overlap extension PCR (Horton et al., 2013). The IRES leads to the translation of the downstream sequence (CEBPA or CEBPB in this case) as it provides direct entry for ribosome. The co-expression of PU.1 and CEBPA/CEBPB was proposed to check for any potential requirements of both the LDTFs in the regulation of the RLR pathway genes. The initial products obtained in the 1st reaction (Figure 4.5A) were used as primers in the 2nd reaction along with the full length CDS primers from the start to stop codon that led to the generation of the final product containing PU.1, IRES and CEBPA/CEBPB genes corresponding to the size of ~2.5 kb (Figure 4.5B).

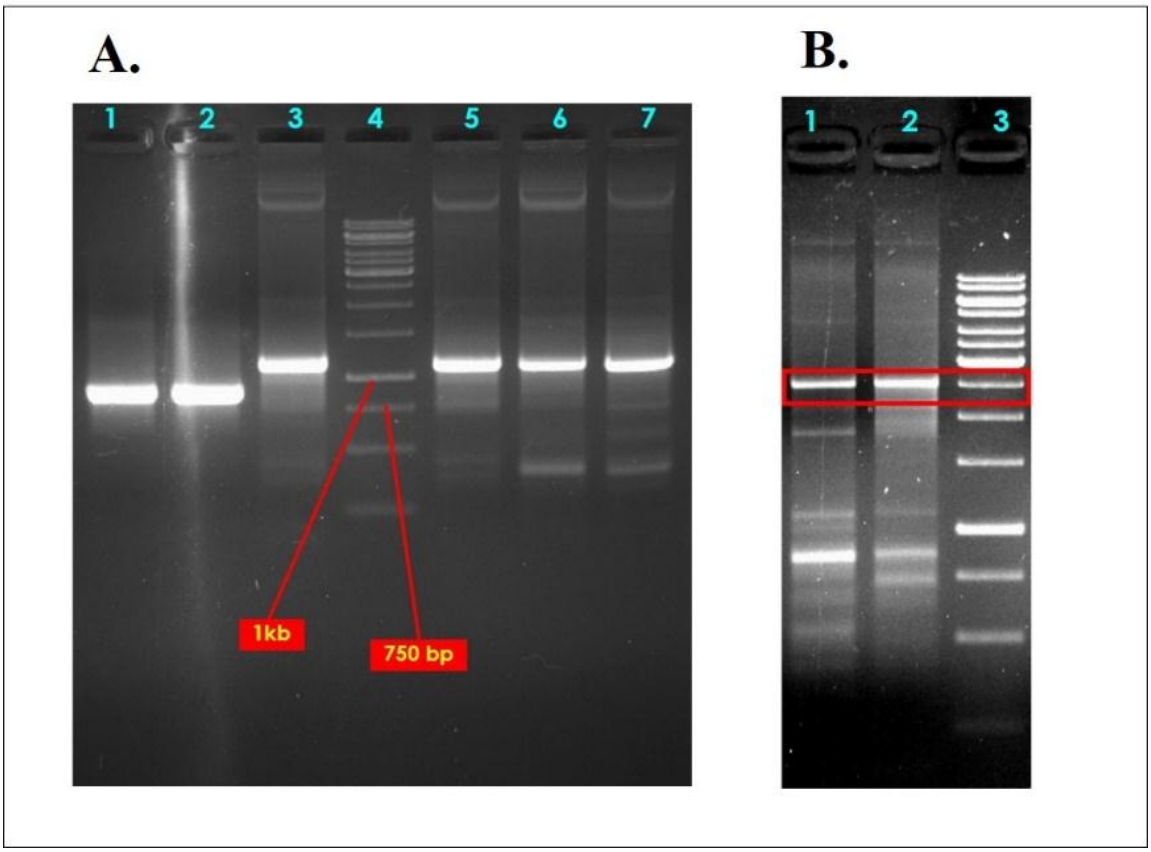


Figure 4.5: Overlap extension PCR reactions for joining of PU.1 with IRES followed by CEBP proteins. A. 1st reaction of the overlap extension PCR to generate initial products where *Lane 1,2- PCR product of PU.1 to be cloned upstream of IRES, Lane 3,5- PCR product of CEBPA to be cloned downstream of IRES, Lane 4- GeneRuler 1kb ladder, Lane 6,7- PCR product of CEBPB to be cloned downstream of IRES.* **B.** 2nd reaction of overlap extension PCR to join the two genes with IRES element where *Lane 1- Joint PU.1-IRES-CEBPA PCR product, Lane 2- Joint PU.1-IRES-CEBPB PCR product, Lane 3- GeneRuler 1kb ladder (the highlighted red region corresponds to the joint product corresponding to the ladder size of 2.5 kb)*

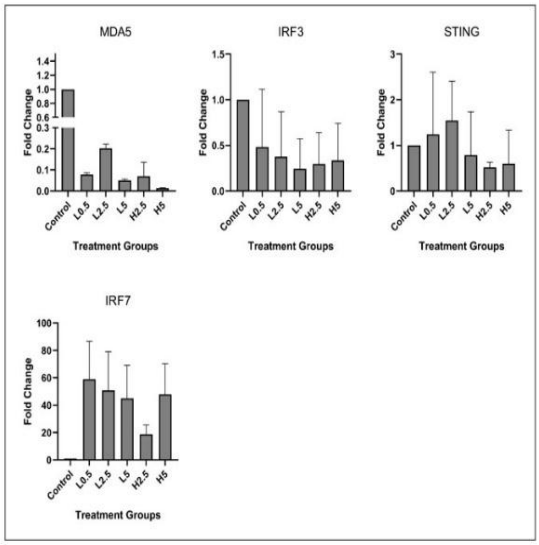
The individual products, the joined products and the pIRES-EGFP-Puro vector backbone were double restriction digested with NheI and XhoI followed by ligation of the products with the vector backbone.

4.4. MDBK cell line is a good model for exogenous gene expression but is not a suitable model for the study of nucleic acid sensing pathways

MDBK is a transformed cell line derived from kidney epithelial cells of cattle. The MDBK cell line when transfected with either LMW or HMW poly(I:C) for 12 hours did not exhibit induction of the RLR pathway genes (Figure 4.6A). The only gene found to be induced was IRF7 transcription factor. MDA5 was found to be down-regulated while other genes were not analyzed due to their very high Ct values which were out of the

range (Figure 4.6B). Although, the MDBK cell line has proved to be a good model for viral infection and propagation (Kopliku et al., 2015; C. Liu et al., 2019; Zhou et al., 2017) but it failed to generate appropriate immune response against the dsRNA analog poly(I:C).

A.



B.

☑	B1	Ctrl IFNB	Unknown	
☑	B2	Ctrl IFNB	Unknown	35.00
☑	B3	LMW 0.5 IFNB	Unknown	
☑	B4	LMW 0.5 IFNB	Unknown	
- Late Cp call (last five cycles) has higher uncertainty, ? - Detector Call uncertain				
☑	B5	LMW 2.5 IFNB	Unknown	35.00
☑	B6	LMW 2.5 IFNB	Unknown	
☑	B7	LMW 5 IFNB	Unknown	
☑	B8	LMW 5 IFNB	Unknown	
☑	B9	HMW IFNB	Unknown	
☑	B10	HMW 2.5 IFNB	Unknown	35.00
☑	B11	HMW 5 IFNB	Unknown	35.00
☑	B12	HMW 5 IFNB	Unknown	
☑	C1	Ctrl RIG1	Unknown	35.00
☑	C2	Ctrl RIG1	Unknown	35.00
☑	C3	LMW 0.5 RIG1	Unknown	35.00
☑	C4	LMW 0.5 RIG1	Unknown	35.00
☑	C5	LMW 2.5 RIG1	Unknown	35.00
☑	C6	LMW 2.5 RIG1	Unknown	35.00
☑	C7	LMW 5 RIG1	Unknown	35.00
☑	C8	LMW 5 RIG1	Unknown	
☑	C9	HMW 2.5 RIG1	Unknown	35.00
☑	C10	HMW 2.5 RIG1	Unknown	35.00
☑	C11	HMW 5 RIG1	Unknown	35.00
☑	C12	HMW 5 RIG1	Unknown	35.00
☑	E1	Ctrl IFI16	Unknown	35.00
☑	E2	Ctrl IFI16	Unknown	
☑	E3	LMW 0.5 IFI16	Unknown	
☑	E4	LMW 0.5 IFI16	Unknown	
☑	E5	LMW 2.5 IFI16	Unknown	
☑	E6	LMW 2.5 IFI16	Unknown	35.00
☑	E7	LMW 5 IFI16	Unknown	35.00
☑	E8	LMW 5 IFI16	Unknown	
☑	E9	HMW 2.5 IFI16	Unknown	
☑	E10	HMW 2.5 IFI16	Unknown	35.00
☑	E11	HMW 5 IFI16	Unknown	35.00
☑	E12	HMW 5 IFI16	Unknown	35.00

Figure 4.6: Semi-quantitative real time PCR of RLR pathway genes in MDBK cell line post poly(I:C) stimulation for 12 hours. A. *MDA5 was found to be down-regulated while IRF7 was drastically up-regulated in MDBK cells treated with either LMW or HMW poly(I:C). In the x-axis labels, 'L' denotes LMW while 'H' denotes HMW poly(I:C) and the numerical value denotes the concentration of poly(I:C) in µg/ml.* **B.** *Screenshot of the raw qPCR file from Roche LightCycler 480 II denoting the out of the range Ct values for various RLR pathway genes. The rows with letter B correspond to IFN-β, letter C- RIG-I and letter E-IFI16.*

Nevertheless, the MDBK cell line was able to exhibit high levels of exogenous gene expression (LDTFs in this case) which suggests that MDBK is a good model for gene expression (Figure 4.7). PU.1 was up-regulated by ~25038 fold in PU.1 expressing cells, ~19641 fold in PU.1-IRES-CEBPA expressing cells and ~13753 fold in PU.1-IRES-CEBPB expressing cells. CEBPA was induced by ~4619 fold in CEBPA expressing cells and ~3512 fold in PU.1-IRES-CEBPA expressing cells. In case of CEBPB, up-regulation

by ~1738 fold in CEBPB expressing cells and ~2244 fold in PU.1-IRES-CEBPB expressing cells were observed. The ability of LDTF expression to boost the immune response against poly(I:C) was assessed in MDBK cells and the results suggested that no such phenomenon takes place as the Ct values of most genes were again out of the range (Figure 4.8). This further emphasizes on the inability of MDBK to stand out as a good model for RLR pathway stimulation in response to poly(I:C). As a result, primary buffalo fibroblasts as well as endometrial epithelial cells were used as the next choice of model for this study.

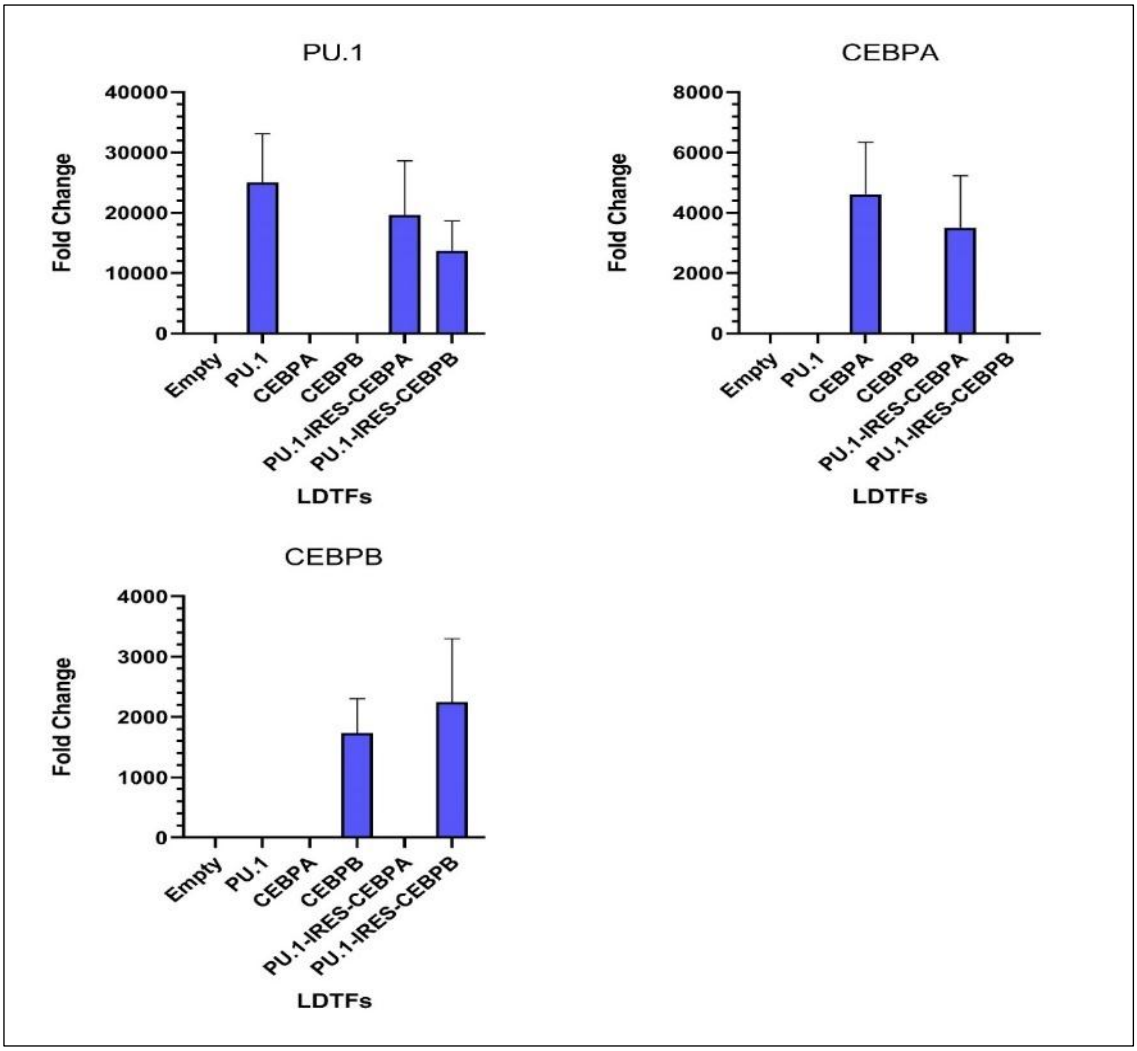
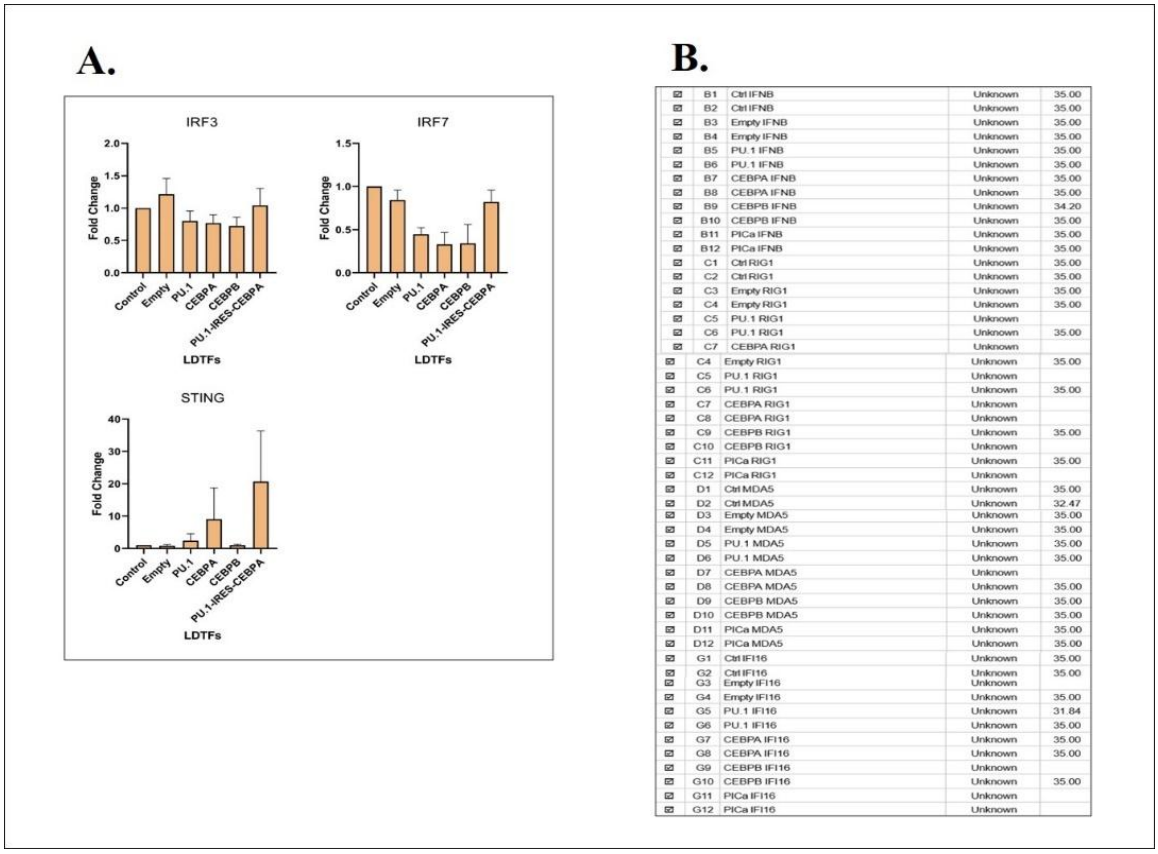


