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Attenuated Responses To Inflammatory Cytokines In Mouse Embryonic Stem Cells: Biological Implications And The Molecular Basis

Bohan Chen
University of Southern Mississippi

ATTENUATED RESPONSES TO INFLAMMATORY CYTOKINES IN MOUSE EMBRYONIC STEM CELLS: BIOLOGICAL IMPLICATIONS AND THE MOLECULAR BASIS

by

Bohan Chen

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Approved by:

Dr. Yan-Lin Guo, Committee Chair
Dr. Alex Flynt
Dr. Fengwei Bai

Dr. Yan-Lin Guo Committee Chair	Dr. Jake Schaefer Director of School	Dr. Karen S. Coats Dean of the Graduate School

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ABSTRACT

Embryonic stem cells (ESCs) have attracted intense interest due to their great potential for regenerative medicine. However, their immune property is an overlooked but a significant issue that needs to be thoroughly investigated not only to resolve the concern for therapeutic applications but also for further understanding the early stage of organismal development. Recent studies demonstrated that ESCs are deficient in innate immune responses to viral/bacterial infections and inflammatory cytokines. Inflammatory conditions generally inhibit cell proliferation, which could be detrimental to ESCs, since cell proliferation is their dedicated task during early embryogenesis. Thus, I hypothesize that the attenuated innate immunity in ESCs could allow them to evade the cytotoxicity caused by immune reactions and is, therefore, a self-protective mechanism during early embryogenesis. We have differentiated mouse ESCs (mESCs) to fibroblast-like cells (mESC-FBs) which were proved to have partially developed innate immunity. Using these cells as a model for comparison with mESCs, the insensitivity of mESCs to the cytotoxic effects from IFNγ, which is an inflammatory cytokine highly presented during early embryogenesis, and other inflammatory conditions were demonstrated, including attenuated expressions of inflammatory and signaling molecules, inactivated transcription factor and unaffected cell viability. Furthermore, basal expressions of protein phosphatases that inhibit IFNγ pathway were higher in mESCs than mESC-FBs. Treating mESCs with protein phosphatases inhibitor upregulated the expression of IFNγ induced signaling molecule. In all, the attenuated inflammatory responses are beneficial for mESCs, and the inhibition effects from protein phosphatases could, at least, partially explain their attenuated responses to IFNγ.

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DEDICATION

I am grateful to my grandparents, uncles, aunts, and cousins, who have always been supportive and believing in me. Deepest gratitude goes to my parents, whose unwavering love, encouragement and support have always been unconditional in whatever I pursue.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

LIST OF ABBREVIATIONS

ESC	Embryonic Stem Cell
ESC-FB	ESC Derived Fibroblast-Like Cells
PRR	Pattern Recognition Receptor
PAMP	Pathogen Associated Molecular Pattern
IFN	Interferon
TNFα	Tumor Necrosis Factor Alpha
iNOS	Inducible Nitric Oxide Synthase
TLR	Toll-Like Receptor
NFκB	Nuclear Factor Kappa Light Chain Enhancer of Activated B Cells
dsRNA	Double-Stranded Ribonucleic Acid
IKK	Inhibitor of NFκB Kinase
ISG	Interferon Stimulated Gene
PTP	Protein Tyrosine Phosphatase
DSP	Dual Specificity Phosphatase
MKP	Mitogen-Activated Protein Kinase Phosphatase
Vn	Vanadate
CM	Conditioned Medium
LPS	Lipopolysaccharide
IRF	Interferon Regulatory Factor
SOCS	Suppressor of Cytokine Signaling

siRNA	Small Interfering Ribonucleic Acid
FBS	Fetal Bovine Serum
LIF	Leukemia Inhibitory Factor
RA	Retinoic Acid
TB	Toluidine Blue
PI	Propidium Iodide
STAT	Signaling Transducer and Activator of Transcription
P-STAT	Phosphorylated STAT
JAK	Janus Kinase

CHAPTER I - INTRODUCTION

Overview of Innate Immunity

The immune system in vertebrates is composed of two major components, innate and adaptive immunity. The innate immune system is a primitive defense system found in all multicellular organisms, and it is known as the first line of defense against the invasion of different pathogens in an immediate and non-specific manner (Kawai & Akira, 2011; Medzhitov & Janeway, 2000). In contrast, the adaptive immune system is pathogen-specific and has only been found in vertebrates that can utilize specialized immune cells, including T cells and B cells (Hoffmann, Kafatos, Janeway, & Ezekowitz, 1999; Kumar, Kawai, & Akira, 2009a).

The innate immune system is a complex network in which different defending mechanisms are involved through various signaling pathways. It has been well-established that innate immune responses play vital roles in eliminating different pathogenic invasions. Antibacterial, antiviral, and inflammatory responses are the primary mechanisms responsible for cellular innate immunity (Medzhitov & Janeway, 2000), which is mainly mediated by large numbers of specific receptors on the surface of cells or in the cytosol that are known as the pattern recognition receptors (PRRs). In the meantime, different types of pathogens were suggested to have different conserved motifs termed pathogen-associated molecular patterns (PAMPs) (Janeway, 1989; Medzhitov, 2009; Mogensen, 2009). When the pathogen invasion occurs, their PAMPs can be detected by the specific PRRs, and thus initiate the innate immune recognition. These PRRs then transduce the signals and activate downstream signaling pathway through the activation of different transcription factors, initiating the activation of a network of innate

immune responses that fight off different pathogens and secret different cytokines, chemokines, and other immunomodulators to alert other tissue cells as well as recruiting innate immune cells such as dendritic cells, natural killer (NK) cells, and macrophages (Akira, Uematsu, & Takeuchi, 2006; Hayden, West, & Ghosh, 2006).

PRRs can be divided into different subtypes in terms of their different functions and recognition of various pathogens. For example, the retinoic acid-inducible gene-1 (RIG-1) receptors (RLRs) and the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are both cytosolic and can only respond to intracellular pathogens (Creagh & O'Neill, 2006). RLRs can be activated by double-stranded RNA (dsRNA) derived from different viral pathogens (Schlee, 2013) while NLRs respond to bacterial peptidoglycan (Kanneganti, Lamkanfi, & Núñez, 2007). The toll-like receptors (TLRs) family are a major group of PRRs, and ten different TLRs have been identified so far expressing on the cell surface or in the endosomes in humans and mice (designated as TLR1 through 10) (Beutler, 2009; Kawai & Akira, 2011). For example, TLR3 is activated by dsRNA generated during viral replication (Alexopoulou, Holt, Medzhitov, & Flavell, 2001) and a synthetic dsRNA analog, the polyinosinic-polycytidylic acid (poly I:C), while TLR4 detects lipopolysaccharide (LPS), a major component of gram-negative bacterial cell membranes (Medzhitov & Janeway, 1997). Another TLR named TLR11 has only been found playing an essential role in mice against parasite and bacterium (Lauw, Caffrey, & Golenbock, 2005).

Different PRRs bind to their specific ligands and transduce signals into the cytoplasm, where the downstream transcription factors such as interferon regulatory factor (IRF) family and nuclear factor-κB (NF-κB) family is activated. Those

transcription factors are considered as the master switch of the innate immune responses and can lead to the production of interferons, inflammatory cytokines, chemokines, cell cycle regulators and other immunomodulators involved in varieties of immune and inflammatory responses (Hayden et al., 2006; Kato, Forero, Fenton, & Hidalgo, 2011; Kawai & Akira, 2011).

Embryonic Stem Cells

The mammal body is composed of trillions of cells, and every cell is descended from the inner cell mass (ICM) in the early stage embryo, called ESCs. The early stage embryo, which is developed from a fertilized egg, is named blastocyst. It consists of an outer layer of trophoblasts, called trophectoderm, which will eventually form the placenta and the ICM that will develop into the embryo (Irie, Tang, & Azim Surani, 2014).

ESCs have two distinctive characteristics from somatic cells: 1) self-renewal: the unlimited ability of self-replication in vitro, which means they can proliferate indefinitely while maintaining stem cell properties under the proper growth condition; 2) pluripotency: the capability to differentiate into any specialized cell types of the three primary germ layers (endoderm, mesoderm, and ectoderm) (Brook & Gardner, 1997; Wobus & Boheler, 2005). These properties make in vitro cultured ESCs a great cell source for regenerative medicine and tissue transplantation therapy as well as a great model to study reproductive and stem cell biology (L. Chen & Daley, 2008). The derivation and differentiation of ESCs are illustrated in Fig. 1.