Figure 4.7: LDTFs expression analysis in MDBK cell line. *In the x-axis, Empty denotes the cells transfected with pIRES-EGFP-Puro backbone. MDBK cell line exhibited very high expression levels of PU.1, CEBPA and CEBPB genes at individual level as well as in PU.1-IRES-CEBPA and PU.1-IRES-CEBPB multi-gene expression system. This indicates that MDBK is good model for gene expression. The expression was monitored 16 hours post transfection.*



☑	B1	Ctrl IFNB	Unknown	35.00
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☑	B5	PU.1 IFNB	Unknown	35.00
☑	B6	PU.1 IFNB	Unknown	35.00
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☑	D11	PICa MDA5	Unknown	35.00
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☑	G1	Ctrl IFI16	Unknown	35.00
☑	G2	Ctrl IFI16	Unknown	35.00
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☑	G4	Empty IFI16	Unknown	35.00
☑	G5	PU.1 IFI16	Unknown	31.84
☑	G6	PU.1 IFI16	Unknown	35.00
☑	G7	CEBPA IFI16	Unknown	35.00
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☑	G9	CEBPB IFI16	Unknown	
☑	G10	CEBPB IFI16	Unknown	35.00
☑	G11	PICa IFI16	Unknown	
☑	G12	PICa IFI16	Unknown	

Figure 4.8: Semi-quantitative real time PCR of RLR pathway genes in LDTF transfected MDBK cells that were stimulated with poly(I:C) for 6 hours. A. *In the x-axis, Empty denotes the pIRES-EGFP-Puro backbone transfection without any insert. The only gene found to get induced was STING and that too in CEBPA and PU.1-IRES-CEBPA transfected groups stimulated with 1 μg/ml LMW poly(I:C).* **B.** *The screenshot of raw qPCR file depicting the out of the range Ct values for various RLR pathway genes. The rows with letter B correspond to IFN-β, letter C- RIG-I, letter D-MDA5 and letter G-IFI16.*

4.5. PU.1 is the master regulator of the RLR pathways in buffalo fetal fibroblasts and endometrial epithelial cells

After the failure of MDBK cells to exhibit RLR pathway activation, two primary non-immune cell lineages i.e., mesenchymal fibroblasts and epithelial cells were used to study the effects of LDTFs expression on RLR and DNA sensing pathways. The transfection of LDTFs cloned in pIRES-EGFP-Puro in both types of cells led to high levels of their gene expression (Figure 4.9A and B). PU.1 levels were induced by ~2544 fold in PU.1 expressing fibroblasts, ~2069 fold in PU.1-IRES-CEBPA expressing fibroblasts and ~1855 fold in PU.1-IRES-CEBPB expressing fibroblasts. CEBPA fold change induction by ~2143 and ~2540 were observed for CEBPA and PU.1-IRES-CEBPA expressing fibroblasts respectively. CEBPB induction by ~1830 and ~1537 fold were observed for fibroblasts expressing CEBPB and PU.1-IRES-CEBPB respectively. For PU.1 induction levels in endometrial epithelial cells, ~1693, ~1404 and ~1455 fold

change in induction were observed in PU.1, PU.1-IRES-CEBPA and PU.1-IRES-CEBPB expressing cells respectively. For CEBPA, ~1779 and ~2007 fold induction were observed in CEBPA and PU.1-IRES-CEBPA expressing endometrial epithelial cells respectively. For CEBPB, ~1604 and ~1284 fold induction were observed in CEBPB and PU.1-IRES-CEBPB expressing cells respectively. The levels of expression were also monitored in fibroblasts through immunofluorescence microscopy for GFP expression (Figure 4.9C). In case of fetal fibroblasts, it was observed that PU.1 and its combination with either CEBPA or CEBPB were able to up-regulate the RLR pathway genes to very high extent. CEBPA alone also exhibited similar effects but to a lesser extent while CEBPB was not found to play any major role in the regulation of the pathway genes (Figure 4.10A). The major up-regulation in PU.1 (also when PU.1 is in combination with CEBPA or CEBPB) expressing fibroblasts was observed for RIG-I, MDA5, IRF7 and cGAS while these were less up-regulated in case of CEBPA expressing cells. For PU.1, PU.1-IRES-CEBPA, PU.1-IRES-CEBPB and CEBPA expressing fibroblasts respectively, ~65, ~77, ~72 and ~26 fold induction for RIG-I, ~20, ~55, ~53 and ~11 fold induction for MDA5; ~8, ~11, ~10 and ~3 fold induction for IFI16-l; ~61, ~56, ~53 and ~9 fold induction for cGAS; ~27, ~31, ~29 and ~10 fold induction for IRF7 and ~7, ~12, ~13 and ~3 fold induction for LGP2 were observed. In case of MDA5, the fibroblast cells expressing only PU.1 exhibited lower induction when compared to PU.1 in combination with CEBPA or CEBPB indicating the necessity of cooperative binding of PU.1 and CEBP LDTFs in MDA5 gene transcription. Similar effects were observed in endometrial epithelial cells where PU.1 was found to drastically induce the RLR pathway genes but, in combination with CEBPA or CEBPB, the induction was further elevated compared to PU.1 alone (Figure 4.10B). For PU.1, PU.1-IRES-CEBPA, PU.1-IRES-CEBPB and CEBPA expressing epithelial cells respectively, ~22, ~36, ~32 and ~10 fold induction for RIG-I, ~15, ~31, ~33 and ~6 fold induction for MDA5; ~6, ~9, ~7 and ~2 fold induction for IFI16-l; ~20, ~49, ~48 and ~3 fold induction for cGAS and ~8, ~17, ~14 and ~4 fold induction for IRF7 and ~3, ~7, ~7 and ~2 fold induction for LGP2 were observed. This observation was in line with a previous report in which, both PU.1 and CEBPA play essential roles to establish monocytic enhancer landscape in HeLa (epithelial cancer) cell line (Jin et al., 2011). As was seen in the fibroblasts, CEBPB was not majorly involved in the regulation of expression of the RLR pathway genes in endometrial epithelial cells as well. Some previous reports have also suggested the PU.1 mediated up-regulation of IRF7 (Goto et al., 2017; Ueno et al., 2009) and the ability of PU.1 to strongly activate the promoter of *MB21D1* gene which encodes cGAS

(Rothenberg et al., 2019) and this is in line with our observations. The only genes that remained unaffected were the adaptor protein MAVS, transcription factor IRF3 and Interferon-β. LGP2 and IFI16-l genes were found to be moderately up-regulated in the fibroblast and epithelial cells expressing PU.1 and its combination with CEBPA or CEBPB. The induction levels of IFI16-l in PU.1 expressing endometrial epithelial cells was similar to that of PU.1-IRES-CEBPA/CEBPB expressing endometrial epithelial cells which indicates that CEBP proteins are not playing any cooperative role with PU.1 for regulating IFI16-l gene. The pathway genes up-regulation in case of CEBPA transfected endometrial epithelial cells was quite less as compared to CEBPA transfected fetal fibroblasts. Although, the similar pattern of expression in both cell types indicate that PU.1 and to a lesser extent, CEBPA, are playing a significant role in the up-regulation of the RLR pathway genes.

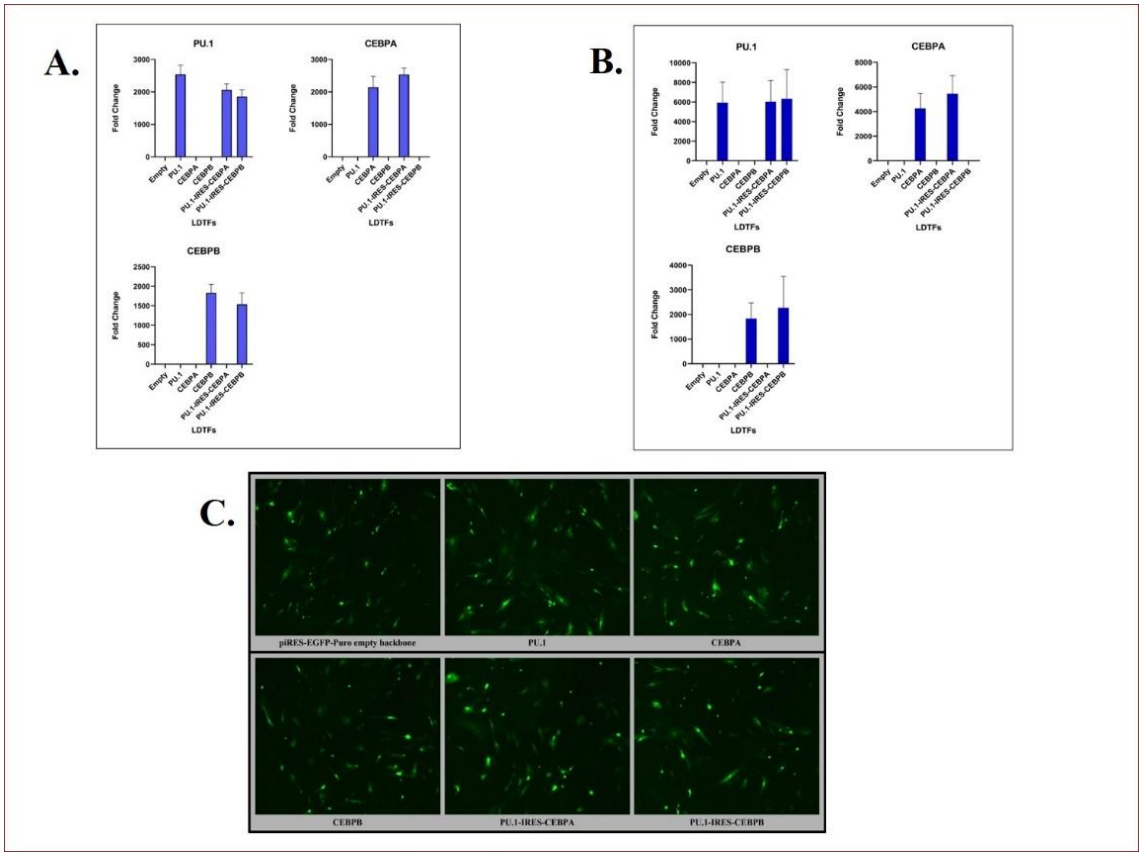

Figure 4.9: Expression analysis of LDTFs in buffalo fetal fibroblasts and endometrial epithelial cells. A. *Semi-quantitative real time PCR for measuring the relative gene expression of LDTFs in buffalo fetal fibroblasts ('Empty' on x-axis denote the cells transfected with empty backbone of pIRES-EGFP-Puro vector).* **B.** *Semi-quantitative real time PCR for measuring the relative gene expression of LDTFs in buffalo endometrial epithelial cells ('Empty' on x-axis denote the cells transfected with empty backbone of pIRES-EGFP-Puro vector).* **B.** *GFP expression analysis under the fluorescence microscopy for fetal fibroblasts transfected with LDTFs.*

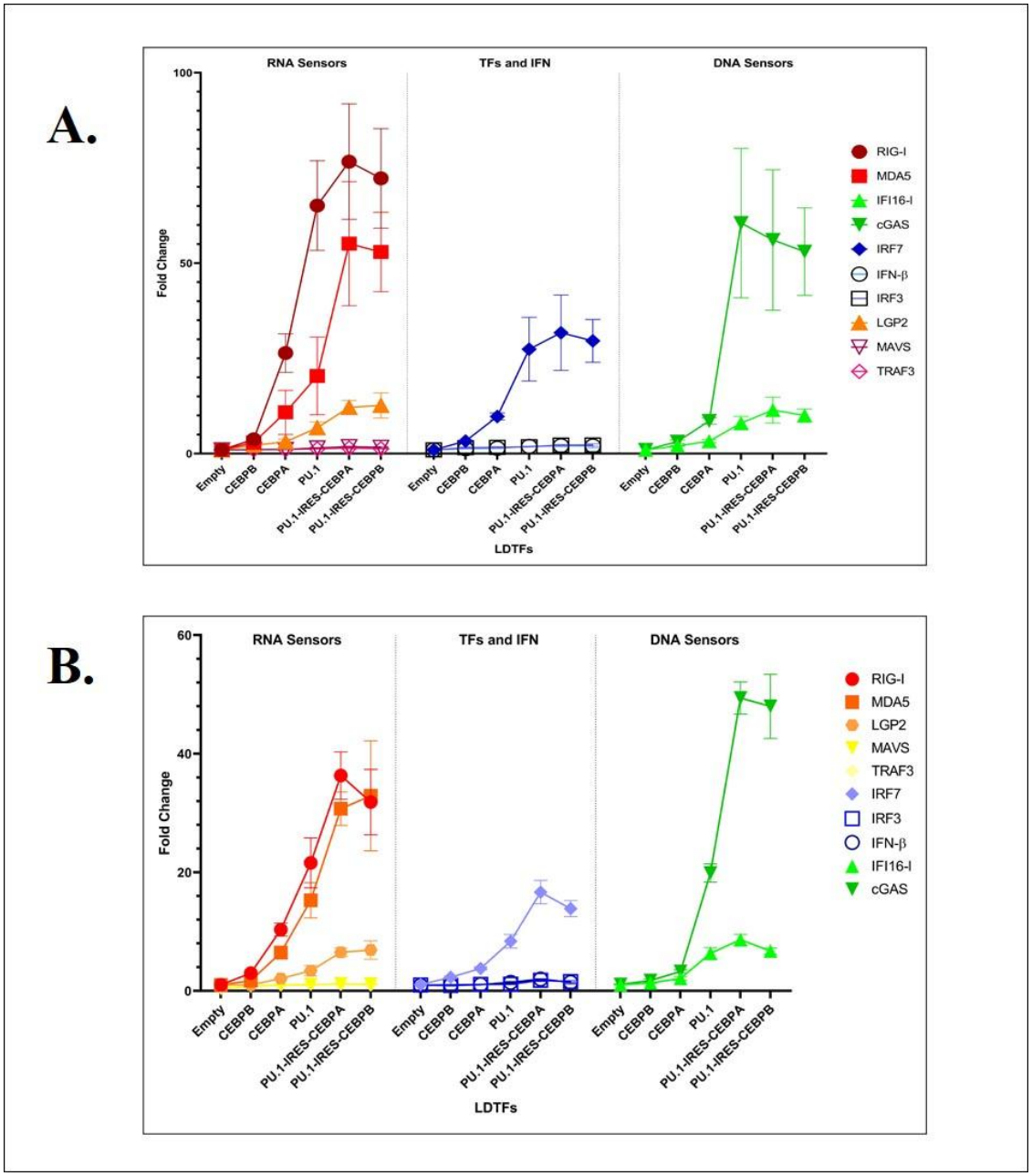

Figure 4.10: Expression analysis of RLR pathway and DNA sensor genes under the influence of LDTFs expression. A. *Relative qPCR of RLR pathway and DNA sensor genes in buffalo fetal fibroblasts subjected to exogenous LDTFs expression. The p-values obtained from One way ANOVA test have been mentioned in the Table 10 of Annexure-IV.* **B.** *Relative qPCR of RLR pathway and DNA sensor genes in buffalo endometrial epithelial cells subjected to exogenous LDTFs expression. The p-values obtained from One way ANOVA test have been mentioned in the Table 11 of Annexure-IV.*

4.6. PU.1 has a significant role in priming of non-immune cells to generate robust immune response against viral dsRNA mimic poly(I:C)

The effect of LDTFs expression on the cellular ability to generate immune response against poly(I:C) was assessed in fetal fibroblasts and endometrial epithelial cells as was done in BoMAC subjected to knock-down of LDTFs. After 6 hours of LMW poly(I:C)

treatment of the fetal fibroblasts expressing the LDTFs, it was observed that PU.1 or combination of PU.1 with CEBPA/CEBPB generated very high immune response via the RLR pathways (Figure 4.11A). The genes RIG-I, MDA5, cGAS, IRF7, IFN-β, ISG54 and ISG56 were induced to very high levels. For PU.1, PU.1-IRES-CEBPA, PU.1-IRES-CEBPB, CEBPA and CEBPB expressing fibroblasts respectively, ~71, ~65, ~61 ~16 and ~2 fold induction for RIG-I; ~31, ~31, ~30, ~17 and ~7 fold induction for MDA5; ~10, ~20, ~27, ~4 and 1 fold induction for IFI16-l; ~121, ~147, ~156, ~20 and ~5 fold induction for cGAS; ~22, ~32, ~35, ~10 and ~2 fold induction for IRF7; ~15, ~23, ~27, ~5 and ~2 fold induction for IFN-β; ~78, ~73, ~75, ~21 and ~8 fold induction for ISG54 and ~81, ~85, ~82, ~29 and ~13 fold induction for ISG56 were observed. In endometrial epithelial cells, PU.1 combined with CEBP proteins exhibited higher immune response than PU.1 alone which was similar to the pattern observed in epithelial cells without poly(I:C) treatment (Figure 4.11B). For PU.1, PU.1-IRES-CEBPA, PU.1-IRES-CEBPB, CEBPA and CEBPB expressing epithelial cells respectively, ~22, ~31, ~31 ~9 and ~3 fold induction for RIG-I, ~19, ~24, ~22, ~7 and ~4 fold induction for MDA5; ~9, ~13, ~13, ~6 and ~3 fold induction for IFI16-l; ~24, ~38, ~37, ~15 and ~6 fold induction for cGAS; ~7, ~13, ~14, ~4 and ~3 fold induction for IRF7 and ~17, ~26, ~27, ~7 and ~6 fold induction for IFN-β were observed. CEBPA expression was also able to prime both the cell types for generating immune response but to a lesser extent as expected from the previous results. CEBPB was the least involved in the up-regulation of the RLR pathway genes in response to poly(I:C). In fetal fibroblasts expressing PU.1 or PU.1-IRES-CEBPA/CEBPB, the highest induction within the category of RNA and DNA sensors was found for RIG-I and cGAS respectively. LGP2 and IFI16-l were found to be less induced. Transcription factor IRF7 was also up-regulated and IFN-β was also induced drastically which is a characteristic of poly(I:C) stimulation. However, PU.1 or PU.1-IRES-CEBPA/CEBPB were not able to up-regulate IFN-β on their own which may indicate that some SDTFs (Signal Dependent Transcription Factors) are playing role in the transcriptional up-regulation of IFN-β that are only induced under the influence of immune pathway stimulation by a signaling molecule as reported previously (Gosselin and Glass, 2014; Heinz et al., 2015; Ostuni et al., 2013). Interferon stimulated genes like ISG54 and ISG56 were also significantly up-regulated in PU.1 and PU.1-IRES-CEBPA/CEBPB expressing fibroblasts while IRF3 and MAVS remained unaffected in all the LDTFs transfected fibroblasts stimulated with poly(I:C). In case of endometrial epithelial cells, up-regulation pattern was similar to what was observed in fibroblasts viz.

the highest up-regulation of pathway genes was observed in cells transfected with PU.1 and PU.1-IRES-CEBPA/CEBPB and then to a lesser extent, with CEBPA. CEBPB exhibited least induction of the RLR pathway genes after poly(I:C) stimulation as was the case in fetal fibroblasts. The RNA sensors RIG-I and MDA5, DNA sensor cGAS were greatly induced while LGP2 and IFI16-l were moderately up-regulated. There was no effect on MAVS gene expression. IRF7 was also up-regulated, IRF3 was unaffected while there was robust induction of IFN-β. Overall, these data indicate that PU.1 is a significant player in the immune response against poly(I:C) which acts through the RLR pathways and some DNA sensors. Activation of PU.1 in non-immune cells may help in boosting the immunity at early stages of infection or prior to infection by up-regulating the expression of nucleic acid sensors which in turn, may aid in the early detection of virus and generation of appropriate immune response. Chemotherapeutic drugs like DNA methyl transferase (DNMT) inhibitor decitabine (5 aza-2'-deoxycytidine) in combination with a histone deacetylase (HDAC) inhibitor chidamide has been shown to up-regulate PU.1 in lymphoma cells (T. Jiang et al., 2017). In another report, decitabine was shown to up-regulate RNA and DNA sensors including RIG-I, MDA5, IFI16 and cGAS in various ovarian cancer cell lines (Chiappinelli et al., 2015). PU.1 may be playing a key role in this DNMT inhibitor mediated up-regulation of RNA and DNA sensing pathways but further studies are required to get more clarity in this regard. Although, prolonged immune response can lead to cytokine storms which is the major cause of mortality in viral infections (Xu et al., 2020). Many chemical agents have been reported to inhibit PU.1 expression and its DNA binding activity which can also be used to suppress inflammation. Calcitriol (active form of vitamin D) is shown to negatively regulate PU.1 expression (Vyas et al., 2020). Vitamin D is also a modulator of immune response which up-regulates anti-inflammatory cytokines as well as reduces tissue injury, fibrosis and expression of pro-inflammatory cytokines that give rise to cytokine storms which is a major cause of multi organ failures followed by death (Kalia et al., 2021; Sari et al., 2019). The molecule DB1976 (a selenophene) has been shown to efficiently inhibit PU.1-DNA binding (Stephens et al., 2016) and it has proved potent in reducing inflammation, steatosis and fibrosis in liver and fibroblasts (Liu et al., 2020; Wohlfahrt et al., 2019). Another class of molecules called hairpin polyamides have also been reported to inhibit PU.1-DNA binding (B. Liu et al., 2019). HDAC inhibitors like TSA (Trichostatin A) and SAHA (suberoylanilide hydroxamic acid) are involved in the down-regulation of PU.1 expression and reduction of cytokine storms (Li et al., 2016, 2008).

SAHA and to a lesser extent, 5 aza-2'-deoxycytidine cause attenuation of poly(I:C) mediated antiviral responses by down-regulating the expression of PRRs and ultimately, their downstream signaling (Hennessy et al., 2019) which is in contrast to other reports discussed previously. Although, PU.1 plays a significant role in the regulation of the RLR pathway genes, still further studies would be required to find the exact roles of various epigenetic modifiers in context to innate immunity and their dependence on PU.1 activity.

It is also worth noting that in the esiRNA-mediated knock-down of LDTFs in BoMAC (Objective 1), CEBPA knock-down was found to suppress the RLR pathway genes expression. Although, PU.1 knock-down failed, however, the observations in the objective 2 indicate that PU.1 is playing more important role than CEBPA in the regulation of RLR pathway genes expression. Successful knock-down of PU.1 would have further confirmed its role in the regulation of the RLR and DNA sensing pathway gene regulation.

Summary of Objective 2

In this objective, the non-immune cells namely, MDBK cell line, primary buffalo fibrobasts and endometrial epithelial cells were subjected to ectopic expression of the LDTFs PU.1, CEBPA and CEBPB. Following observations were deduced from the objective 2:

- MDBK cell line was observed to be a good model for ectopic LDTF expression however, it failed to stand as a good model for the RLR pathway studies due to its unresponsiveness against poly(I:C) stimulation.
- In fetal fibroblasts, PU.1 was found to be the master transcription factor in the regulation of the RLR pathway genes as well as the DNA sensors.
- In fibroblasts, combination of PU.1 with CEBPA or CEBPB also caused up-regulation of the genes to similar extent as PU.1 alone. CEBPA also up-regulated the pathway genes but to a lesser extent while CEBPB did not play any major role in the regulation.
- In epithelial cells, PU.1 in combination with CEBPA or CEBPB was found to up-regulate the RLR and DNA sensing genes to the highest extent followed by PU.1 alone. CEBPA also up-regulated the genes to a lesser extent while CEBPB did not play any role in the regulation.

- PU.1, its combination with CEBPA/CEBPB and to a lesser extent, CEBPA were also able to prime the fibroblasts and the epithelial cells to generate a robust immune response against poly(I:C).

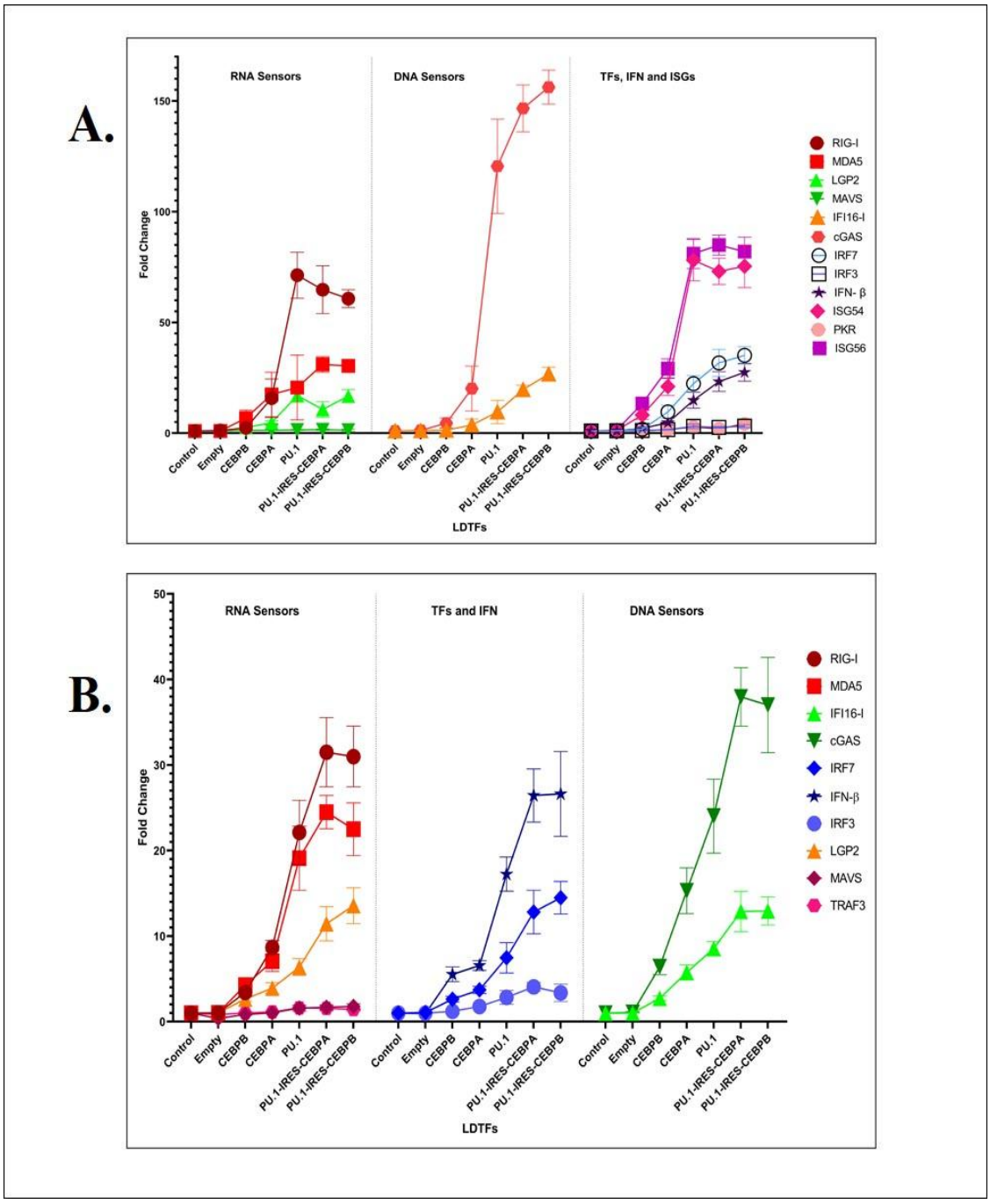

Figure 4.11: Expression analysis of RLR pathway and DNA sensor genes when the cells under the influence of LDTFs expression are stimulated with poly(I:C). A. *Relative qPCR of RLR pathway and DNA sensor genes in buffalo fetal fibroblasts subjected to LDTFs expression followed by poly(I:C) stimulation for 6 hours. The p-values obtained from One way ANOVA test have been mentioned in the Table 12 of Annexure-IV.* **B.** *Relative qPCR of RLR pathway and DNA sensor genes in buffalo endometrial epithelial cells subjected to LDTFs expression followed by poly(I:C) stimulation for 6 hours. The p-values obtained from One way ANOVA test have been mentioned in the Table 13 of Annexure-IV.*

CHAPTER –5

Summary and Conclusions

SUMMARY & CONCLUSIONS

The study was aimed to explore the role of PU.1 and CEBP transcription factors in the regulation of the antiviral RIG-I like receptor pathways in bovine immune and non-immune cells. The knock-down of PU.1, CEBPA, and CEBPB in immune cells BoMAC (macrophage) was achieved by transfecting esiRNAs against the corresponding genes. CEBPA knock-down was effective, however CEBPB knock-down was unsuccessful. However, in the case of PU.1, strong up-regulation rather than down-regulation was seen, and a complete understanding of the underlying process was outside the scope of this research. Most RLR pathway genes were down-regulated when CEBPA was knocked-down (additionally including DNA sensors like IFI16-l, cGAS and DAI). The knock-down significantly reduced the inflammatory response in BoMAC triggered with dsRNA virus mimic poly(I:C), as observed by decreased expression of RLR pathway effector genes such as IFN-β and many ISGs. As a result, CEBPA could be used to modulate immunological responses in immune cells. MDBK was chosen as the cellular model of choice for transfection of LDTF genes (cloned in mammalian expression vector pIRES-EGFP-Puro) in non-immune cells and it demonstrated good exogenous LDTF expression but did not respond to poly(I:C) stimulation. Therefore, primary cells were chosen as the new models for the linked research because LDTF expression, which was predicted to promote up-regulation of the RLR pathways, did not cause any such alterations in the MDBK cell line. PU.1 was discovered to be the master regulator of the RLR pathway receptors, transcription factors, and certain DNA sensors (like IFI16-l and cGAS) in buffalo fetal fibroblasts and endometrial epithelial cells.