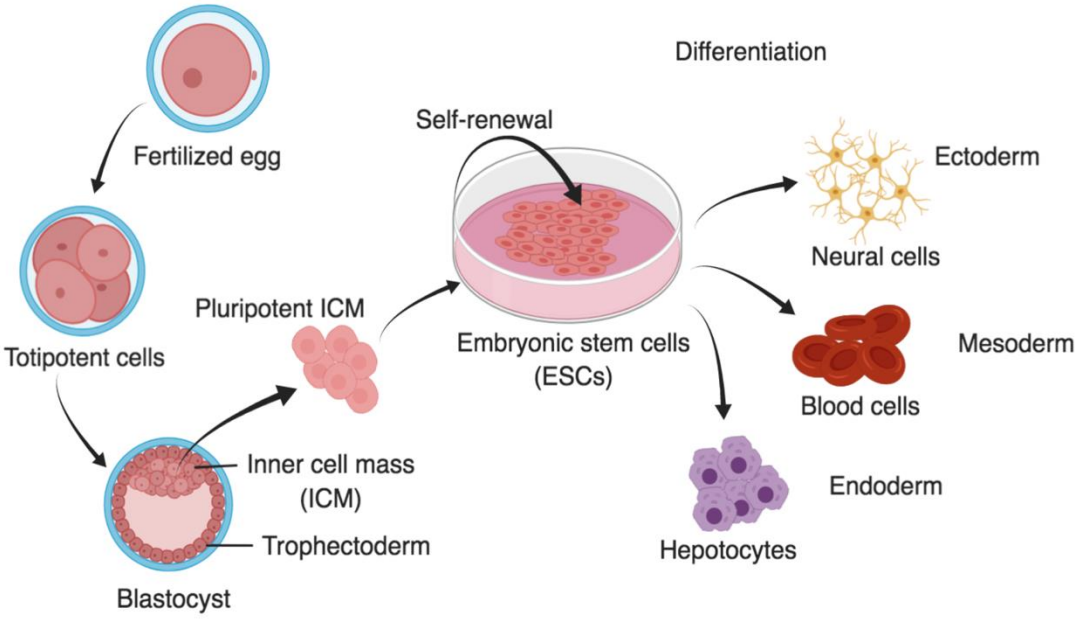

Figure 1. Derivation and differentiation of ESCs.

The blastocyst, which is developed from the fertilized egg, is comprised of an outer layer of trophoblasts, called trophectoderm and ICM. ICM can be isolated and cultured in vitro to yield a population of ESCs, which have the unlimited ability of self-renewal and are capable of differentiating into all the cell types from each of the three germ layers.

The ability of self-renewal and pluripotency of ESCs are maintained by several pluripotent markers, which are a group of transcription factors, including Nanog, Oct4, Sox2, and other signaling modulators. Under the continuous presence and expression of those molecules, the development and differentiation-promoting genes in ESCs are negatively regulated (Pinney & Emerson, 1989; Smith, 2001). Recent research indicates that the characteristics of ESCs are also governed by a group of ESC specific micro RNAs, such as miR-290 cluster (L. Chen & Daley, 2008; Lüningschrör, Stöcker, Kaltschmidt, & Kaltschmidt, 2012; Tiscornia & Izpisua Belmonte, 2010). Therefore, the unique properties of ESCs are maintained by the network of different molecules at both mRNA and protein level.

Innate Immunity in Embryonic Stem Cells

ESCs have been considered as the most promising source for the application of regenerative medicine. For the last two decades, researchers have concentrated on studying the differentiation from ESCs to desirable cell types as well as optimizing conditions for specific cell differentiation. However, less attention has been paid to the immune property of ESCs and their differentiated cells, although this is a critical issue that will undoubtedly affect the fate and the functionality of the transplanted cells or tissues when used in regenerative medicine (Guo et al., 2015). Recent studies have revealed that unlike somatic cells, where innate immunity has been well developed (Kumar, Kawai, & Akira, 2009b; Sen, 2001), ESCs from both humans (L.-L. Chen, Yang, & Carmichael, 2010; Földes et al., 2010) and mice (D'Angelo et al., 2017; Wang et al., 2013; Yu, Rossi, Hale, Goulding, & Dougan, 2009) lack or have attenuated innate immune responses under the exposure of various infectious agents such as viruses, bacteria, and inflammatory cytokines. Induced pluripotent stem cells (iPSCs) are also reported to have the similar underdeveloped innate immune properties (G.-Y. Chen et al., 2012; Hong & Carmichael, 2013), indicating that the attenuated innate immune system is intrinsic properties in pluripotent cells. However, the biological implications of the underdeveloped innate immunity in ESCs are still unclear, and the molecular mechanisms remain to be further elucidated.

While the modulation of the innate immunity involves complex networks, some findings from different investigators could partially explain the molecular mechanisms behind the attenuated innate immunity in ESCs. The studies from our research group have shown that the expression level of receptors for viral dsRNA, bacterial endotoxin,

and several inflammatory cytokines are substantially lower in ESCs (Guo et al., 2015), and some receptors are not even functional in protein level (D'Angelo et al., 2017; Zampetaki, Xiao, Zeng, Hu, & Xu, 2006). Furthermore, transcription factor NF-κB (a family of proteins including p65/RelA, RelB, c-Rel, p50 and p52), which has been known as a master regulator of innate immune responses as mentioned previously, was not activated in ESCs under the presence of different infectious agents (D'Angelo et al., 2016, 2017). One of the key ESC markers Nanog was reported to inhibit the transcriptional activity of NF-κB by specifically binding to NF-κB protein (Torres & Watt, 2008). A group of ESC specific micro RNAs was also reported to inhibit the continuous p65/RelA activation by silencing its mRNA transcription (Lüningschrör et al., 2012). Taken together, ESCs have intrinsic attenuated innate immune property, which could be important and necessary for maintaining their self-renewal and pluripotent capability. This study is intended to provide experimental evidence to support this hypothesis.

Inflammatory Responses

Inflammatory responses usually occur during the infections caused by the invasion of different pathogens or around the wounded areas in the body. Some non-infectious diseases such as cancer, diabetes, and arthritis could also induce inflammation. Thus, the inflammatory responses are considered a series of self-healing reactions to restore the tissue inner environment to homeostasis (Medzhitov, 2010). The inflammatory responses are mainly regulated by a group of innate immune cells, which are also called tissue-resident sentinel cells, such as macrophages, mast cells, NK cells, and dendritic cells. The acute inflammation is initiated by those cells either through the direct sensation

of different PAMPs or by the chemical factors that secreted from other infected cells through a paracrine manner. Then, those innate immune cells can further produce different types of inflammatory cytokines, chemokines, and free radicals that limit the spread of infections and activate adaptive immune system (Conner & Grisham, 1996). It should be noted that while the inflammatory responses can defend the organism against the invading pathogens, they can also have detrimental effects on tissue cells such as cell proliferating inhibition, cell damage, and apoptosis (Hertzog, Hwang, & Kola, 1994; Kotredes & Gamero, 2013; Sedger & McDermott, 2014). Thus, the inflammation response is considered to be a double-edged sword.

TNFα and IFNγ Signaling and Their Roles in Inflammatory Responses

Among the various inflammatory cytokines, TNFα and IFNγ are particularly known for causing cytotoxicity and inflammation. TNFα is one of the most potent pro-inflammatory cytokines produced by activated innate immune cells such as macrophages and NK cells during the acute inflammatory reaction as well as non-immune cells such as fibroblasts and endothelial cells (Falvo, Tsytsykova, & Goldfeld, 2010). As initially characterized, TNFα can signal apoptosis but mainly in tumor cells, virus-infected cells or cells under cell cycle arrest. In those scenarios, the extracellular TNFα binds to TNFR and induce the release of silencer of death domain (SODD) protein, resulting the recruitment of death-inducing signaling complex (DISC) proteins, which include TNFR-associated death domain (TRADD), TNFR-associated factor (TRAF), Fas-associated death domain (FADD) and receptor interacting protein (RIP). Upon the recruitment of DISC proteins, they can further recruit procaspase-8 and procaspase-3 that will subsequently release the activated caspase-8 and caspase-3, inducing the caspase-

activated DNase (CAD), which can degrade genomic DNA, cause DNA fragmentation and induce caspase-dependent cell death. While in normal tissue cells, TNFα does not usually cause apoptosis. Instead, it can activate the global trans-activator NF-κB and other non-apoptotic signaling pathways such as mitogen-activated protein kinase (MAPK) and cJun N-terminal kinase (JNK) and induce a panel of chemokines and inflammatory mediators, which will further monitor the immune microenvironment (Horssen, Hagen, & Eggermont, 2006; Sedger & McDermott, 2014). NF-κB signaling activation, in particular, is the major event of TNFα induction and plays significant roles in regulating cell survival and inflammatory responses. Upon binding of TNFα ligand with its receptor TNFR, a complex of inhibitor of NF-κB (IκB) kinase (IKK), which comprises IKKα, IKKβ, and Nemo/IKKγ, is activated and can subsequently phosphorylate and degrade IκB. IκB binds to NF-κB and inhibits its translocation to the nucleus in inactivated cells. With the release from IκB, NF-κB p50 and p65/Rel can translocate to the nucleus and bind to their binding sites on the target gene promoters, inducing the transcription of various genes involved in immune and inflammatory responses such as interferons, different chemokines, and cell adhesion molecules. (Mak & Yeh, 2002). TNFα signaling regulation is schematically illustrated in Fig. 2. It is noted that TNFα can induce and has synergistic effects with interferons, and interferons can, in turn, upregulate the expression levels of TNFR, which could partially explain their synergy (Sedger & McDermott, 2014).