In fibroblasts, PU.1 alone or in conjunction with CEBPA/CEBPB were both able to up-regulate the RLR pathway genes to equal levels, while in endometrial epithelial cells, PU.1 in combination with CEBPA/CEBPB was able to up-regulate the genes to higher levels than PU.1 alone. CEBPA was able to up-regulate the pathway genes in both cell types to some extent, but CEBPB was found to play no function in the regulation. In none of the transfected groups, IFN-β was upregulated. When stimulated with poly(I:C), the cells expressing PU.1 or its combination with CEBPA/CEBPB were able to prime the fetal fibroblasts for the generation of a robust immunological response via RLR pathways. When endometrial epithelial cells expressing the LDTFs were stimulated with

poly(I:C), a similar response was seen. IFN-β was up-regulated to very high levels, implying the involvement of some additional signal-dependent transcription factors. CEBPA was able to up-regulate the epithelial cells' RLR pathway genes, albeit to a lesser extent than PU.1 and CEBPB did not play a significant role in the epithelial cell RLR pathway gene regulation. In non-immune cells, PU.1 was a better target for immune modulation than CEBPA, but because its knock-down in macrophage cells failed, it cannot be claimed that it is not a better target in immune cells, and more research is needed to gain insights into this conundrum.

The conclusions drawn from the study are as follows:

- CEBPA knockdown causes RLR pathway sensors and transcription factors to be down-regulated in the myeloid cell line BoMAC. CEBPA-deficient cells can also exhibit diminished inflammatory response to poly(I:C). This suggests that CEBPA could be used to modulate immune responses during cytokine storms.
- The MDBK cell line is a suitable model for protein expression but not for RLR pathway research.
- In non-immune cells, PU.1 is the dominant lineage determining transcription factor that regulates the RLR pathway genes. In fibroblasts, PU.1 alone is adequate for dramatically increasing the RLR pathway genes and DNA sensors, however in epithelial cells, PU.1 cooperates with either CEBPA or CEBPB to further up-regulate the gene expression. CEBPA, like PU.1, can trigger pathway genes in non-immune cells, but to a lesser level, whereas CEBPB has no effect on the regulation.
- PU.1 can stimulate non-immune cells such as fibroblasts and epithelial cells to produce a powerful immune response against dsRNA viral mimic poly(I:C). In both types of cells, CEBPA is a weaker up-regulator of the genes, but CEBPB has no effect. As a result, PU.1 might be targeted before viral infections and assessed for its potential to eliminate viral loads quickly in the event of a disease outbreak. It may also be suppressed later in the infection to prevent excessive cytokine storms, which are the leading cause of viral infection mortality.

Future Directions

- The up-regulation of PU.1 in macrophage cells transfected with esiRNA against it remains a mystery and could be a research topic. Other proteins' effects on PU.1 regulation can be investigated by utilizing transcription factor binding assays such as ChIP-sequencing (chromatin immunoprecipitation with sequencing) and study of PU.1 promoters and enhancers. Mechanistic insights into the esiRNA-mediated up-regulation of the PU.1 gene could also be gained through protein quantification investigations.
- ChIP-Seq and chromosomal conformation capture technologies can be used to investigate the exact molecular basis of the regulatory mechanisms of the RLR pathway genes through PU.1 and CEBP LDTFs.
- Several medicines (as noted in the results and discussion) affect the expression of the LDTFs and, as a result, they may also affect the RLR pathways in animal models.

Bibliography

BIBLIOGRAPHY

Andersson, R., Sandelin, A., 2020. Determinants of enhancer and promoter activities of regulatory elements. Nat. Rev. Genet. https://doi.org/10.1038/s41576-019-0173-8

Atar, O., Levi, B.Z., 2005. PU.1 silencing leads to terminal differentiation of erythroleukemia cells. Biochem. Biophys. Res. Commun. 329, 1288–1292. https://doi.org/10.1016/j.bbrc.2005.02.109

Axtner, J., Sommer, S., 2009. Validation of internal reference genes for quantitative real-time PCR in a non-model organism, the yellow-necked mouse, Apodemus flavicollis. BMC Res. Notes 2. https://doi.org/10.1186/1756-0500-2-264

Banerjee, S., Pal, Aruna, Pal, Abantika, Mandal, S.C., Chatterjee, P.N., Chatterjee, J.K., 2021. RIG-I Has a Role in Immunity Against Haemonchus contortus, a Gastrointestinal Parasite in Ovis aries: A Novel Report. Front. Immunol. 11. https://doi.org/10.3389/fimmu.2020.534705

Bardhan, D., Kumar, S., Anandsekaran, G., Chaudhury, J.K., Meraj, M., Singh, R.K., Verma, M.R., Kumar, D., Kumar, N., Lone, S.A., Mishra, V., Mohanty, B.S., Korade, N., De, U.K., 2017. The economic impact of peste des petits ruminants in India. OIE Rev. Sci. Tech. 36, 245–264. https://doi.org/10.20506/rst.36.1.2626

Bhargavan, B., Woollard, S.M., Kanmogne, G.D., 2016. Toll-like receptor-3 mediates HIV-1 transactivation via NFκB and JNK pathways and histone acetylation, but prolonged activation suppresses Tat and HIV-1 replication. Cell. Signal. 28, 7–22. https://doi.org/10.1016/j.cellsig.2015.11.005

Borkenhagen, L.K., Salman, M.D., Ma, M.J., Gray, G.C., 2019. Animal influenza virus infections in humans: A commentary. Int. J. Infect. Dis. https://doi.org/10.1016/j.ijid.2019.08.002

Briard, B., Place, D.E., Kanneganti, T.D., 2020. DNA sensing in the innate immune response. Physiology. https://doi.org/10.1152/physiol.00022.2019

Brubaker, S.W., Bonham, K.S., Zanoni, I., Kagan, J.C., 2015. Innate immune pattern recognition: A cell biological perspective. Annu. Rev. Immunol. 33, 257–290. https://doi.org/10.1146/annurev-immunol-032414-112240

Cai, D.H., Wang, D., Keefer, J., Yeamans, C., Hensley, K., Friedman, A.D., 2008. C/EBPα:AP-1 leucine zipper heterodimers bind novel DNA elements, activate the PU.1 promoter and direct monocyte lineage commitment more potently than C/EBPα homodimers or AP-1. Oncogene 27, 2772–2779. https://doi.org/10.1038/sj.onc.1210940

Carneiro, L.C., Bedford, C., Jacca, S., Rosamilia, A., de Lima, V.F., Donofrio, G., Martin Sheldon, I., Cronin, J.G., 2017. Coordinated role of toll-like receptor-3 and retinoic acid-inducible gene-I in the innate response of bovine endometrial cells to virus. Front. Immunol. 8. https://doi.org/10.3389/fimmu.2017.00996

Carty, M., Guy, C., Bowie, A.G., 2021. Detection of Viral Infections by Innate Immunity. Biochem. Pharmacol. 183, 114316. https://doi.org/10.1016/j.bcp.2020.114316

Chang, X., Shi, X., Zhang, X., Wang, L., Li, X., Wang, A., Deng, R., Zhou, E., Zhang, G., 2019. IFI16 inhibits porcine reproductive and respiratory syndrome virus 2 replication in a MAVS-dependent manner in MARC-145 cells. Viruses 11. https://doi.org/10.3390/v11121160

Chauveau, E., Doceul, V., Lara, E., Adam, M., Breard, E., Sailleau, C., Viarouge, C., Desprat, A., Meyer, G., Schwartz-Cornil, I., Ruscanu, S., Charley, B., Zientara, S., Vitour, D., 2012. Sensing and Control of Bluetongue Virus Infection in Epithelial Cells via RIG-I and MDA5 Helicases. J. Virol. 86, 11789–11799. https://doi.org/10.1128/jvi.00430-12

Chen, H.M., Ray-Gallet, D., Zhang, P., Hetherington, C.J., Gonzalez, D.A., Zhang, D.E., Moreau-Gachelin, F., Tenen, D.G., 1995. PU.1 (Spi-1) autoregulates its expression in myeloid cells. Oncogene 11, 1549–1560.

Chen, S.N., Zou, P.F., Nie, P., 2017. Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) in fish: current knowledge and future perspectives. Immunology. https://doi.org/10.1111/imm.12714

Chiappinelli, K.B., Strissel, P.L., Desrichard, A., Li, H., Henke, C., Akman, B., Hein, A., Rote, N.S., Cope, L.M., Snyder, A., Makarov, V., Buhu, S., Slamon, D.J., Wolchok, J.D., Pardoll, D.M., Beckmann, M.W., Zahnow, C.A., Mergoub, T., Chan, T.A., Baylin, S.B., Strick, R., 2015. Inhibiting DNA Methylation Causes an Interferon Response in Cancer via dsRNA Including Endogenous Retroviruses. Cell 162, 974–986. https://doi.org/10.1016/j.cell.2015.07.011

Christensen, M.H., Paludan, S.R., 2017. Viral evasion of DNA-stimulated innate immune responses. Cell. Mol. Immunol. https://doi.org/10.1038/cmi.2016.06

De Vlugt, C., Sikora, D., Pelchat, M., 2018. Insight into influenza: A virus cap-snatching. Viruses. https://doi.org/10.3390/v10110641

Deng, X., Hackbart, M., Mettelman, R.C., O'Brien, A., Mielech, A.M., Yi, G., Kao, C.C., Baker, S.C., 2017. Coronavirus nonstructural protein 15 mediates evasion of dsRNA sensors and limits apoptosis in macrophages. Proc. Natl. Acad. Sci. U. S. A. 114, E4251–

E4260. https://doi.org/10.1073/pnas.1618310114

Dhawan, P., Wei, R., Sun, C., Gombart, A.F., Koeffler, H.P., Diamond, G., Christakos, S., 2015. C/EBPα and the vitamin D receptor cooperate in the regulation of cathelicidin in lung epithelial cells. J. Cell. Physiol. 230, 464–472. https://doi.org/10.1002/jcp.24729

Dong, X.Y., Liu, W.J., Zhao, M.Q., Wang, J.Y., Pei, J.J., Luo, Y.W., Ju, C.M., Chen, J.D., 2013. Classical swine fever virus triggers RIG-I and MDA5-dependent signaling pathway to IRF-3 and NF-κB activation to promote secretion of interferon and inflammatory cytokines in porcine alveolar macrophages. Virol. J. 10. https://doi.org/10.1186/1743-422X-10-286

Eaglesham, J.B., Kranzusch, P.J., 2020. Conserved strategies for pathogen evasion of cGAS–STING immunity. Curr. Opin. Immunol. https://doi.org/10.1016/j.coi.2020.04.002

Fay, P.C., Cook, C.G., Wijesiriwardana, N., Tore, G., Comtet, L., Carpentier, A., Shih, B., Freimanis, G., Haga, I.R., Beard, P.M., 2020. Madin-Darby bovine kidney (MDBK) cells are a suitable cell line for the propagation and study of the bovine poxvirus lumpy skin disease virus. J. Virol. Methods 285. https://doi.org/10.1016/j.jviromet.2020.113943

Feng, R., Desbordes, S.C., Xie, H., Tillo, E.S., Pixley, F., Stanley, E.R., Graf, T., 2008. PU.1 and C/EBPα/β convert fibroblasts into macrophage-like cells. Proc. Natl. Acad. Sci. U. S. A. 105, 6057–6062. https://doi.org/10.1073/pnas.0711961105

Forbes-Hernández, T.Y., Cianciosi, D., Ansary, J., Mezzetti, B., Bompadre, S., Quiles, J.L., Giampieri, F., Battino, M., 2020. Strawberry (: Fragaria × ananassa cv. Romina) methanolic extract promotes browning in 3T3-L1 cells, in: Food and Function. pp. 297–304. https://doi.org/10.1039/c9fo02285f

García-Nicolás, O., V'kovski, P., Vielle, N.J., Ebert, N., Züst, R., Portmann, J., Stalder, H., Gaschen, V., Vieyres, G., Stoffel, M., Schweizer, M., Summerfield, A., Engler, O., Pietschmann, T., Todt, D., Alves, M.P., Thiel, V., Pfaender, S., 2018. The small-compound inhibitor K22 displays broad antiviral activity against different members of the family Flaviviridae and offers potential as a panviral inhibitor. Antimicrob. Agents Chemother. 62. https://doi.org/10.1128/AAC.01206-18

Gasparrini, M., Forbes-Hernandez, T.Y., Giampieri, F., Afrin, S., Mezzetti, B., Quiles, J.L., Bompadre, S., Battino, M., 2017. Protective effect of strawberry extract against inflammatory stress induced in human dermal fibroblasts. Molecules 22. https://doi.org/10.3390/molecules22010164

Gethmann, J., Probst, C., Conraths, F.J., 2020. Economic Impact of a Bluetongue Serotype 8 Epidemic in Germany. Front. Vet. Sci. 7. https://doi.org/10.3389/fvets.2020.00065

Ghisletti, S., Barozzi, I., Mietton, F., Polletti, S., De Santa, F., Venturini, E., Gregory, L., Lonie, L., Chew, A., Wei, C.L., Ragoussis, J., Natoli, G., 2010. Identification and Characterization of Enhancers Controlling the Inflammatory Gene Expression Program in Macrophages. Immunity 32, 317–328. https://doi.org/10.1016/j.immuni.2010.02.008

Gitlin, L., Barchet, W., Gilfillan, S., Cella, M., Beutler, B., Flavell, R.A., Diamond, M.S., Colonna, M., 2006. Essential role of mda-5 in type I IFN responses to polyriboinosinic: polyribocytidylic acid and encephalomyocarditis picornavirus. Proc. Natl. Acad. Sci. U. S. A. 103, 8459–8464. https://doi.org/10.1073/pnas.0603082103

Gosselin, D., Glass, C.K., 2014. Epigenomics of macrophages. Immunol. Rev. 262, 96–112. https://doi.org/10.1111/imr.12213

Goto, H., Kariya, R., Kudo, E., Okuno, Y., Ueda, K., Katano, H., Okada, S., 2017. Restoring PU.1 induces apoptosis and modulates viral transactivation via interferon-stimulated genes in primary effusion lymphoma. Oncogene 36, 5252–5262. https://doi.org/10.1038/onc.2017.138

Goubau, D., Schlee, M., Deddouche, S., Pruijssers, A.J., Zillinger, T., Goldeck, M., Schuberth, C., Van Der Veen, A.G., Fujimura, T., Rehwinkel, J., Iskarpatyoti, J.A., Barchet, W., Ludwig, J., Dermody, T.S., Hartmann, G., Reis E Sousa, C., 2014. Antiviral immunity via RIG-I-mediated recognition of RNA bearing 59-diphosphates. Nature 514, 372–375. https://doi.org/10.1038/nature13590

Guanglan, L.I., Wenke, H.A.O., Wenxue, H.U., 2020. Transcription factor PU.1 and immune cell differentiation (Review). Int. J. Mol. Med. 46, 1943–1950. https://doi.org/10.3892/ijmm.2020.4763

Haehnel, V., Schwarzfischer, L., Fenton, M.J., Rehli, M., 2002. Transcriptional Regulation of the Human Toll-Like Receptor 2 Gene in Monocytes and Macrophages . J. Immunol. 168, 5629–5637. https://doi.org/10.4049/jimmunol.168.11.5629

Hagman, J., 2015. Transcriptional Regulation of Early B Cell Development, in: Molecular Biology of B Cells: Second Edition. pp. 35–53. https://doi.org/10.1016/B978-0-12-397933-9.00003-5

Hansen, K., Prabakaran, T., Laustsen, A., Jørgensen, S.E., Rahbæk, S.H., Jensen, S.B., Nielsen, R., Leber, J.H., Decker, T., Horan, K.A., Jakobsen, M.R., Paludan, S.R., 2014.

Listeria monocytogenes induces IFNβ expression through an IFI16- , cGAS- and STING- dependent pathway . EMBO J. 33, 1654–1666. https://doi.org/10.15252/embj.201488029

Hardy, S., Jackson, B., Goodbourn, S., Seago, J., 2021. Classical Swine Fever Virus N pro Antagonizes IRF3 To Prevent Interferon-Independent TLR3- and RIG-I-Mediated Apoptosis . J. Virol. 95. https://doi.org/10.1128/jvi.01136-20

Hartenian, E., Nandakumar, D., Lari, A., Ly, M., Tucker, J.M., Glaunsinger, B.A., 2020. The molecular virology of coronaviruses. J. Biol. Chem. https://doi.org/10.1074/jbc.REV120.013930

Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y.C., Laslo, P., Cheng, J.X., Murre, C., Singh, H., Glass, C.K., 2010. Simple Combinations of Lineage-Determining Transcription Factors Prime cis-Regulatory Elements Required for Macrophage and B Cell Identities. Mol. Cell 38, 576–589. https://doi.org/10.1016/j.molcel.2010.05.004

Heinz, S., Romanoski, C.E., Benner, C., Glass, C.K., 2015. The selection and function of cell type-specific enhancers. Nat. Rev. Mol. Cell Biol. https://doi.org/10.1038/nrm3949

Hennessy, C., O'Connell, S., Egan, L.J., McKernan, D.P., 2019. Inhibition of anti-viral responses in intestinal epithelial cells by epigenetic modifying drugs is mediated by a reduction in viral pattern recognition receptor expression and activity. Immunopharmacol. Immunotoxicol. 41, 527–537. https://doi.org/10.1080/08923973.2019.1661430

Hornung, V., Ellegast, J., Kim, S., Brzózka, K., Jung, A., Kato, H., Poeck, H., Akira, S., Conzelmann, K.K., Schlee, M., Endres, S., Hartmann, G., 2006. 5′-Triphosphate RNA is the ligand for RIG-I. Science (80-.). 314, 994–997. https://doi.org/10.1126/science.1132505

Horton, R.M., Cai, Z., Ho, S.N., Pease, L.R., 2013. Gene splicing by overlap extension: Tailor-made genes using the polymerase chain reaction. Biotechniques 54, 528–535. https://doi.org/10.2144/000114017

Hosokawa, H., Rothenberg, E. V., 2021. How transcription factors drive choice of the T cell fate. Nat. Rev. Immunol. https://doi.org/10.1038/s41577-020-00426-6

Hu, H.M., Baer, M., Williams, S.C., Johnson, P.F., Schwartz, R.C., 1998. Redundancy of C/EBPα, -β, and -δ in supporting the lipopolysaccharide-induced transcription of IL-6 and monocyte chemoattractant protein-1. J. Immunol. 160, 2334–2342.

Hung, Y.F., Chen, C.Y., Shih, Y.C., Liu, H.Y., Huang, C.M., Hsueh, Y.P., 2018. Endosomal

TLR3, TLR7, and TLR8 control neuronal morphology through different transcriptional programs. J. Cell Biol. 217, 2727–2742. https://doi.org/10.1083/jcb.201712113

Hybiak, J., Jankowska, K., Machaj, F., Rosik, J., Broniarek, I., Żyluk, A., Hilderman, G.C., Małecki, A., Łos, M.J., Urasińska, E., 2020. Reprogramming and transdifferentiation - two key processes for regenerative medicine. Eur. J. Pharmacol. https://doi.org/10.1016/j.ejphar.2020.173202

Jiang, T., Wang, F., Hu, L., Cheng, X., Zheng, Y., Liu, T., Jia, Y., 2017. Chidamide and decitabine can synergistically induce apoptosis of Hodgkin lymphoma cells by up-regulating the expression of PU.1 and KLF4. Oncotarget 8, 77586–77594. https://doi.org/10.18632/oncotarget.20659

Jiang, Y., Zhu, Y., Liu, Z.J., Ouyang, S., 2017. The emerging roles of the DDX41 protein in immunity and diseases. Protein Cell. https://doi.org/10.1007/s13238-016-0303-4

Jin, F., Li, Y., Ren, B., Natarajan, R., 2011. PU.1 and C/EBPα synergistically program distinct response to NF-κB activation through establishing monocyte specific enhancers. Proc. Natl. Acad. Sci. U. S. A. 108, 5290–5295. https://doi.org/10.1073/pnas.1017214108

Jin, T., Perry, A., Jiang, J., Smith, P., Curry, J.A., Unterholzner, L., Jiang, Z., Horvath, G., Rathinam, V.A., Johnstone, R.W., Hornung, V., Latz, E., Bowie, A.G., Fitzgerald, K.A., Xiao, T.S., 2012. Structures of the HIN Domain: DNA Complexes Reveal Ligand Binding and Activation Mechanisms of the AIM2 Inflammasome and IFI16 Receptor. Immunity 36, 561–571. https://doi.org/10.1016/j.immuni.2012.02.014

Jung, S., Lee, M.S., Choi, A.J., Kim, C.T., Kim, Y., 2019. Anti-inflammatory effects of high hydrostatic pressure extract of mulberry (morus alba) fruit on lps-stimulated RAW264.7 Cells. Molecules 24. https://doi.org/10.3390/molecules24071425

Kadkhodaeian, H.A., 2021. Mesenchymal stem cells: Signaling pathways in transdifferentiation into retinal progenitor cells. Basic Clin. Neurosci. https://doi.org/10.32598/bcn.9.10.510

Kaikkonen, M.U., Spann, N.J., Heinz, S., Romanoski, C.E., Allison, K.A., Stender, J.D., Chun, H.B., Tough, D.F., Prinjha, R.K., Benner, C., Glass, C.K., 2013. Remodeling of the enhancer landscape during macrophage activation is coupled to enhancer transcription. Mol. Cell 51, 310–325. https://doi.org/10.1016/j.molcel.2013.07.010

Kalia, V., Studzinski, G.P., Sarkar, S., 2021. Role of vitamin D in regulating COVID-19 severity—An immunological perspective. J. Leukoc. Biol.

https://doi.org/10.1002/JLB.4COVR1020-698R

Kasumba, D.M., Grandvaux, N., 2019. Therapeutic Targeting of RIG-I and MDA5 Might Not Lead to the Same Rome. Trends Pharmacol. Sci. https://doi.org/10.1016/j.tips.2018.12.003

Kato, H., Takahasi, K., Fujita, T., 2011. RIG-I-like receptors: Cytoplasmic sensors for non-self RNA. Immunol. Rev. https://doi.org/10.1111/j.1600-065X.2011.01052.x

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K.J., Yamaguchi, O., Otsu, K., Tsujimura, T., Koh, C.S., Reis E Sousa, C., Matsuura, Y., Fujita, T., Akira, S., 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 441, 101–105. https://doi.org/10.1038/nature04734

Katz, I.S.S., Guedes, F., Fernandes, E.R., dos Ramos Silva, S., 2017. Immunological aspects of rabies: a literature review. Arch. Virol. https://doi.org/10.1007/s00705-017-3484-0

Kaur, B.P., Secord, E., 2019. Innate Immunity. Pediatr. Clin. North Am. https://doi.org/10.1016/j.pcl.2019.06.011

Kende, M., Paragas, J., Salazar, A.M., 2019. The efficacy of poly-ICLC against Ebola-Zaire virus (EBOV) infection in mice and cynomolgus monkeys. Antiviral Res. 163, 179–184. https://doi.org/10.1016/j.antiviral.2018.12.020

Kerur, N., Veettil, M.V., Sharma-Walia, N., Bottero, V., Sadagopan, S., Otageri, P., Chandran, B., 2011. IFI16 acts as a nuclear pathogen sensor to induce the inflammasome in response to Kaposi Sarcoma-associated herpesvirus infection. Cell Host Microbe 9, 363–375. https://doi.org/10.1016/j.chom.2011.04.008

Khodarev, N.N., 2019. Intracellular RNA Sensing in Mammalian Cells: Role in Stress Response and Cancer Therapies, in: International Review of Cell and Molecular Biology. pp. 31–89. https://doi.org/10.1016/bs.ircmb.2018.08.005

Kikkert, M., 2020. Innate Immune Evasion by Human Respiratory RNA Viruses. J. Innate Immun. https://doi.org/10.1159/000503030

Kim, H.J., Lee, M.J., Jang, J.Y., Lee, S.H., 2019. Allium hookeri root extract inhibits adipogenesis by promoting lipolysis in high fat diet-induced obese mice. Nutrients 11. https://doi.org/10.3390/nu11102262

Kim, S., Kaang, B.K., 2017. Epigenetic regulation and chromatin remodeling in learning and memory. Exp. Mol. Med. https://doi.org/10.1038/emm.2016.140

Koehler, H.S., Feng, Y., Mandal, P., Mocarski, E.S., 2020. Recognizing limits of Z-nucleic acid

binding protein (ZBP1/DAI/DLM1) function. FEBS J. https://doi.org/10.1111/febs.15242

Kopliku, L., Relmy, A., Romey, A., Gorna, K., Zientara, S., Bakkali-Kassimi, L., Blaise-Boisseau, S., 2015. Establishment of persistent foot-and-mouth disease virus (FMDV) infection in MDBK cells. Arch. Virol. 160, 2503–2516. https://doi.org/10.1007/s00705-015-2526-8

Kumaki, Y., Salazar, A.M., Wandersee, M.K., Barnard, D.L., 2017. Prophylactic and therapeutic intranasal administration with an immunomodulator, Hiltonol® (Poly IC:LC), in a lethal SARS-CoV-infected BALB/c mouse model. Antiviral Res. 139, 1–12. https://doi.org/10.1016/j.antiviral.2016.12.007

Kumar, S., Chera, J.S., Vats, A., De, S., 2019. Nature of selection varies on different domains of IFI16-like PYHIN genes in ruminants. BMC Evol. Biol. 19. https://doi.org/10.1186/s12862-018-1334-7

Kuriakose, T., Kanneganti, T.D., 2018. ZBP1: Innate Sensor Regulating Cell Death and Inflammation. Trends Immunol. https://doi.org/10.1016/j.it.2017.11.002

Kurotaki, D., Yoshida, H., Tamura, T., 2020. Epigenetic and transcriptional regulation of osteoclast differentiation. Bone. https://doi.org/10.1016/j.bone.2020.115471

Laiosa, C. V., Stadtfeld, M., Xie, H., de Andres-Aguayo, L., Graf, T., 2006. Reprogramming of Committed T Cell Progenitors to Macrophages and Dendritic Cells by C/EBPα and PU.1 Transcription Factors. Immunity 25, 731–744. https://doi.org/10.1016/j.immuni.2006.09.011

Lee, H.C., Chathuranga, K., Lee, J.S., 2019. Intracellular sensing of viral genomes and viral evasion. Exp. Mol. Med. https://doi.org/10.1038/s12276-019-0299-y

Lee, J.-E., Schmidt, H., Lai, B., Ge, K., 2019. Transcriptional and Epigenomic Regulation of Adipogenesis. Mol. Cell. Biol. 39. https://doi.org/10.1128/mcb.00601-18

Lee, M.S., Kim, Y., 2020. Mulberry Fruit Extract Ameliorates Adipogenesis via Increasing AMPK Activity and Downregulating MicroRNA-21/143 in 3T3-L1 Adipocytes. J. Med. Food 23, 266–272. https://doi.org/10.1089/jmf.2019.4654

Li, C., Tao, Y., Liu, B., Liu, J., Wang, G., Liu, H., 2016. PU.1-Bim axis is involved in Trichostatin A-induced apoptosis in murine pro-B lymphoma FL5.12 cells. Acta Biochim. Biophys. Sin. (Shanghai). 48, 850–855. https://doi.org/10.1093/abbs/gmw067

Li, C., Xu, M.M., Wang, K., Adler, A.J., Vella, A.T., Zhou, B., 2018. Macrophage polarization and meta-inflammation. Transl. Res. https://doi.org/10.1016/j.trsl.2017.10.004

Li, K., Wang, C., Yang, F., Cao, W., Zhu, Z., Zheng, H., 2021. Virus–Host Interactions in Foot-and-Mouth Disease Virus Infection. Front. Immunol. https://doi.org/10.3389/fimmu.2021.571509

Li, N., Zhao, D., Kirschbaum, M., Zhang, C., Lin, C.L., Todorov, I., Kandeel, F., Forman, S., Zeng, D., 2008. HDAC inhibitor reduces cytokine storm and facilitates induction of chimerism that reverses lupus in anti-CD3 conditioning regimen. Proc. Natl. Acad. Sci. U. S. A. 105, 4796–4801. https://doi.org/10.1073/pnas.0712051105

Li, P., Zhu, Z., Cao, W., Yang, F., Ma, X., Tian, H., Zhang, K., Liu, X., Zheng, H., 2021. Dysregulation of the RIG-I–like Receptor Pathway Signaling by Peste des Petits Ruminants Virus Phosphoprotein. J. Immunol. 206, 566–579. https://doi.org/10.4049/jimmunol.2000432

Li, Q., Liu, C., Yue, R., El-Ashram, S., Wang, J., He, X., Zhao, D., Zhou, X., Xu, L., 2019. Cgas/sting/tbk1/irf3 signaling pathway activates bmdcs maturation following mycobacterium bovis infection. Int. J. Mol. Sci. 20. https://doi.org/10.3390/ijms20040895

Li, Y., 2021. Modern epigenetics methods in biological research. Methods. https://doi.org/10.1016/j.ymeth.2020.06.022

Li, Z., Kang, H., You, Q., Ossa, F., Mead, P., Quinton, M., Karrow, N.A., 2018. In vitro bioassessment of the immunomodulatory activity of Saccharomyces cerevisiae components using bovine macrophages and Mycobacterium avium ssp. paratuberculosis. J. Dairy Sci. 101, 6271–6286. https://doi.org/10.3168/jds.2017-13805