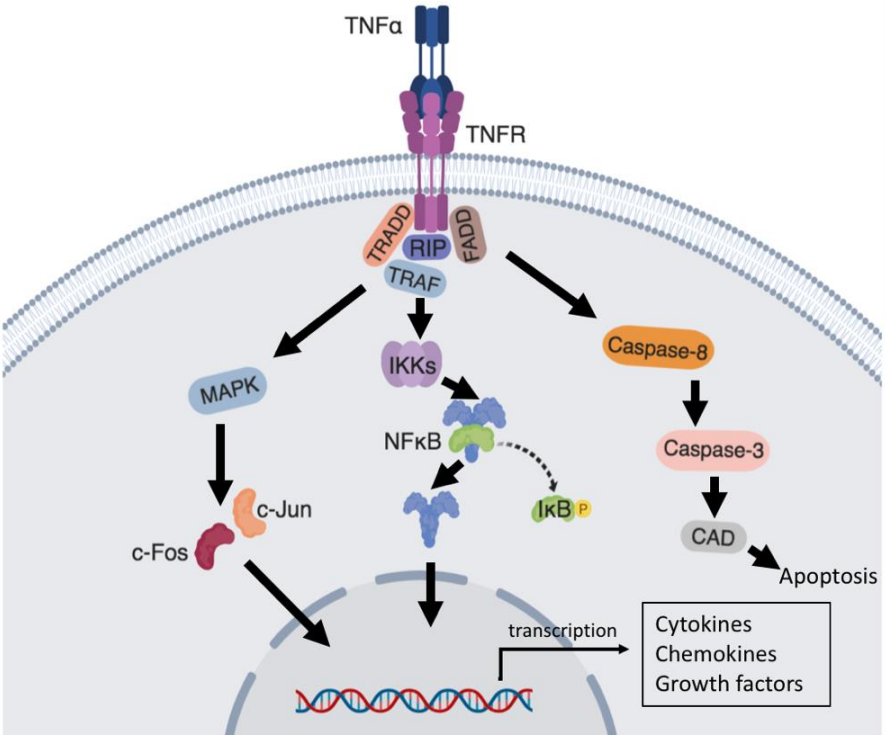

Figure 2. Schematic illustration of TNFα signaling pathway.

TNFα regulates both apoptosis and survival pathways. The apoptosis signaling induced by TNFα is caspase-dependent and only occur when there is an aberration within the cells. In normal tissue cells, TNF can induce non-death signaling pathway via the activation of transcription factors NF-κB and cFos/cJun. NF-κB is an important global trans-activator. During its activation, the IKK complex is initially activated through binding of TNFα with their receptors. Activated IKK complex then induces subsequent ubiquitination and degradation of IκB, which enable NF-κB proteins to translocate to the nucleus and induce transcription of various inflammatory mediators such as inflammatory cytokines, chemokines, and genes that regulate cell proliferation.

IFNγ is the only member of the type II class interferon and is one of the most commonly found inflammatory cytokines at the site of pathogen infections (Boehm, Klamp, Groot, & Howard, 1997; Stark, Kerr, Williams, Silverman, & Schreiber, 1998) and in the placenta during the early stage of fetal development in mammals (Ashkar, Di Santo, & Croy, 2000; Platt & Hunt, 1998). It is mainly produced by T lymphocytes and NK cells, and involves in multiple processes of innate and adaptive immune responses via autocrine and paracrine manner, including antigen presentation, inhibition of cell

proliferation, and cell apoptosis (Schroder, Hertzog, Ravasi, & Hume, 2004). The signal transduction of IFNγ is regulated by the JAK-STAT pathway (Fig. 3). Binding of IFNγ to its receptors on the cell surface, which are composed of IFNγR1 and IFNγR2, leads to the activation of the receptor-associated tyrosine kinase JAK1 and JAK2. The activated JAKs induce the phosphorylation of the tyrosine residue at the IFNγR1 intracellular domain. STAT1 are then subsequently recruited to the intracellular portion of the IFNγR complex and get phosphorylated on the tyrosine residue (Tyr) 701 by the JAKs. Tyrosine phosphorylation of STAT1 leads to their homodimerization and translocation to the nucleus. By binding at the IFNγ-activating sequence (GAS) in the nucleus, phosphorylated STAT1 can activate the transcription of different primary response genes that contain the GAS within their promoters, one of which is the gene encoding IRF1 (Boehm et al., 1997; Darnell, 1997; Platanias, 2005). The elevated amount of IRF1 can bind to interferon-stimulated response element (ISRE), which is another specific nucleotide sequence, inducing the transcription of secondary response genes (Kröger, Köster, Schroeder, Hauser, & Mueller, 2002). Excessive and uncontrolled IFNγ responses could cause deleterious effects on tissue cells, but this can be limited by negative regulators including suppressor of cytokine signaling 1 (SOCS1), protein tyrosine phosphatase (PTP), dual specificity phosphatase (DSP), and protein inhibitor of activated STAT (PIAS) (Greenhalgh & Hilton, 2001; Shuai & Liu, 2003; T. R. Wu et al., 2002).

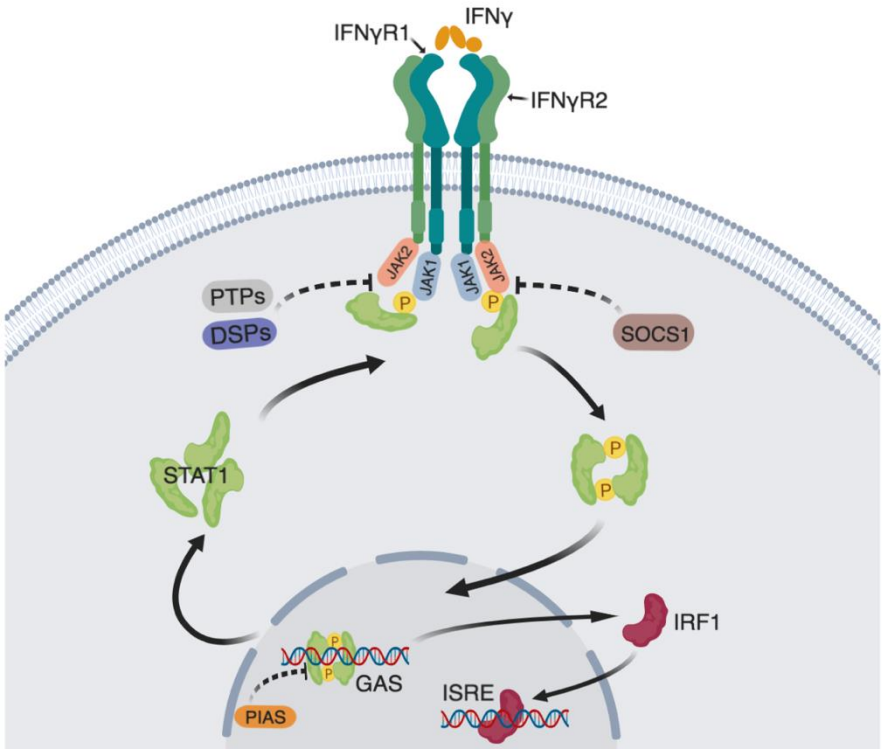

Figure 3. Schematic illustration of IFNγ signaling pathway.

Binding of IFNγ to the extracellular domain of the IFNγR complex leads to the phosphorylation of JAKs and the intracellular domain of the receptors. STAT1 are then subsequently recruited to the receptors and are phosphorylated, which leads the homodimerization and nucleus translocation of p-STAT1. Inside the nucleus, p-STAT1 homodimers bind to GAS and induce the primary transcription of different genes, including IRF1. By binding to ISRE, IRF1 can further activate the transcription of secondary response genes.

Transcription factors act like "switches" to different inflammation signaling pathways (Oeckinghaus & Ghosh, 2009). Our previous studies have demonstrated that NF-κB, a transcription factor responsible for TNFα responses, could not be activated in mESCs (D'Angelo et al., 2017). It was reported by other researchers that Nanog, a critical pluripotent marker of ESCs (Torres & Watt, 2008), and a group of ESC-specific miRNA (Lüningschrör et al., 2012) are inhibiting the transcriptional activity of NF-κB in mESCs. These results provide possible explanations for the lack of TNFα responses in

mESCs. However, the reason behind the lack of responses to IFNγ in mESCs is still not clear.

Innate Immune Responses During Early Embryogenesis

As an evolutionarily conservative defense system in vertebrates, the innate immune responses have been known to be the prominent events through the fetal development in the uterus (Warning, McCracken, & Morris, 2011; Wira, Fahey, Sentman, Pioli, & Shen, 2005). There are elevated amounts of uterine immune cells (e.g., NK cells) and inflammatory molecules especially before and during the process of implantation (Cram, Zapata, Toy, & Baker, 2002; Lamont, 2003; PrabhuDas et al., 2015). The abnormal change of the levels of different cytokines and chemokines, which play pivotal roles in balancing the microenvironment in the uterus, is one of the main factors that cause failed pregnancy and recurrent miscarriages (Mor, Cardenas, Abrahams, & Guller, 2011; Murphy, Thompson, & Belov, 2009; Sykes et al., 2012). Therefore, the precise regulation of the innate immune responses in the cells during the early embryonic stages is crucial for successful embryo and fetus development. Recent studies have revealed that ESCs, as the progenitors of all the tissue cells, have attenuated innate immune responses to a wide range of inflammatory cytokines (Burke, Graham, & Lehman, 1978; D'Angelo et al., 2017; Hong & Carmichael, 2013; Wang et al., 2014). IFNs and other inflammatory cytokines are well-known to have anti-proliferation effects and can induce cell apoptosis as mentioned previously. While ESCs are only transiently existed in limited numbers in the blastocyst, the damage or loss of ESCs could cause severe developmental deficiencies (Naeye & Blanc, 1965). For this reason, the attenuated responses to inflammatory cytokines could be beneficial for ESCs and the early embryo

to evade the detrimental effects caused by inflammatory responses, which is the major hypothesis of this study.