Liu, B., Bashkin, J.K., Poon, G.M.K., Wang, Shuo, Wang, Siming, Wilson, W.D., 2019. Modulating DNA by polyamides to regulate transcription factor PU.1-DNA binding interactions. Biochimie 167, 1–11. https://doi.org/10.1016/j.biochi.2019.08.009

Liu, C., Liu, Y., Liang, L., Cui, S., Zhang, Y., 2019. RNA-Seq based transcriptome analysis during bovine viral diarrhoea virus (BVDV) infection. BMC Genomics 20. https://doi.org/10.1186/s12864-019-6120-4

Liu, C., Wang, M., Sun, W., Cai, F., Geng, S., Su, X., Shi, Y., 2017. PU.1 serves a critical role in the innate defense against Aspergillus fumigatus via dendritic cell-associated C-type lectin receptor-1 and toll-like receptors-2 and 4 in THP-1-derived macrophages. Mol. Med. Rep. 15, 4084–4092. https://doi.org/10.3892/mmr.2017.6504

Liu, Q., Yu, J., Wang, L., Tang, Y., Zhou, Q., Ji, S., Wang, Y., Santos, L., Haeusler, R.A., Que, J., Rajbhandari, P., Lei, X., Valenti, L., Pajvani, U.B., Qin, J., Qiang, L., 2020. Inhibition of PU.1 ameliorates metabolic dysfunction and non-alcoholic steatohepatitis. J. Hepatol. 73, 361–370. https://doi.org/10.1016/j.jhep.2020.02.025

Liu, Y., Olagnier, D., Lin, R., 2017. Host and viral modulation of RIG-I-mediated antiviral immunity. Front. Immunol. https://doi.org/10.3389/fimmu.2016.00662

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262

Maina, T., Prysliak, T., Perez-Casal, J., 2019. Mycoplasma bovis delay in apoptosis of macrophages is accompanied by increased expression of anti-apoptotic genes, reduced cytochrome C translocation and inhibition of DNA fragmentation. Vet. Immunol. Immunopathol. 208, 16–24. https://doi.org/10.1016/j.vetimm.2018.12.004

Malik, G., Zhou, Y., 2020. Innate immune sensing of influenza a virus. Viruses. https://doi.org/10.3390/v12070755

McClellan, J.S., Dove, C., Gentles, A.J., Ryan, C.E., Majeti, R., 2015. Reprogramming of primary human Philadelphia chromosome-positive B cell acute lymphoblastic leukemia cells into nonleukemic macrophages. Proc. Natl. Acad. Sci. U. S. A. 112, 4074–4079. https://doi.org/10.1073/pnas.1413383112

Mehraj, U., Nisar, S., Qayoom, H., Bhat, B.A., Sheikh, B.A., Hamdani, S.S., Mir, M.A., 2019. The innate and adaptive immunity, in: Basics and Fundamentals of Immunology. pp. 117–138.

Mehrizi, A.A., Rezvani, N., Zakeri, S., Gholami, A., Babaeekhou, L., 2018. Poly(I:C) adjuvant strongly enhances parasite-inhibitory antibodies and Th1 response against Plasmodium falciparum merozoite surface protein-1 (42-kDa fragment) in BALB/c mice. Med. Microbiol. Immunol. 207, 151–166. https://doi.org/10.1007/s00430-018-0535-4

Menachery, V.D., Eisfeld, A.J., Schäfer, A., Josset, L., Sims, A.C., Proll, S., Fan, S., Li, C., Neumann, G., Tilton, S.C., Chang, J., Gralinski, L.E., Long, C., Green, R., Williams, C.M., Weiss, J., Matzke, M.M., Webb-Robertson, B.J., Schepmoes, A.A., Shukla, A.K., Metz, T.O., Smith, R.D., Waters, K.M., Katze, M.G., Kawaoka, Y., Baric, R.S., 2014. Pathogenic influenza viruses and coronaviruses utilize similar and contrasting approaches to control interferon-stimulated gene responses. MBio 5. https://doi.org/10.1128/mBio.01174-14

Moriyama, M., Chino, S., Ichinohe, T., 2017. Consecutive inoculations of influenza virus vaccine and poly(I:C) protects mice against homologous and heterologous virus challenge. Vaccine 35, 1001–1007. https://doi.org/10.1016/j.vaccine.2017.01.025

Morrison, A.J., 2020. Chromatin-remodeling links metabolic signaling to gene expression. Mol. Metab. https://doi.org/10.1016/j.molmet.2020.100973

Motwani, M., Pesiridis, S., Fitzgerald, K.A., 2019. DNA sensing by the cGAS–STING pathway in health and disease. Nat. Rev. Genet. https://doi.org/10.1038/s41576-019-0151-1

Mueller, F.S., Richetto, J., Hayes, L.N., Zambon, A., Pollak, D.D., Sawa, A., Meyer, U., Weber-Stadlbauer, U., 2019. Influence of poly(I:C) variability on thermoregulation, immune responses and pregnancy outcomes in mouse models of maternal immune activation. Brain. Behav. Immun. 80, 406–418. https://doi.org/10.1016/j.bbi.2019.04.019

Nguyen, M.L.T., Jones, S.A., Prier, J.E., Russ, B.E., 2015. Transcriptional enhancers in the regulation of T cell differentiation. Front. Immunol. https://doi.org/10.3389/fimmu.2015.00462

Niu, W., Zang, T., Zou, Y., Fang, S., Smith, D.K., Bachoo, R., Zhang, C.L., 2013. In vivo reprogramming of astrocytes to neuroblasts in the adult brain. Nat. Cell Biol. 15, 1164–1175. https://doi.org/10.1038/ncb2843

Nogales, A., Martinez-Sobrido, L., Topham, D.J., Dediego, M.L., 2018. Modulation of innate immune responses by the influenza a NS1 and PA-X proteins. Viruses. https://doi.org/10.3390/v10120708

Oh, Y., Jang, J., 2019. Directed differentiation of pluripotent stem cells by transcription factors. Mol. Cells. https://doi.org/10.14348/molcells.2019.2439

Okamoto, M., Kouwaki, T., Fukushima, Y., Oshiumi, H., 2018. Regulation of RIG-I Activation by K63-Linked Polyubiquitination. Front. Immunol. https://doi.org/10.3389/fimmu.2017.01942

Okuno, Y., Huang, G., Rosenbauer, F., Evans, E.K., Radomska, H.S., Iwasaki, H., Akashi, K., Moreau-Gachelin, F., Li, Y., Zhang, P., Göttgens, B., Tenen, D.G., 2005. Potential Autoregulation of Transcription Factor PU.1 by an Upstream Regulatory Element. Mol. Cell. Biol. 25, 2832–2845. https://doi.org/10.1128/mcb.25.7.2832-2845.2005

Olschewski, S., Cusack, S., Rosenthal, M., 2020. The Cap-Snatching Mechanism of Bunyaviruses. Trends Microbiol. https://doi.org/10.1016/j.tim.2019.12.006

Orsini, M., Chateauvieux, S., Rhim, J., Gaigneaux, A., Cheillan, D., Christov, C., Dicato, M., Morceau, F., Diederich, M., 2019. Sphingolipid-mediated inflammatory signaling

leading to autophagy inhibition converts erythropoiesis to myelopoiesis in human hematopoietic stem/progenitor cells. Cell Death Differ. 26, 1796–1812. https://doi.org/10.1038/s41418-018-0245-x

Ostuni, R., Piccolo, V., Barozzi, I., Polletti, S., Termanini, A., Bonifacio, S., Curina, A., Prosperini, E., Ghisletti, S., Natoli, G., 2013. Latent enhancers activated by stimulation in differentiated cells. Cell 152, 157–171. https://doi.org/10.1016/j.cell.2012.12.018

Owen, K.L., Brockwell, N.K., Parker, B.S., 2019. Jak-stat signaling: A double-edged sword of immune regulation and cancer progression. Cancers (Basel). https://doi.org/10.3390/cancers11122002

Pang, S.H.M., de Graaf, C.A., Hilton, D.J., Huntington, N.D., Carotta, S., Wu, L., Nutt, S.L., 2018. PU.1 is required for the developmental progression of multipotent progenitors to common lymphoid progenitors. Front. Immunol. 9. https://doi.org/10.3389/fimmu.2018.01264

Park, S.Y., Lee, S.W., Baek, S.H., Lee, C.W., Lee, W.S., Rhim, B.Y., Hong, K.W., Kim, C.D., 2013. Suppression of PU.1-linked TLR4 expression by cilostazol with decrease of cytokine production in macrophages from patients with rheumatoid arthritis. Br. J. Pharmacol. 168, 1401–1411. https://doi.org/10.1111/bph.12021

Patel, M. V., Shen, Z., Wira, C.R., 2018. Poly (I:C) and LPS induce distinct immune responses by ovarian stromal fibroblasts. J. Reprod. Immunol. 127, 36–42. https://doi.org/10.1016/j.jri.2018.05.001

Patrick, K.L., Bell, S.L., Watson, R.O., 2016. For Better or Worse: Cytosolic DNA Sensing during Intracellular Bacterial Infection Induces Potent Innate Immune Responses. J. Mol. Biol. https://doi.org/10.1016/j.jmb.2016.04.030

Pichlmair, A., Schulz, O., Tan, C.P., Näslund, T.I., Liljeström, P., Weber, F., Reis E Sousa, C., 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. Science (80-.). 314, 997–1001. https://doi.org/10.1126/science.1132998

Pimenova, A.A., Herbinet, M., Gupta, I., Machlovi, S.I., Bowles, K.R., Marcora, E., Goate, A.M., 2021. Alzheimer's-associated PU.1 expression levels regulate microglial inflammatory response. Neurobiol. Dis. 148. https://doi.org/10.1016/j.nbd.2020.105217

Pouzolles, M., Oburoglu, L., Taylor, N., Zimmermann, V.S., 2016. Hematopoietic stem cell lineage specification. Curr. Opin. Hematol. https://doi.org/10.1097/MOH.0000000000000260

Qiao, Y., Zhu, S., Deng, S., Zou, S.S., Gao, B., Zang, G., Wu, J., Jiang, Y., Liu, Y.J., Chen, J., 2021. Human Cancer Cells Sense Cytosolic Nucleic Acids Through the RIG-I–MAVS Pathway and cGAS–STING Pathway. Front. Cell Dev. Biol. 8. https://doi.org/10.3389/fcell.2020.606001

Qin, X., Jiang, Q., Nagano, K., Moriishi, T., Miyazaki, T., Komori, H., Ito, K., von der Mark, K., Sakane, C., Kaneko, H., Komori, T., 2020. Runx2 is essential for the transdifferentiation of chondrocytes into osteoblasts. PLoS Genet. 16. https://doi.org/10.1371/journal.pgen.1009169

Rathinam, V.A.K., Jiang, Z., Waggoner, S.N., Sharma, S., Cole, L.E., Waggoner, L., Vanaja, S.K., Monks, B.G., Ganesan, S., Latz, E., Hornung, V., Vogel, S.N., Szomolanyi-Tsuda, E., Fitzgerald, K.A., 2010. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. Nat. Immunol. 11, 395–402. https://doi.org/10.1038/ni.1864

Rehwinkel, J., Gack, M.U., 2020. RIG-I-like receptors: their regulation and roles in RNA sensing. Nat. Rev. Immunol. https://doi.org/10.1038/s41577-020-0288-3

Roger, T., Miconnet, I., Schiesser, A.L., Kai, H., Miyake, K., Calandra, T., 2005. Critical role for Ets, AP-1 and GATA-like transcription factors in regulating mouse Toll-like receptor 4 (Tlr4) gene expression. Biochem. J. 387, 355–365. https://doi.org/10.1042/BJ20041243

Rola-Łuszczak, M., Materniak, M., Pluta, A., Hulst, M., Kuźmak, J., 2014. Transcriptomic microarray analysis of BoMac cells after infection with bovine foamy virus. Arch. Virol. 159, 1515–1519. https://doi.org/10.1007/s00705-013-1959-1

Rosa, F.F., Pires, C.F., Kurochkin, I., Ferreira, A.G., Gomes, A.M., Palma, L.G., Shaiv, K., Solanas, L., Azenha, C., Papatsenko, D., Schulz, O., Sousa, C.R.E., Pereira, C.F., 2018. Direct reprogramming of fibroblasts into antigen-presenting dendritic cells. Sci. Immunol. 3. https://doi.org/10.1126/sciimmunol.aau4292

Rothbart, S.B., Strahl, B.D., 2014. Interpreting the language of histone and DNA modifications. Biochim. Biophys. Acta - Gene Regul. Mech. https://doi.org/10.1016/j.bbagrm.2014.03.001

Rothenberg, E. V., Hosokawa, H., Ungerbäck, J., 2019. Mechanisms of action of hematopoietic transcription factor PU.1 in initiation of T-cell development. Front. Immunol. 10. https://doi.org/10.3389/fimmu.2019.00228

Sanchez David, R.Y., Combredet, C., Najburg, V., Millot, G.A., Beauclair, G., Schwikowski, B., Léger, T., Camadro, J.M., Jacob, Y., Bellalou, J., Jouvenet, N., Tangy, F.,

Komarova, A. V., 2019. LGP2 binds to PACT to regulate RIG-I– and MDA5-mediated antiviral responses. Sci. Signal. 12. https://doi.org/10.1126/scisignal.aar3993

Sapre, S.U., Nair, P., 2020. Potentiality of DNA sensors in activating immune system in emerging viral infectious diseases, in: Dynamics of Immune Activation in Viral Diseases. pp. 243–260. https://doi.org/10.1007/978-981-15-1045-8_16

Sari, D.C.R., Putri, M.W., Leksono, T.P., Chairunnisa, N., Reynaldi, G.N., Simanjuntak, B.C., Debora, J., Yunus, J., Arfian, N., 2019. Calcitriol ameliorates kidney injury through reducing podocytopathy, tubular injury, inflammation and fibrosis in 5/6 subtotal nephrectomy model in rats. Kobe J. Med. Sci. 65, E153–E163.

Schoggins, J.W., 2019. Interferon-Stimulated Genes: What Do They All Do? Annu. Rev. Virol. 6, 567–584. https://doi.org/10.1146/annurev-virology-092818-015756

Shimizu, T., Nakamura, H., Kawakami, A., 2021. Role of the innate immunity signaling pathway in the pathogenesis of sjögren's syndrome. Int. J. Mol. Sci. https://doi.org/10.3390/ijms22063090

Stabel, J.R., Stabel, T.J., 1995. Immortalization and characterization of bovine peritoneal macrophages transfected with SV40 plasmid DNA. Vet. Immunol. Immunopathol. 45, 211–220. https://doi.org/10.1016/0165-2427(94)05348-V

Stephens, D.C., Kim, H.M., Kumar, A., Farahat, A.A., Boykin, D.W., K Poon, G.M., 2016. Pharmacologic efficacy of PU.1 inhibition by heterocyclic dications: A mechanistic analysis. Nucleic Acids Res. 44, 4005–4013. https://doi.org/10.1093/nar/gkw229

Streicher, F., Jouvenet, N., 2019. Stimulation of Innate Immunity by Host and Viral RNAs. Trends Immunol. https://doi.org/10.1016/j.it.2019.10.009

Sultana, T., Mamede, J.I., Saito, A., Ode, H., Nohata, K., Cohen, R., Nakayama, E.E., Iwatani, Y., Yamashita, M., Hope, T.J., Shioda, T., 2019. Multiple Pathways To Avoid Beta Interferon Sensitivity of HIV-1 by Mutations in Capsid. J. Virol. 93. https://doi.org/10.1128/jvi.00986-19

Takahashi, K., Yamanaka, S., 2006. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell 126, 663–676. https://doi.org/10.1016/j.cell.2006.07.024

Tan, X., Sun, L., Chen, J., Chen, Z.J., 2018. Detection of Microbial Infections Through Innate Immune Sensing of Nucleic Acids. Annu. Rev. Microbiol. 72, 447–478. https://doi.org/10.1146/annurev-micro-102215-095605

Thomas, M., Wang, Z., Sreenivasan, C.C., Hause, B.M., Renukaradhya, G.J., Li, F., Francis, D.H., Kaushik, R.S., Khatri, M., 2015. Poly I: C adjuvanted inactivated swine influenza vaccine induces heterologous protective immunity in pigs. Vaccine 33, 542–548. https://doi.org/10.1016/j.vaccine.2014.11.034

Thompson, M.R., Kaminski, J.J., Kurt-Jones, E.A., Fitzgerald, K.A., 2011. Pattern recognition receptors and the innate immune response to viral infection. Viruses. https://doi.org/10.3390/v3060920

Toka, F.N., Dunaway, K., Smaltz, F., Szulc-Dabrowska, L., Drnevich, J., Mielcarska, M.B., Bossowska-Nowicka, M., Schweizer, M., 2019. Bacterial and viral pathogen-associated molecular patterns induce divergent early transcriptomic landscapes in a bovine macrophage cell line. BMC Genomics 20. https://doi.org/10.1186/s12864-018-5411-5

Tsatsanis, C., Androulidaki, A., Alissafi, T., Charalampopoulos, I., Dermitzaki, E., Roger, T., Gravanis, A., Margioris, A.N., 2006. Corticotropin-Releasing Factor and the Urocortins Induce the Expression of TLR4 in Macrophages via Activation of the Transcription Factors PU.1 and AP-1. J. Immunol. 176, 1869–1877. https://doi.org/10.4049/jimmunol.176.3.1869

Uehata, T., Takeuchi, O., 2020. RNA Recognition and Immunity-Innate Immune Sensing and Its Posttranscriptional Regulation Mechanisms. Cells. https://doi.org/10.3390/cells9071701

Ueno, S., Tatetsu, H., Hata, H., Iino, T., Niiro, H., Akashi, K., Tenen, D.G., Mitsuya, H., Okuno, Y., 2009. PU.1 induces apoptosis in myeloma cells through direct transactivation of TRAIL. Oncogene 28, 4116–4125. https://doi.org/10.1038/onc.2009.263

Unterholzner, L., Keating, S.E., Baran, M., Horan, K.A., Jensen, S.B., Sharma, S., Sirois, C.M., Jin, T., Latz, E., Xiao, T.S., Fitzgerald, K.A., Paludan, S.R., Bowie, A.G., 2010. IFI16 is an innate immune sensor for intracellular DNA. Nat. Immunol. 11, 997–1004. https://doi.org/10.1038/ni.1932

Vats, A., Gautam, D., Maharana, J., Singh Chera, J., Kumar, S., Rout, P.K., Werling, D., De, S., 2020. Poly I:C stimulation in-vitro as a marker for an antiviral response in different cell types generated from Buffalo (Bubalus bubalis). Mol. Immunol. 121, 136–143. https://doi.org/10.1016/j.molimm.2020.03.004

Vyas, S.P., Hansda, A.K., Kaplan, M.H., Goswami, R., 2020. Calcitriol Regulates the Differentiation of IL-9–Secreting Th9 Cells by Modulating the Transcription Factor PU.1. J. Immunol. 204, 1201–1213. https://doi.org/10.4049/jimmunol.1901205

Wang, J., Li, S., Li, H., Zhou, X., Wen, H., Lai, B., 2021. IRF4 overexpression promotes the transdifferentiation of tregs into macrophage- like cells to inhibit the development of colon cancer. Cancer Cell Int. 21. https://doi.org/10.1186/s12935-021-01766-6

Wang, M., Liu, Z., Liu, C., Wu, T., Cai, F., Wang, Q., Su, X., Shi, Y., 2016. PU.1 is involved in the immune response to Aspergillus fumigatus through upregulating Dectin-1 expression. BMC Infect. Dis. 16. https://doi.org/10.1186/s12879-016-1632-x

Weis, S., Te Velthuis, A.J.W., 2021. Influenza virus rna synthesis and the innate immune response. Viruses. https://doi.org/10.3390/v13050780

Wohlfahrt, T., Rauber, S., Uebe, S., Luber, M., Soare, A., Ekici, A., Weber, S., Matei, A.E., Chen, C.W., Maier, C., Karouzakis, E., Kiener, H.P., Pachera, E., Dees, C., Beyer, C., Daniel, C., Gelse, K., Kremer, A.E., Naschberger, E., Stürzl, M., Butter, F., Sticherling, M., Finotto, S., Kreuter, A., Kaplan, M.H., Jüngel, A., Gay, S., Nutt, S.L., Boykin, D.W., Poon, G.M.K., Distler, O., Schett, G., Distler, J.H.W., Ramming, A., 2019. PU.1 controls fibroblast polarization and tissue fibrosis. Nature 566, 344–349. https://doi.org/10.1038/s41586-019-0896-x

Wolff, G., Melia, C.E., Snijder, E.J., Bárcena, M., 2020. Double-Membrane Vesicles as Platforms for Viral Replication. Trends Microbiol. https://doi.org/10.1016/j.tim.2020.05.009

Woolsey, C., Menicucci, A.R., Cross, R.W., Luthra, P., Agans, K.N., Borisevich, V., Geisbert, J.B., Mire, C.E., Fenton, K.A., Jankeel, A., Anand, S., Ebihara, H., Geisbert, T.W., Messaoudi, I., Basler, C.F., 2019. A VP35 Mutant Ebola Virus Lacks Virulence but Can Elicit Protective Immunity to Wild-Type Virus Challenge. Cell Rep. 28, 3032-3046.e6. https://doi.org/10.1016/j.celrep.2019.08.047

Wu, J.J., Li, W., Shao, Y., Avey, D., Fu, B., Gillen, J., Hand, T., Ma, S., Liu, X., Miley, W., Konrad, A., Neipel, F., Stürzl, M., Whitby, D., Li, H., Zhu, F., 2015. Inhibition of cGAS DNA Sensing by a Herpesvirus Virion Protein. Cell Host Microbe 18, 333–344. https://doi.org/10.1016/j.chom.2015.07.015

Xu, X.W., Ding, B.W., Zhu, C.R., Ji, W., Li, J.S., 2013. PU.1-silenced dendritic cells prolong allograft survival in rats receiving intestinal transplantation. World J. Gastroenterol. 19, 7766–7771. https://doi.org/10.3748/wjg.v19.i43.7766

Xu, Z., Shi, L., Wang, Y., Zhang, J., Huang, L., Zhang, C., Liu, S., Zhao, P., Liu, H., Zhu, L., Tai, Y., Bai, C., Gao, T., Song, J., Xia, P., Dong, J., Zhao, J., Wang, F.S., 2020. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. Lancet Respir. Med. 8, 420–422. https://doi.org/10.1016/S2213-2600(20)30076-X

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., Slukvin, I.I., Thomson, J.A., 2007. Induced pluripotent stem cell lines derived from human somatic cells. Science (80-.). 318, 1917–1920. https://doi.org/10.1126/science.1151526

Zhang, C., Wang, S.H., Liao, C.P., Shao, S., Lasbury, M.E., Durant, P.J., Lee, C.H., 2010. Downregulation of PU.1 leads to decreased expression of dectin-1 in alveolar macrophages during Pneumocystis pneumonia. Infect. Immun. 78, 1058–1065. https://doi.org/10.1128/IAI.01141-09

Zhang, X., Paget, M., Wang, C., Zhu, Z., Zheng, H., 2020. Innate immune evasion by picornaviruses. Eur. J. Immunol. https://doi.org/10.1002/eji.202048785

Zhou, Y., Ren, Y., Cong, Y., Mu, Y., Yin, R., Ding, Z., 2017. Autophagy induced by bovine viral diarrhea virus infection counteracts apoptosis and innate immune activation. Arch. Virol. 162, 3103–3118. https://doi.org/10.1007/s00705-017-3482-2

Zhu, H., Zheng, C., 2020. The Race between Host Antiviral Innate Immunity and the Immune Evasion Strategies of Herpes Simplex Virus 1. Microbiol. Mol. Biol. Rev. 84. https://doi.org/10.1128/mmbr.00099-20

Zhu, L., Ding, X., Zhang, D., Yuan, C.H., Wang, J., Ndegwa, E., Zhu, G., 2015. Curcumin inhibits bovine herpesvirus type 1 entry into MDBK cells. Acta Virol. 59, 221–227. https://doi.org/10.4149/av_2015_03_221

Zhu, L., Yuan, C., Zhang, D., Ma, Y., Ding, X., Zhu, G., 2016. BHV-1 induced oxidative stress contributes to mitochondrial dysfunction in MDBK cells. Vet. Res. 47. https://doi.org/10.1186/s13567-016-0332-2

Annexures

ANNEXURE-I

1. **Primers used for LDTF amplification. The underlined regions are the sites for restriction enzymes (NheI in case of forward and XhoI in case of reverse primers). 4 and 5 additional nucleotides were added at 5' end of NheI and XhoI sites respectively to facilitate efficient enzyme binding on DNA.**

Primer name	Sequence	Length (bp)
PU.1 FW	ATATGCTAGCATGTTACAGGCGTGCAAAATGGAAGGGTTTC	41
PU.1 RV	AATAACTCGAGTCAGTGGGGCGGGTGGCGC	30
PU.1 3' UTR RV	CGAAGGGGTTAATGCTATGGCCAACG	26
CEBPA FW	ATATGCTAGCATGGAGTCGGCCGACTTCTACGAG	34
CEBPA RV	AATAACTCGAGTCACGCGCAGTTGCCCATGGCC	33
CEBPB FW	ATATGCTAGCATGCAACGCCTGGTGGTCTGGGA	33
CEBPB RV	AATAACTCGAGCTAGCAGTGGCCGGAGGAGGC	32

2. **Primers used for the overlap extension PCR to join PU.1 with IRES element followed by either CEBPA or CEBPB. The underlined region is complementary to the respective gene while the other part of the sequence is complementary to the IRES element present in the pIRES-EGFP-Puro vector.**

Primer name	Sequence	Length (bp)
PU.1 RV IRES	CGCGGTACCGTCGACTGCAGTCAGTGGGGCGGGTGGCGC	39
CEBPA FW IRES	TTTGAAAAACACGATGATAATATGGCCACAACCATGGAGTCGGCCGACTTCTACGAG	57
CEBPB FW IRES	TTTGAAAAACACGATGATAATATGGCCACAACCATGCAACGCCTGGTGGTCTGGGA	56

3. **Primers used for semi-quantitative real time PCR.**

Primer name	Sequence	Length (bp)
PU.1 FW	GCAGTGATGGAGAAAGCCACAGCG	24
PU.1 RV	CACGTGCATCTGCTCCAGCTCCA	23
CEBPA FW	ATGGAGTCGGCCGACTTCTACGAG	24
CEBPA RV	GTCGATGTAGGCGCTGATGTCGATG	25
CEBPB FW	GGACTGCAAGCGGAAGGAGGAG	22
CEBPB RV	CTGGACGACGAGGACGTGGAC	21

RIG-I FW	CTTGCAAGAGGAATACCACTTAAACCCAGAGAC	33
RIG-I RV	TTCTGCCACGTCCAGTCAATATGCCAGGTTT	31
MDA5 FW	TCTGCTTATCGCTACCACAGTGGCAGA	27
MDA5 RV	TGCTCTCATCAGCTCTGGCTCGACC	25
LGP2 FW	CCACCACGTCAATGTGAACCCCAACTTC	28
LGP2 RV	TGAGCACTGGCAGCTTCACTGACTTGTAGAT	31
IRF7 FW	GCCTCCTGGAAAACCAACTTCCGCTG	26
IRF7 RV	GCAGATGGTCCTCCAAGCAGCTCTG	25
IRF3 FW	GATACTGCCCTGGCTGATATCTCAGCTG	28
IRF3 RV	GTTCAGGGCAGACCGGAAATTCCTCTTC	28
IFN-β FW	CACCACAGCTCTTTCCAGGAGCTACA	26
IFN-β RV	GGAACTGCTGTTCTTGCTTCATCTCCTC	28
ISG54 FW	AAGTGCACGGCAATCATGAGTGAGACCA	28
ISG54 RV	CCTCTACCAAGTTCCAGGTGAAATGGC	27
ISG56 FW	CTATGTGAAACACCTGAGAGGCCAGAATGA	30
ISG56 RV	CAGGCATAGTTGCCCCAGGTAACCAG	26
OAS FW	CGACATCATCTGCAGTTTCCTGAAGGAGA	29
OAS RV	GGTCGTGCCTTTGCCTGAGGAGC	23
PKR FW	ACTGGTCGTTCACCATGTTTCTACATAGAAG	31
PKR RV	CCTTCTTTGATCTACCTTCAGCCTTTGGAT	30
IFI16-l FW	GAGAATTGCCTCCAGACTCCTCAGAAGCT	29
IFI16-l RV	GTTCCGTTGCCTTCAGCACCATCACTTC	28
cGAS FW	GGGGGAATCTTATCGGCTTCTAAGATGCTGT	31
cGAS RV	AGGCCAGCTGCTTTTTGACTCCAAAGCCA	29
DAI FW	GGGATGAAGACAGCGAAGGAAGTCAACC	28
DAI RV	GGTCCCTTTCTGACAGATCATGTTGATTGG	30
DDX41 FW	CATGAACGTGTCCGGAAGAAGTACCACATC	30
DDX41 RV	CCTGGATCTGAATGGGTGTTGGGTGG	26
STING FW	GGCTCATGGGCTGGCCTGGTC	21
STING RV	CGGGAAGAGGATGTGCAGCCG	21
RPS18 FW	TGCGAGTACTCAACACCAACATCGATGG	28
RPS18 RV	GGATTCTGCATAATGGTGATCACACGTTCC	30

ANNEXURE-II

4. PCR reaction components for amplification of the LDTF genes

Components	Genes		
	PU.1*	**CEBPA**	**CEBPB**
5X Q5 Reaction Buffer	2.5 µl	2.5 µl	2.5 µl
dNTP Mix (2 mM each)	0.7 µl	0.7 µl	0.7 µl
Forward primer (10 µM)	0.25 µl	0.25 µl	0.25 µl
Reverse primer (10 µM)	0.25 µl	0.25 µl	0.25 µl
Template	2 µl (100 ng cDNA)	2 µl (100 ng DNA)	2 µl (100 ng DNA)
Q5 Polymerase	0.25 µl	0.25 µl	0.25 µl
Q5 5X GC Enhancer	-	5 µl	5 µl
Water (upto 25 µl)	19.05 µl	14.05 µl	14.05 µl

*The second reaction for PU.1 had all the same reaction components except for template (0.25 µl) and water (20.8 µl)

5. Thermal cycler conditions for the PCR of LDTF genes

Step	Temperature	Time
Initial Denaturation	98°C	60 seconds
***PCR amplification (30 cycles) :**		
Denaturation	98°C	10 seconds
Annealing	63°C	10 seconds
Extension	72°C	20 seconds
Final Extension	72°C	120 seconds
Hold	4°C	-