CHAPTER II - HYPOTHESIS AND SIGNIFICANCE

Based on the unlimited ability of self-replication and the potential to be differentiated into any specialized cell type in the body, ESCs and their derived cells have been considered as one of the most promising sources for applications in tissue transplantation and regenerative medicine (Keller, 2005; Soria et al., 2000; Wobus & Boheler, 2005). While researchers have been concentrating on optimizing in vitro differentiation conditions from ESCs to various desirable cell types for therapeutic application, innate immune response, which is the first line of defense against the invasion of different pathogens and have been widely studied in somatic cells, are often overlooked on ESCs. Recent studies from our group and other investigators demonstrated that ESCs from both mouse and human intrinsically lack or have underdeveloped innate immune responses. They exhibit little or no responses to varieties of infections, including viruses (Wang et al., 2013), bacteria (Földes et al., 2010), and various inflammatory cytokines (D'Angelo et al., 2017; Kim et al., 2008). Although it has been proven that the innate immunity is being developed during ESC differentiation process, it is still substantially underdeveloped in in vitro differentiated ESCs compared with their naturally differentiated counterparts (Guo et al., 2015). Understanding the innate immune system in ESCs becomes much more important especially for this special group of cells to be used for clinical treatment. While these properties of ESCs not only raise concerns for their therapeutic use, they also represent fundamental questions in stem cell and developmental biology that need to be reevaluated.

In this study, I further demonstrated the effects of various inflammatory conditions on mouse ESCs, including the cytotoxicity, induction of the inflammatory

genes, and the activation of transcription factors. The potential molecular mechanisms that account, at least in part, for the attenuated innate immune responses in ESCs were also demonstrated. The immunological properties of ESCs described in this study will help us assess their clinical application as well as understand the fundamental biological questions at the early stage of the development.

CHAPTER III - MATERIALS AND METHODS

Cell Culture and mESC Differentiation

Two commonly used mESCs cell lines: D3 and DBA252 mESCs were cultured in the standard mESC medium as described before (Wang et al., 2013). mESC-FBs were differentiated from D3 and DBA252 mESCs through a retinoic acid (RA) induced differentiation protocol and purified by reseeding them to an uncoated dish, to which they will quickly adhere (Wang et al., 2014). The medium was changed to remove the unattached cells after 10 min of seeding. Since both D3 and DBA252 cells and their differentiated cells (D3 or DBA252 mESC-FBs) share similar properties as characterized from previous studies (Wang et al., 2013), the experiments in this study were mainly performed with D3 mESCs and their differentiated mESC-FBs. mESC-FBs between passage 10-35 were used for this study. Some key experiments were confirmed with DBA252 mESCs. RAW264.7 cells (a murine macrophage cell line) were obtained from ATCC. mESC-FBs and RAW264.7 were cultured in 10% fetal bovine serum (FBS) DMEM with 100 units/ml penicillin and 100μg/ml streptomycin. In some specific experiments, medium was changed to 2% FBS DMEM before the treatments were added to maximize the responses of cells. Different conditions will be described for individual experiments. All cells were maintained at 37°C in a humidified incubator with 5% CO_2.

Preparation of Conditioned Medium, Heat Killed Bacteria, and Cell Treatment

RAW264.7 cells (50-70% confluence) were treated with LPS (1μg/ml, isolated from *E. coli* O111: B4, Sigma) for 4 h. Then the medium was removed, and cells were thoroughly washed twice with PBS. Fresh medium was added to culture cells for an additional 24 h. The CM was collected and designated as LPS CM. CM prepared from

RAW264.7 cells that without any treatment was used as control (Con CM). Heat killed *E. coli* (O157: H7, ATCC) (HKE) were prepared by heating bacteria at 80 ^{o}C for one hour (Koziel et al., 2009). mESCs and mESC-FBs were treated with CM (1:1 diluted with 10%FBS DMEM), HKE (bacterial:mESCs or mESC-FBs at a ratio of 200:1), TNFα, or INFγ (20ng/ml, Peprotech. Supplement of IFNγ were added at 5ng/ml) under the condition as described in each individual experiment.

Quantitative Analysis of Cell Viability

Cells were fixed with cold methanol for 10 min at room temperature, followed by staining with 1% toluidine blue (TB, Sigma) in dH_2O for 30 min. Cells were then rinsed with tap water to remove excess TB, and 2% SDS (Sigma) was added to extract the staining. Optical density at 630 nm, which correlates with the number of cells, was measured with a BioTek ELx800 microplate reader.

RT-qPCR

Cells were collected using TRI-reagent (Sigma), followed by phenol-chloroform extraction of the whole RNA. Total RNA concentration in each sample was determined by a Thermo Genesys 10 Bio UV-Vis spectrophotometer. 1μg RNA from each sample was used for reverse transcription to generate cDNA with Moloney murine leukemia virus (MML-V) reverse transcriptase (Promega). RT-qPCR was performed using SYBR green supermix (Bio-Rad) on a Stratagene Mx3000P real-time PCR system with gene-specific primers. β-actin was used as a calibrator to normalize different genes for comparison. The expression levels of mRNA were either normalized with controls (designated as 1) and expressed as fold change activation, or normalized with β-actin

(designated as 1) and expressed as relative levels. The sequences of the primer sets utilized for RT-qPCR are listed in Table 1.

Table 1

Primer sequences of mouse genes for RT-qPCR analysis

Gene	Sequence (forward)	Sequence (reverse)
β-actin	CATGTACGTAGCCATCCAGGC	CTCTTTGATGTCACGCACGAT
iNOS	CAGCACAGGAAATGTTTCAGC	TAGCCAGCGTACCGGATGA
Cox2	TGAGCAACTATTCCAAACCAGC	GCACGTAGTCTTCGATCACTATC
STAT1	GCTGCCTATGATGTCTCGTTT	TGCTTTTCCGTATGTTGTGCT
IRF-1	ATGCCAATCACTCGAATGCG	TTGTATCGGCCTGTGTGAATG
SOCS1	CTGCGGCTTCTATTGGGGAC	AAAAGGCAGTCGAAGGTCTCG
IFNγR1	CTGGCAGGATGATTCTGCTGG	GCATACGACAGGGTTCAAGTTAT
IFNγR2	TCCTCGCCAGACTCGTTTTC	GTCTTGGGTCATTGCTGGAAG
JAK1	ACGCTCCGAACCGAATCATC	GTGCCAGTTGGTAAAGTAGAACC
JAK2	TTGTGGTATTACGCCTGTGTATC	ATGCCTGGTTGACTCGTCTAT
MKP1	ATGCAGCTCCTGTAGTACCC	ATATCCTTCCGAGAAGCGTGA
PTPN2	GCAGTGAGAGCATTCTACGGA	TGACACAAACCCCATCTTAGTGA
SHP-1	GGACTTCTATGACCTGTACGGA	CGAGCAGTTCAGTGGGTACTT
SHP-2	AGAGGGAAGAGCAAATGTGTCA	CTGTGTTTCCTTGTCCGACCT

Flow Cytometry

For cell cycle analysis, cells were collected and fixed with 80% ethanol for 1 h, then stained with 50 μg/ml propidium iodide (PI). For cellular protein analysis, either control cells or treated cells were collected and fixed with 3% paraformaldehyde. The cells were incubated with antibodies against specific proteins, including antibodies for iNOS (sc-7271), IFNγR1 (sc-12755), JAK1 (sc-1677), JAK2 (sc-390539), p16 (sc-1661), p21 (sc-6246), MKP1 (sc-370), MKP2 (sc-1200), SHP-1 (sc-7289), SHP-2 (sc-7384), SOCS1 (sc-9021)(Santa Cruz Biotechnology), and IFNγR2 (#559917, BD Biosciences). Then the cells were incubated with secondary antibodies conjugated with fluorescence dye and examined with an Accuri C6 flow cytometer (BD Biosciences). The results were

generated by a CFlow software where protein expression was determined by their different fluorescence intensity.

Immunocytochemistry

Immunostaining was performed in a mESCs and mESC-FBs co-culture model (D'Angelo et al., 2016) where both cells can be easily distinguished by their different cell morphology. Both cells will be seeded on the top of cover glasses in a cell culture plate followed by designated treatments. Cells will then be fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton-X100. The cellular location of STAT1 was determined by P-STAT1 antibody specific to Tyr 701 phosphorylated site (#7649, Cell Signaling Technology) and a secondary antibody conjugated with fluorescein isothiocyanate (FITC). The cells were visualized under a Zeiss LSM510 laser-scanning confocal microscope.

Statistical Analysis

Statistical analysis was performed using a 2-tailed and paired student t-test. Differences are considered statistically significant when $*p < 0.05$ and statistically highly significant when $** p < 0.01$.

CHAPTER IV – RESULTS

The research in our lab has been concentrated on studying the innate immunity of mouse ESCs (mESCs), and we found that mESCs lack or have the attenuated ability to mount the innate immune responses in the presence of bacterial/virus infections and inflammatory cytokines (D'Angelo et al., 2017; Wang et al., 2014, 2013). Along with studies from other researchers on both human ESCs (hESCs) and mESCs (L.-L. Chen et al., 2010; Földes et al., 2010; Yu et al., 2009), we have concluded that the lack of innate immune responses is an intrinsic property of ESCs. However, the rationales of lacking such fundamental abilities in ESCs and the implications of their underdeveloped innate immunity are still not clear. To understand the molecular mechanisms underlying these findings, I used a differentiated model of mESCs called mESC-FBs, which were proved to have partially developed innate immune responses in comparison with mESCs. Briefly, I removed LIF (a cytokine that inhibits differentiation of mESCs) from the mESCs culture medium and treated mESCs with 1 μM retinoic acid (a vitamin A metabolite that can induce differentiation). After 10 days of' spontaneous culture, the morphology of a large portion of the cells turned into a spindle shape and became flatter than the original mESCs colonies. Those cells were then purified by reseeding to a new dish and changing the medium within 10-15 min to remove unattached cells. The pure fibroblast morphology was shown on the remaining cells, which are designated as mESC-FBs (Wang et al., 2013). The subject of this research project is to demonstrate the implications of the attenuated responses to various inflammatory conditions in mESCs and the possible molecular regulating mechanisms. The results are summarized below.

mESCs Are Insensitive to the Cytotoxicity of IFNγ and TNFα

Somatic cells and immune cells can secrete large numbers of IFNs and inflammatory cytokines when they have encountered viral, bacterial, or other types of infections to defend themselves and nearby infected cells (Mogensen, 2009; O'Shea & Murray, 2008). TNFα and IFNγ are the two most common inflammatory cytokines found in inflammation conditions. Large amounts of TNFα or IFNγ production caused by infections could negatively affect the functionality and propagation of cells (Buntinx et al., 2004; Ohmori, Schreiber, & Hamilton, 1997). Inflammatory cytokines like TNFα or IFNγ alone usually does not cause apparent effects on cell viability within a short time frame, but when they are presented together, they can synergistically potentiate their cytotoxicity and cause cell death. By treating mESCs and mESC-FBs with either TNFα and IFNγ by themselves or in combination, I first aimed to test the effects of this artificially created inflammatory condition on cells viability. After incubating for 48 h in 2% FBS DMEM, TNFα or IFNγ alone did not have significant effects on cell viability in both mESCs and mESC-FBs, but the combination of these two cytokines caused significant cell proliferation inhibition in mESC-FBs (~70% of cells lost viability by 48 h; toxicity was apparent as early as 24 h), while no effects occurred in mESCs (Fig. 4A). To determine the impacts from IFNγ alone on both cell types for a longer time, cells were then treated for 4 days under the same culture condition as in Fig. 4A, and once again, ~50% loss of viability in mESC-FBs was observed, but there were no effects in mESCs (Fig. 4B). To further confirm the effects of TNFα and IFNγ, both mESCs and mESC-FBs were treated with either of the two cytokines alone or their combination for 24 h and

analyzed their cell cycle by propidium iodide (PI) using a flow cytometer. As shown in Fig. 4C, decreased cell numbers in the S and G2 phase were only found in TNFα and IFNγ treated mESC-FBs, which confirmed the cytotoxicity from TNFα and IFNγ only occurred in mESC-FBs.

p16 and p21 are two primary cell proliferation regulators and are widely used as senescent markers (Harada, Taniguchi, & Tanaka, 1998; Stark et al., 1998). To further demonstrate the effects of TNFα and IFNγ on the cellular level, protein expressions of p16 and p21 in mESCs and mESC-FBs were examined by flow cytometry after 24 h treatment of both cytokines. As shown in Fig. 4D, both p16 and p21 expressions clearly increased in mESC-FBs after treatment of cytokines, but again, no effects were detected in mESCs. All the results above indicate that by lacking responses to those inflammatory cytokines, mESCs could potentially evade from the harmful effects caused by inflammation and protect their pluripotent identity.

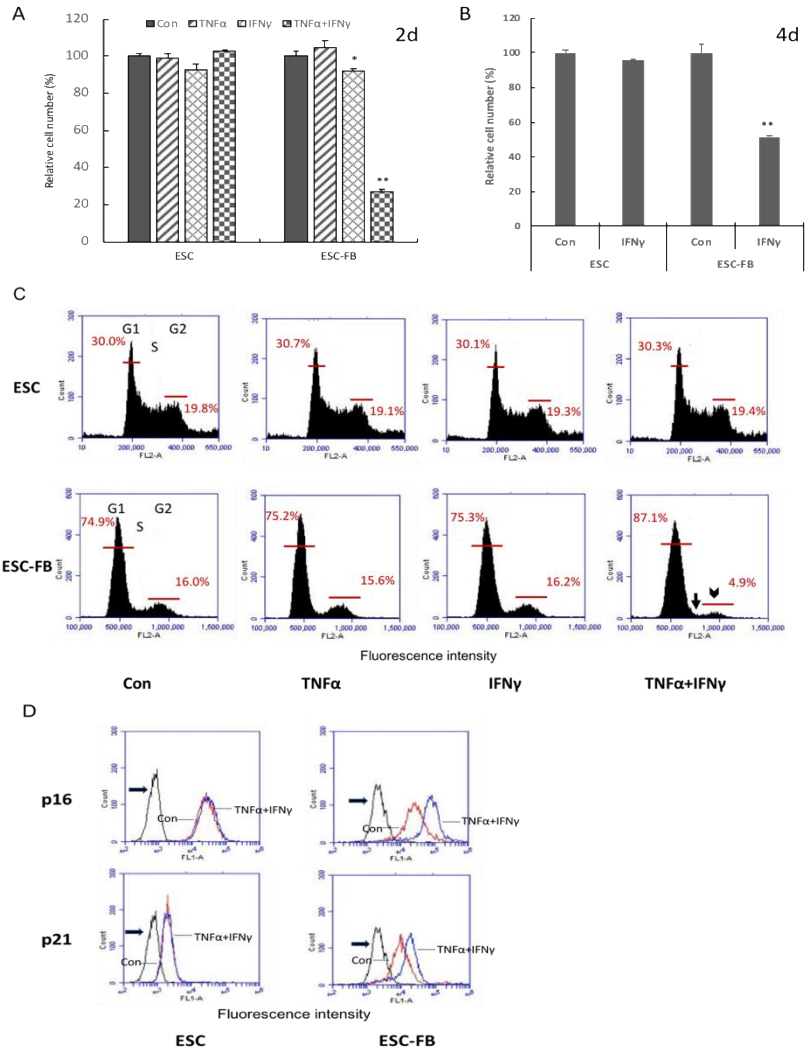

Figure 4. Effects of IFNγ and TNFα on the viability of mESCs and mESC-FBs.

(*A*) ESCs and ESC-FBs were treated with either TNFα (20ng/ml), IFNγ (20ng/ml) alone or TNFα plus IFNγ. After 48h treatments, both cells were fixed and stained with toluidine blue as an indicator of cell viability analysis. The cell number in control (Con) was defined as 100%. (*B*) ESCs and ESC-FBs were treated with IFNγ (20ng/ml) for 4 days. Quantitative analysis of the cell viability was performed as discussed above. Data are mean ± SD of a representative experiment that was performed in triplicate. $*P < 0.05$, $**P < 0.01$. (*C*) ESCs and ESC-FBs were treated with TNFα, IFNγ or their combination for 24 h, then collected and stained with 50μg/ml propidium iodide for cell cycle analysis by flow cytometry. Reduction of the S and G2/M phase cells were indicated by arrow and arrowhead. (*D*) Both cells were treated with TNFα plus IFNγ for 24 h, then the expression of p16 and p21 were analyzed by flow cytometry. The lines denoted by arrows are negative controls that were only stained with secondary antibody. Flow cytometry data was derived from a representative experiment that was performed twice yielding similar results.

mESCs Have Attenuated Responses to IFNγ and TNFα

From our previous studies, we have already demonstrated that mESCs lack responses to TNFα and the responses could be gradually developed during differentiation of mESCs to mESC-FBs (D'Angelo et al., 2016). In Fig. 4, IFNγ alone or with the combination of TNFα did not have any significant effect on the cell viability of mESCs. However, whether IFNγ itself or the combination with TNFα can alter the expression of signaling or inflammatory genes of mESCs is still not clear. Thus, the effects of TNFα and IFNγ alone or their combination on the mRNA expression of signaling molecules in mESCs and mESC-FBs were first determined. iNOS is an important cytokine-inducible factor synthesized by cells in response to different infections (Zamora, Vodovotz, & Billiar, 2000). ISG15 can also be induced by IFNγ during immune responses (Cunha, Knight, Haast, Truitt, & Borden, 1996). Both iNOS and ISG15 play essential roles in regulating cell proliferation and responses to the infections. The expression levels of these two inflammatory molecules after the treatment with TNFα and IFNγ were tested first. As shown in Fig. 5A(a), significant effects of IFNγ or its synergistic combination with TNFα were only seen in mESC-FBs, but they were barely changed in mESCs. The dose-dependent treatment of IFNγ further demonstrated the attenuated responses in mESCs (Fig. 5A(b)). The expression of iNOS protein induced by TNFα and IFNγ in mESC-FBs were then confirmed by flow cytometry, where no detectable protein changes were found in mESCs (Fig. 5B). Based on these results, it was further confirmed that mESCs have attenuated responses to IFNγ and did not have significant responses to the synergistic effects from its combination with TNFα.