*****For the 2nd reaction of PU.1, the number of PCR amplification cycles were reduced to 25

ANNEXURE-III

6. Coding sequences of LDTFs obtained through Sanger sequencing

Gene	Sequence
PU.1	ATGTTACAGGCGTGCAAAATGGAAGGGTTTCCCCTCGTCCCCCCTCAGCCATCGGAAGACCTAGTTCCCTATGACACGGA TCTCTACCAACGCCAAACGCACGAATATTACCCCTATCTCAGCAGTGATGGAGAAAGCCACAGCGACCATTACTGGGACT TCCATCCGCACCACGTGCACAGCGAGTTCGAGAGCTTCGCCGAGAACCACTTCACGGAGCTGCAGAGCGTCCAGCCCCCA CAACTGCAGCAGCTCTACCGCCACATGGAGCTGGAGCAGATGCACGTGCTCGACAGCTCCATGGCTCCTCCCCACGCCAG CCTCAGTCACCAGGTCCCCTACCTGTCCCGGATGTGCCTGCCGTACCCCTCGCTGTCTCCCGCCCGGCCCAGCTCGGATG AAGAGGAGGGGGAGCGGCAGAGCCCCCCGCTGGAGGTGTCCGATGGGGAGGCCGACGGCCTGGAGCCAGGGCCTGGCCTC CTGCACGGGGAGACAGGCAGCAAGAAGAAGATCCGCCTGTACCAGTTCCTGCTGGACCTGCTGCGCAGCGGCGACATGAA GGACAGCATCTGGTGGGTGGACAAGGACAAGGGCACGTTCCAGTTCTCGTCCAAGCACAAGGAGGCGCTGGCACACCGCT GGGGCATCCAGAAGGGCAACCGCAAGAAGATGACCTACCAGAAGATGGCCCGAGCGCTGCGCAACTACGGCAAGACGGGC GAGGTCAAGAAGGTCAAGAAGAAGCTCACCTACCAGTTCAGCGGGGAGGTGCTGGGCCGCGGGGGCCTGGCCGAGCGGCG CCACCCGCCCCACTGA
CEBPA	ATGGAGTCGGCCGACTTCTACGAGGCGGAGCCGCGGCCCCCGATGAGCAGCCACCTCCAGAGCCCCCCACACGCGCCCAG CAGCGCCGCTTTCGGCTTTCCCCGGGGCGCGGGCCCCTCGCAGCCCCCCGCCCCACCTGCCGCCCCGGAGCCTCTGGGCG GCATCTGCGAACACGAGACGTCCATCGACATCAGCGCCTACATCGACCCGGCCGCCTTCAACGACGAGTTCCTGGCCGAC CTGTTCCAACACAGCCGGCAGCAGGAGAAGGCCAAGGCGGCCGCGGCCCCCGCAGGAGGCGGCAACGACTTTGACTACCC GGGCGCCCCCGTGGGCCCCGGCGGCGCCGTCATGCCCGGGGGGACGCACGGTCCCCCTCCTGGCTACGGCTGCGCGGCGG CCGGCTACCTGGACAGCAGGCTGGAGCCTCTGTACGAGCGGGTCGGGGCGCCGGCGCTGCGGCCGCTGGTGATCAAGCAG GAGCCGCGCGAGGAGGACGAAGCGAAGCAGCTGGCGCTGGCCGGCCTCTTTCCCTACCAGCCGCCGCCGCCGCCGCCGCC GCCGCACTCGCACCCGCCGCCCGCTCACCTGGCCGCCCCGCACCTGCAGTTCCAGATCGCACACTGCGGCCAGACCACCA TGCACCTGCAGCCCGGCCACCCCACGCCGCCGCCCACGCCCGTGCCCAGCCCGCACCCAGCGCCCGCGCTCGGCGCCGCT GGCCTGCCAGGCCCCGGCGGCGCGCTCAAGGGTCTGGCCGCCGCGCACCCCGACCTCCGTGCGGGCGGTGGCGGCGGCGG CAAAGCCAAGAAGTCCGTGGACAAGAACAGCAACGAGTACCGGGTGCGGCGCGAGCGCAACAACATCGCGGTGCGCAAGA GCCGGGACAAGGCCAAGCAGCGCAACGTGGAGACGCAGCAGAAGGTGCTGGAGCTGACCAGTGACAATGACCGCCTGCGC AAGCGGGTGGAACAACTGAGTCGCGAACTGGACACGCTGCGGGGCATCTTCCGTCAGCTGCCCGAGAGCTCCCTGGTCAA GGCCATGGGCAACTGC
CEBPB	ATGCAACGCCTGGTGGCCTGGGACCCAGCATGTCTCCCCCTGCCGCCGCCGCCGCCCGCCTTTAAATCCATGGAAGTGGC CAACTTCTACTACGAGGCGGACTGCTTGGCTGCTGCGTACGGCGGCAAGGCGGCCCCCGCGGCGCCCCCGGCGGCCAGAC CCGGGCCGCGCCCCCCCGTCGGCGAGCTGGGTAGCATCGGAGAGCACGAGCGCGCCATCGACTTCAGCCCCTACCTGGAG CCGCTGGGCGCGCCGCAGGCCCCGGCACCCACCACGGCCACGGACACCTTCGAGGCGGCTCCGCCCGCGCCCGCCCCCGC GCCCGCCTCCTCCGGGCAGCACCACGACTTCCTCTCCGACCTCTTCTCCGACGACTACGGGGGCAAGAACTGCAAGAAGG CGGCCGAGTACGGCTACGTGAGCCTGGGCCGCCTGGGGGCCGCCAAGGGAGCGCTGCACCCGGGCTGCTTCGCGCCCCTG CACCCGCCGCCCCCGCCGCCGCCGCCGCCGGCCGAGCTCAAGGCGGAGCCGGGCTTCGAGCCCGCGGACTGCAAGCG GAAGGAGGAGGCCGGAGCTCCAGGCGGCGGCGCCGCGGGCATGGCGGCCGGCTTCCCGTACGCGCTGCGCGCCTACCTCG GCTACCAGGCGGTGCCGAGCGGCAGCAGCGGGAGCCTGTCCACGTCCTCGTCGTCCAGCCCGCCCGGCACGCCGAGCCCC GCCGACGCCAAGGCGACCCCGGCCGCCGCCGCCTGTTACGCGGGGGCGGCGCCGGCGCCCTCGCAGGTCAAGAGCAAGGC CAAGAAGACGGTGGACAAGCACAGCGACGAGTACAAGATCCGGCGGGAGCGCAACAACATCGCGGTGCGCAAGAGCCGCG ACAAGGCCAAGATGCGCAACCTGGAGACGCAGCACAAGGTCCTGGAGCTCACGGCCGAGAACGAGCGGCTCCAGAAGAAG GTGGAGCAACTGTCGCGCGAGCTCAGCACCCTGCGGAACTTGTTCAAGCAGCTGCCCGAGCCTCTGCTCGCCTCCTCCGG CCACTGCTAG

7. Sequences of the LDTF genes used for preparing esiRNAs

Gene	Sequence	Position from TSS	Identity %
PU.1	ATGTTACAGGCGTGCAAAATGGAAGGGTTTCCCCTCGTCCCCCCTCAGCCATCGGAAGA CCTAGTTCCCTATGACACGGATCTCTACCAACGCCAAACGCACGAATATTACCCCTATCT CAGCAGTGATGGAGAAAGCCACAGCGACCATTACTGGGACTTTCATCCGCACCACGTGC ACAGCGAGTTTGAGAGCTTCCCCGAGAACCACTTCACGGAGCTGCAGAGCGTCCAGCCC CCACAGCTGCAGCAGC	1-253 (Exons spanned 1-3)	Cow- 100% Buffalo-98.42%
CEBPA	CCCCGCCCCACCTGCCGCCCCGGAGCCTCTGGGCGGCATCTGCGAACACGAGACGTCCA TCGACATCAGCGCCTACATCGACCCGGCCGCCTTCAACGACGAGTTCCTGGCCGACCTG TTCCAACACAGCCGGCAGCAGGAGAAGGCCAAGGCGGCCGCGGCCCCCGCAGGAGGCG GCAACGACTTTGACTACCCGGGCGCCCCCGTGGGCCCCGGCGGCGCCGTCATGCCCGGG GGGACGCACGGTCCCCCTCCTGGCTACGGCTGCGCGGCAGCCGGCTACCTGGACAGCAG GCTGGAGCCTCTGTACGAGCGGG	126-442 (Intronless gene)	Cow- 100% Buffalo-99.68%
CEBPB	CGCCCGCCTTTAAATCCATGGAAGTGGCCAACTTCTACTACGAGGCGGACTGCTTGGCT GCTGCGTACGGCGGAAAGGCGGCCCCCGCGGCGCCCCCGGCGGACAGACCCGGGCCGC GCCCCCCCACCGGCGAGCTGGGTAGCATCGGAGAGCACGAGCGCGCCATCGACTTCAGC CCCTACCTGGAGCCGCTGGGCGCGCCGCAGGCCCCGGCACCCACCACGGCCTCGGACAC CTTCGAGGCGGCTCCGTCCGCCGCCCGCCCCCGTGCCCGCCTCCTCCGGGCAGCACCACG ACTTCCTCTCCGACCTCTTCTCC	53-369 (Intronless gene)	Cow- 100% Buffalo-97.79%

ANNEXURE-IV

8. The p-values for One-way ANOVA test conducted on the qPCR experiments comparing the RLR pathway genes expression between GFP esiRNA and CEBPA esiRNA transfected BoMAC (refer Figure 4.2A of Chapter 4)

Gene	p-value
RIG-I	<0.0001
MDA5	<0.0001
LGP2	0.0016
MAVS	0.3965
IFI16-l	0.0073
cGAS	0.0025
STING	0.3885
DAI	0.0001
IRF3	0.6215
IRF7	0.0008
IFN-β	0.4539
ISG54	<0.0001
ISG56	<0.0001
OAS	0.4187
PKR	0.6233

9. The p-values (<0.05 for significance) for One-way ANOVA test conducted on the qPCR experiments comparing the RLR pathway genes expression after poly(I:C) stimulation between GFP esiRNA and CEBPA esiRNA transfected BoMAC (refer Figure 4.2B of Chapter 4)

Gene	p-value
RIG-I	0.0061
MDA5	<0.0001
LGP2	0.0026
MAVS	0.3965
IFI16-l	0.0169
cGAS	0.0001
STING	0.2175
DAI	0.0007
IRF3	0.0060

IRF7	0.0005
IFN-β	0.0001
ISG54	<0.0001
ISG56	<0.0001
OAS	0.1587
PKR	0.0039

10. The p-values for One-way ANOVA test conducted on the qPCR experiments comparing the RLR pathway genes expression (taking empty vector backbone transfection as control) in buffalo fetal fibroblasts expressing different LDTFs (refer Figure 4.10A of Chapter 4)

Gene	p-values (<0.05 for significance)				
	CEBPB	**CEBPA**	**PU.1**	**PU.1-I-CEBPA**	**PU.1-I-CEBPB**
RIG-I	0.3997	0.0385	<0.0001	<0.0001	<0.0001
MDA5	0.1598	0.0442	0.0023	<0.0001	<0.0001
LGP2	0.2783	0.0436	0.0036	<0.0001	<0.0001
MAVS	0.2953	0.0695	0.0552	0.0473	0.0434
IFI16-l	0.1017	0.0269	<0.0001	<0.0001	<0.0001
cGAS	0.3468	0.0156	<0.0001	<0.0001	<0.0001
IRF3	0.1784	0.1054	0.0498	0.0455	0.0472
IRF7	0.0517	0.0118	<0.0001	<0.0001	<0.0001
IFN-β	0.2384	0.1798	0.0519	0.0491	0.0488

11. The p-values for One-way ANOVA test conducted on the qPCR experiments comparing the RLR pathway genes expression (taking empty vector backbone transfection as control) in buffalo endometrial epithelial cells expressing different LDTFs (refer Figure 4.10B of Chapter 4)

Gene	p-values (<0.05 for significance)				
	CEBPB	**CEBPA**	**PU.1**	**PU.1-I-CEBPA**	**PU.1-I-CEBPB**
RIG-I	0.0197	0.0015	<0.0001	<0.0001	<0.0001
MDA5	0.0371	0.0027	<0.0001	<0.0001	<0.0001
LGP2	0.1054	0.0463	0.0276	0.0019	0.0011
MAVS	0.1784	0.1021	0.0591	0.0482	0.0573
IFI16-l	0.2187	0.0377	0.0039	0.0020	0.0031
cGAS	0.0756	0.0213	<0.0001	<0.0001	<0.0001
IRF3	0.2998	0.1896	0.0786	0.0715	0.0669
IRF7	0.0423	0.0265	<0.0001	<0.0001	<0.0001
IFN-β	0.2105	0.1721	0.0831	0.0675	0.0570

12. The p-values for One-way ANOVA test conducted on the qPCR experiments comparing the RLR pathway genes expression (taking empty vector backbone transfection as control) in buffalo fetal fibroblasts expressing different LDTFs followed by poly(I:C) treatment (refer Figure 4.11A of Chapter 4)

Gene	p-values (<0.05 for significance)				
	CEBPB	**CEBPA**	**PU.1**	**PU.1-I-CEBPA**	**PU.1-I-CEBPB**
RIG-I	0.0537	0.0197	<0.0001	<0.0001	<0.0001
MDA5	0.0349	0.0011	<0.0001	<0.0001	<0.0001
LGP2	0.0879	0.0646	0.0183	0.0219	0.0095
MAVS	0.0793	0.0621	0.0591	0.0482	0.0573
IFI16-l	0.0556	0.0472	0.0056	<0.0001	<0.0001
cGAS	0.0412	0.0194	<0.0001	<0.0001	<0.0001
IRF3	0.0813	0.0792	0.0628	0.0514	0.0537
IRF7	0.0497	0.0313	<0.0001	<0.0001	<0.0001
IFN-β	0.0458	0.0251	<0.0001	<0.0001	<0.0001
ISG54	0.0347	0.0104	<0.0001	<0.0001	<0.0001
ISG56	0.0293	0.0095	<0.0001	<0.0001	<0.0001
PKR	0.0876	0.0801	0.0695	0.0671	0.0587

13. The p-values for One-way ANOVA test conducted on the qPCR experiments comparing the RLR pathway genes expression (taking empty vector backbone transfection as control) in buffalo fetal fibroblasts in buffalo endometrial epithelial cells expressing different LDTFs followed by poly(I:C) treatment (refer Figure 4.11A of Chapter 4)

Gene	p-values (<0.05 for significance)				
	CEBPB	**CEBPA**	**PU.1**	**PU.1-I-CEBPA**	**PU.1-I-CEBPB**
RIG-I	0.0201	0.0103	<0.0001	<0.0001	<0.0001
MDA5	0.0391	0.0196	<0.0001	<0.0001	<0.0001
LGP2	0.0423	0.0312	0.0183	0.0219	0.0095
MAVS	0.0886	0.0723	0.0617	0.0577	0.0501
IFI16-l	0.0605	0.0096	0.0059	<0.0001	<0.0001
cGAS	0.0265	<0.0001	<0.0001	<0.0001	<0.0001
IRF3	0.0913	0.0561	0.0497	0.0381	0.0467
IRF7	0.0554	0.0439	<0.0001	<0.0001	<0.0001
IFN-β	0.0227	0.0184	<0.0001	<0.0001	<0.0001