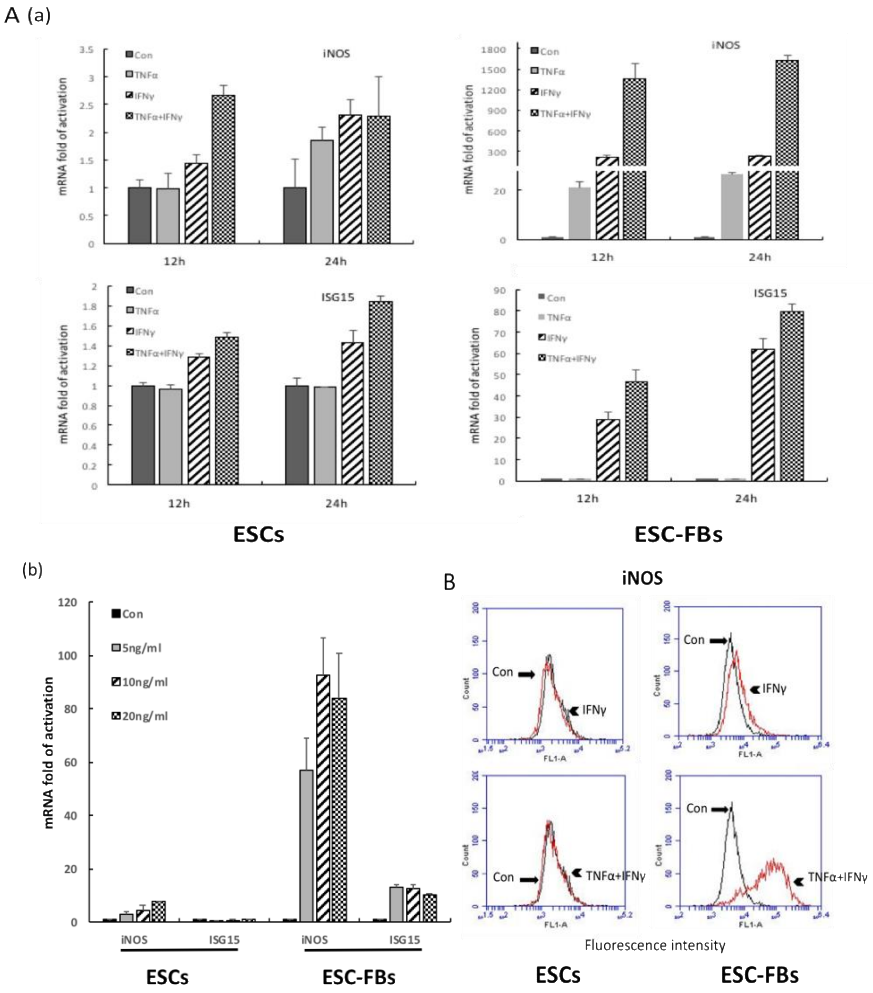

Figure 5. Attenuated responses of mESCs to IFNγ and TNFα.

ESCs and ESC-FBs were treated with TNFα (20ng/ml), IFNγ (20ng/ml) and their combination for 24 h (*A(a)) or* treated with different concentration of IFNγ for 12 h (*A(b)*). The control cells were left untreated. The mRNA level of iNOS and ISG15 was determined by RT-qPCR. The results are presented as fold-activation; the mRNA level in untreated control cells is designated as 1. (*B*) The effect of IFNγ or TNFα plus IFNγ on the protein expression of iNOS in ESCs and ESC-FBs were determined by flow cytometry (denoted by arrowheads). The lines denoted by arrows represent controls. RT-qPCR data are mean ± SD of representative experiments from three independent experiments. Flow cytometry data are derived from a representative experiment that was performed three times yielding similar results.

mESCs Are Insensitive to the Cytotoxicity Associated with Macrophage Activation

It can be concluded from the results above that TNFα and IFNγ did not affect the overall viability of mESCs and barely induced the expression of inflammatory genes.

However, the immune system and signaling pathways in vivo are complicated and are involved in numerous other inflammation molecules. Thus, we created an in vitro inflammation model by making the conditioned medium from macrophages (RAW264.7), which are known to have robust immune responses when activated by infectious agents and secret a large amount of various inflammatory molecules (Funk, Feingold, Moser, & Grunfeld, 1993; Lyu & Park, 2005). Briefly, RAW cells were either left untreated or treated with lipopolysaccharide (LPS; a bacterial endotoxin that strongly induces inflammatory responses) (Hambleton, Weinsteint, Lemt, Defrancots, & Bishop, 1996). Then, those media were collected and named as Con-CM and LPS-CM, respectively. The Con or LPS-CM were mixed with cell culture medium at a 1:1 ratio and were used to culture both cell types for 48 h, followed by the cell viability analysis. As shown in Fig. 6A, similar patterns from LPS-CM were observed compared with the effects from TNFα and IFNγ as indicated by the first two bars in the bar graph of each cell type: LPS-CM caused more than 40% inhibition of cells proliferation in mESC-FBs but had no effects on mESCs. Although it is not clear what the major molecules are in the CMs, there could be synergistic effects under the presence of additional cytokines (Bartee & McFadden, 2013; Cassese et al., 2003). Thus, supplement of IFNγ was added to another group of Con and LPS CM cultured mESCs and mESC-FBs to see if there were synergistic effects. As shown in Fig. 6A, the supplement of IFNγ caused additional toxicity, once again, only in mESC-FBs, under both Con and LPS CM cultured conditions. No significant effects were detected in mESCs. The effects from CM and supplement of IFNγ were further confirmed from the mRNA level by determining the expression of iNOS and COX2, two major inflammatory molecules induced during

inflammation under the same condition as the cell viability experiments. These genes were barely induced in mESCs under all conditions tested (Fig. 6B).

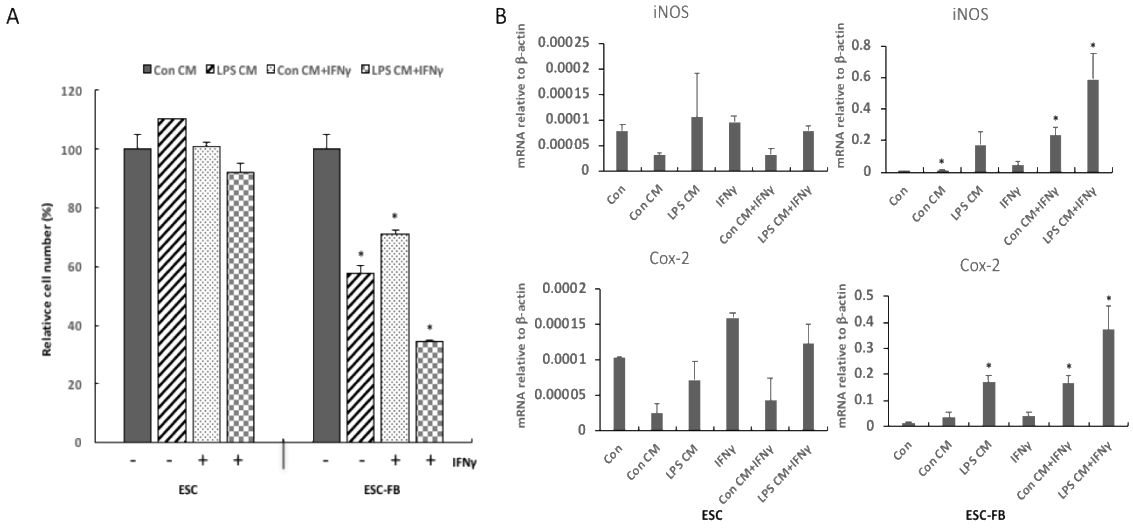

Figure 6. Effects of CM in the presence or absence of IFNγ on the viability and inflammatory gene expression in mESCs and mESC-FBs.

(*A*) ESCs and ESC-FBs were treated either with conditioned medium from untreated (Con CM) or LPS treated RAW cells (LPS CM) 1:1 mixed with cell culture medium (first two bars in each cell type) or with additional IFNγ (5ng/ml) for 48 h. Cells were stained with toluidine blue as an indicator of cell viability analysis. The cell number in controls (Con) were defined as 100%. (*B*) mRNA expression of iNOS and Cox-2 were determined after the same treatment for 24 h as described in (*A*) by RT-qPCR. Data are mean ± SD of a representative experiment that was performed in triplicate or from three independent experiments. $*P < 0.05$.

IFNγ Potentiates the Cytotoxic Effects of Heat Killed E. coli in mESC-FBs but Not in mESCs

To further demonstrate the insensitivity to the cytotoxic effects from various inflammatory conditions in mESCs, HKE was used to illustrate the effects of bacterial infection agents. mESCs and mESC-FBs were incubated with HKE, alone or in combination with IFNγ. As shown in Fig. 7, over a 4-day treatment period, HKE alone had no significant effects on the viability of either cell type, but supplementation of IFNγ

with HKE induced synergistic cytotoxicity in mESC-FBs, though this combination still had no effects on the viability of mESCs.

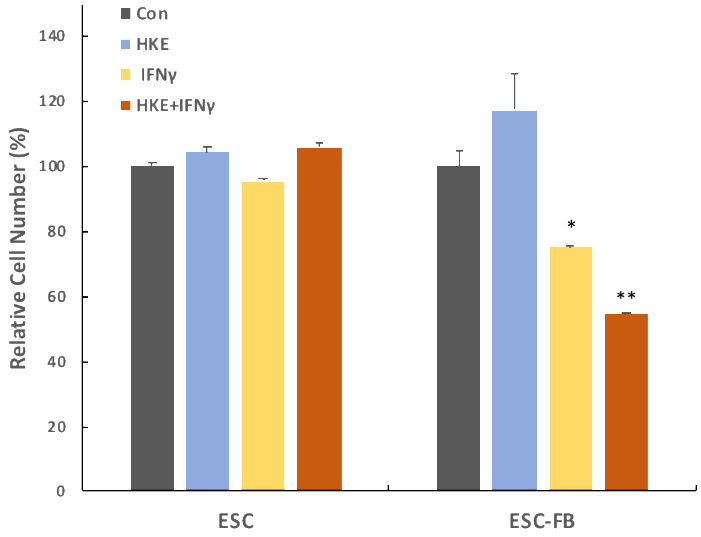

Figure 7. IFNγ potentiates the toxicity of HKE in mESC-FBs but not in mESCs.

ESCs and ESC-FBs were treated either with HKE (200:1 with cells), IFNγ (20ng/ml) alone or their combination for 4 days in 10% FBS DMEM. Cells were stained with toluidine blue as an indicator of cell viability. The cell number in controls (Con) was defined as 100%. Data are mean ± SD of a representative experiment that was performed in triplicate. *$P < 0.05$, **$P < 0.01$.

STAT1 Is Not Activated by IFNγ in mESCs

STAT1 is the initial and major transcription factor that controls interferons responses (Lehtonen, Matikainen, & Julkunen, 1997). The activation of STAT1 can induce the downstream transcription factor IRF-1, which will further regulate the downstream IFNγ pathway (Platanias, 2005). To further determine the molecular basis for the attenuated IFNγ response in mESCs, I first tested IRF-1 and STAT1 mRNA basal level and IFNγ induction level in both mESCs and mESC-FBs. Interestingly, as shown in Fig. 8A(a), mRNA basal levels of both IRF-1 and STAT1 are significantly higher in mESCs than mESC-FBs. However, IFNγ treatment results in a 5-fold increase in mRNA expression of each gene in mESCs, compared with a 25-30 fold increase in mESC-FBs

(Fig. 8A(b)). When STAT1 is activated, it will be phosphorylated (P-STAT1) on both tyrosine and serine sites. P-STAT1 can then be translocated from cytoplasm to the nucleus and bind to their promoter regions of target genes to initiate transcription. To monitor the nuclear translocation of P-STAT1, an antibody-based immunocytochemistry assay and a co-culture model were used, where mESCs can be easily distinguished by their characteristic cell morphology and colonial growth (as indicated within the circled areas) compared with mESC-FBs. As shown in Fig. 8B, P-STAT1 can be detected in the cytoplasm of both cell types in CON. However, nuclear translocation was only detected in mESC-FBs after the treatment of IFNγ, while no detectable changes occurred in mESCs. We previously demonstrated that mESCs could respond to type I IFN, and STAT1 was able to be translocated into the nucleus after IFNα induction, indicating that STAT1 nuclear translocation is functional in mESCs (Wang et al., 2014). Therefore, there must be other mechanisms responsible for the attenuated response to IFNγ in mESCs.

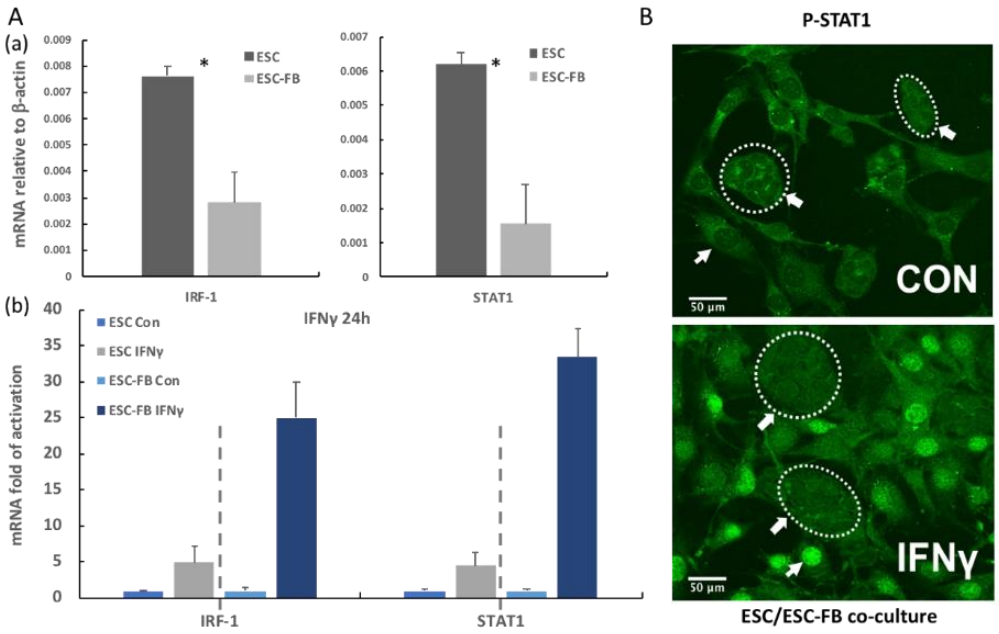

Figure 8. Expression of IRF-1 and STAT1 and the effects of IFNγ on STAT1 activation in mESCs and mESC-FBs.

(*A*) IRF-1 and STAT1 mRNA expression in both ESCs and ESC-FBs. The basal mRNA expression levels of IRF-1 and STAT1 in mESCs and mESC-FBs were determined by RT-qPCR (a). The mRNA induction of IRF-1 and STAT1 by IFNγ was attenuated in mESCs in comparison with mESC-FBs. Both cell types were treated with IFNγ (20ng/ml) for 24 h and then collected for RT-qPCR (b). Data are mean ± SD of representative experiments from three independent experiments. $*P < 0.05$. (*B*) IFNγ induced nuclear translocation of STAT1 in ESC-FBs but not in ESCs. ESCs and ESC-FBs were grown in a co-culture model in which ESCs were identified by their colonial growth (dotted circle area), and ESC-FBs were identified by their flattened large cell morphology. The cells in the co-culture were treated with IFNγ (20ng/ml) for 15 min, and the cellular location of STAT1 was analyzed with Abs against Tyr701 P-STAT1. Arrows indicate the representative nuclei. The images were from representative experiments performed in duplicate. CON represents cells that were not treated.

Relative Expression Levels of IFNγ Signaling Molecules in mESCs and mESC-FBs

We have demonstrated that transcription factor STAT1 was activated by type I IFN (IFNα and β) (Wang et al., 2014), but not by IFNγ in mESCs (Fig. 8). It is uncertain if the signaling receptors responsible for IFNγ are functional. IFNγR1 and IFNγR2 are located at the cell membrane and contain intracellular and extracellular domains. Their extracellular domains are mainly responsible for IFNγ binding, while the intracellular

domains bind with their receptor-associated factors JAK1 and JAK2 to further transduce the signals (Darnell, 1997). Relative mRNA expression of signaling molecules mentioned above in both mESCs and mESC-FBs were analyzed by RT-qPCR. As shown in Fig. 9A, mRNA of these major signaling molecules were expressed in both cell types, although some of them were expressed relatively lower in mESCs compared with mESC-FBs. At the protein level, these signaling molecules were also detectable in both cell types (Fig. 9B), indicating that the major signaling molecules of IFNγ pathway are present in mESCs, and other regulating mechanisms might be responsible for the attenuated IFNγ responses in mESCs.

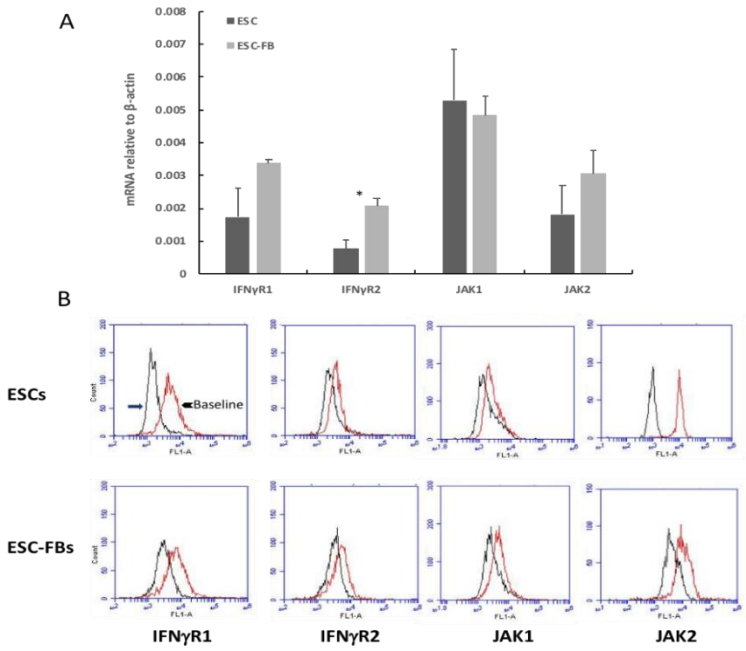

Figure 9. Expression of signaling molecules that regulate IFNγ responses in mESCs and mESC-FBs.

(*A*) Relative basal mRNA level of each gene in ESCs and ESC-FBs was compared after normalization to β-actin mRNA in each cell type. (*B*) Expression of signaling molecules in ESC and ESC-FB was determined by flow cytometry (the lines denoted by arrowheads). The lines denoted by arrows represent negative controls. RT-qPCR data are mean ± SD of representative experiments from three independent experiments. *$p < 0.05$. Flow cytometry data are derived from a representative experiment that was performed three times yielding similar results.

SOCS1 Is Not a Critical Suppressor Responsible for the Attenuated IFNγ Responses in mESCs

SOCS1 plays critical roles in regulating the IFNγ signaling pathway. By interacting with the IFNγ receptor-associated factor JAK2, SOCS1 can inhibit STAT1 phosphorylation by JAK2 and thus inhibit the signal transduction activity (Fig. 3) (Alexander et al., 1999; Davey, Heath, & Starr, 2006; Naka & Fujimoto, 2010). Unlike type I IFN activation, where STAT1 forms a heterodimer with STAT2 before translocation into the nucleus, P-STAT1 will form a homodimer after cell activation by IFNγ. Homodimerization of P-STAT1 is a prerequisite for them to be translocated into the nucleus in response to IFNγ (Platanias, 2005). To determine whether SOCS1-medicated inhibition could be one of the reason that accounts for the attenuated IFNγ responses in mESCs, I first tested the basal mRNA and protein expression level of SOCS1 in mESCs and mESC-FBs. Both mRNA and protein basal expression of SOCS1 in mESCs were found to be lower than mESC-FBs. (Fig. 10A (a and b)). Next, both cells were treated with IFNγ for 15 h or 24 h and then SOCS1 induction was analyzed at the mRNA and protein levels. As shown in Fig. 10B (a), SOCS1 mRNA was significantly upregulated by IFNγ in mESC-FBs, and the induction level was around 4 times more than in mESCs. Consistent with SOCS1 mRNA induction, SOCS1 protein expression was also increased in IFNγ treated mESC-FBs but not in mESCs (Fig. 10B(b)). To further demonstrate the role of SOCS1 in the regulation of responses to IFNγ, mESCs were either transfected with Con siRNA or siSOCS1 (30nM). Optimal transfection conditions were determined first (data not shown). Transfection of siSOCS1 resulted in knockdown

SOCS1 mRNA expression by more than 40% in mESCs at 19 h after transfection. After 19 h transfection, mESCs were treated with IFNγ for another 12 h. The mRNA induction levels of iNOS in Con siRNA- and siSOCS1-transfected mESCs were determined by RT-qPCR. The results indicated that knocking down of SOCS1 had no effect on the induction level of iNOS by IFNγ in mESCs (Fig. 10C). In conclusion, SOCS1 may not be a critical suppressor of mESCs' responses to IFNγ.

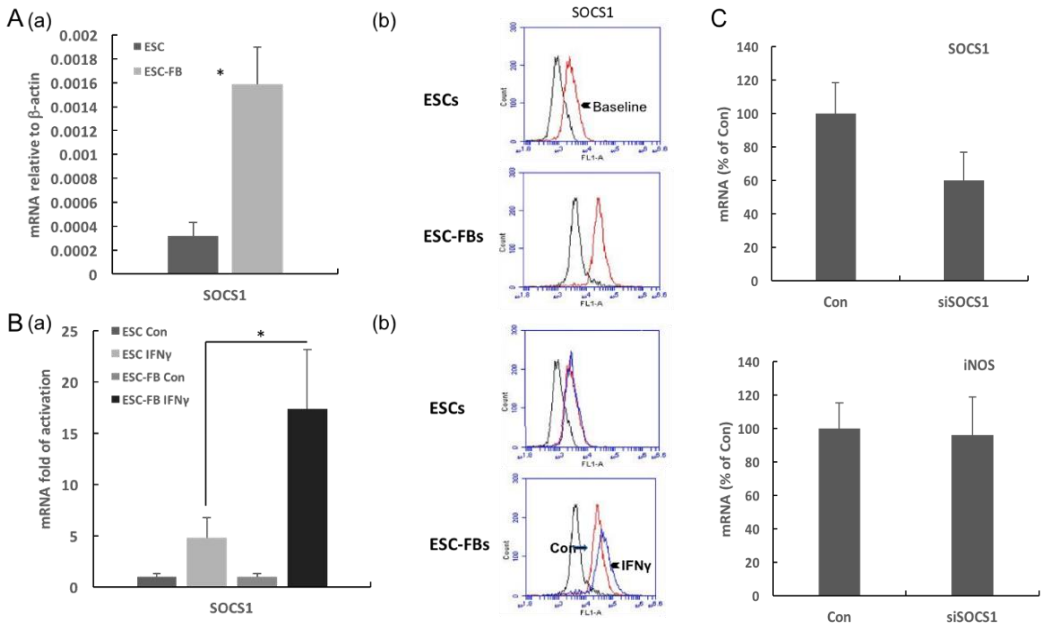

Figure 10. SOCS1 is not a critical suppressor responsible for the attenuated IFNγ responses in mESCs.

(*A*) Relative basal mRNA level of SOCS1 in ESCs and ESC-FBs was compared after normalization to β-actin mRNA in each cell type (a). The basal protein level of SOCS1 was determined by flow cytometry (As indicated by the arrowhead) (b). (*B*) Effects of IFNγ (20ng/ml) on the mRNA and protein expression of SOCS1 in ESCs and ESC-FBs were determined by RT-qPCR and flow cytometry. (*C*) ESCs were transfected either with Con siRNA or siSOCS1 for 19 h. Transfection efficiency was determined by mRNA levels of SOCS1. Cells were then treated with IFNγ for another 12 h. iNOS mRNA expression (%) was determined as normalized with Con. RT-qPCR data are mean ± SD of representative experiments from three independent experiments. *$p < 0.05$. Flow cytometry data are derived from a representative experiment that was performed twice yielding similar results.

Relative Expression Levels of PTPs That Regulate IFNγ Signaling and the Effects of IFNγ on Their Expressions

Protein phosphorylation is essential for many types of signal transduction, including the responses to cytokines, and growth factors. Tyrosine and serine phosphorylation are central regulators of IFNγ responses, which are negatively regulated by PTPs and DSPs (Fig. 3) (Shuai & Liu, 2003; T. R. Wu et al., 2002; Xu & Qu, 2008). As demonstrated in Fig. 8B, P-STAT1 is not detectable in the nucleus after treatment of mESCs with IFNγ. I speculated that PTPs and DSPs could be factors that contribute to the attenuated IFNγ responses in mESCs. To test this possibility, I first examined the basal mRNA and protein expression level of several PTPs and DSPs in mESCs and mESC-FBs. As shown in Fig. 11A (a and b), the basal mRNA levels of the tested protein phosphatases were all relatively higher in mESCs than mESC-FBs. While the basal protein levels of most of the tested protein phosphatases was comparable between both cell types, SHP-2 was more highly expressed in mESCs than mESC-FBs. SHP-2 and other tested protein phosphatase have been reported to tightly regulate STAT1 signal transduction (Greenhalgh & Hilton, 2001; P. T. M. and C. Wu, 1996; T. R. Wu et al., 2002; Xu & Qu, 2008). The effects of IFNγ on the mRNA expression of different protein phosphatases were also determined. As shown in Fig. 11B, IFNγ significantly downregulated the expression of MKP1 and SHP-1 in mESCs, while there were no significant effects in mESC-FBs. Therefore, the relatively high basal mRNA level of tested protein phosphatases and the high expression of SHP-2 in mESCs may be potential factors that limit the mESCs' responses to IFNγ.

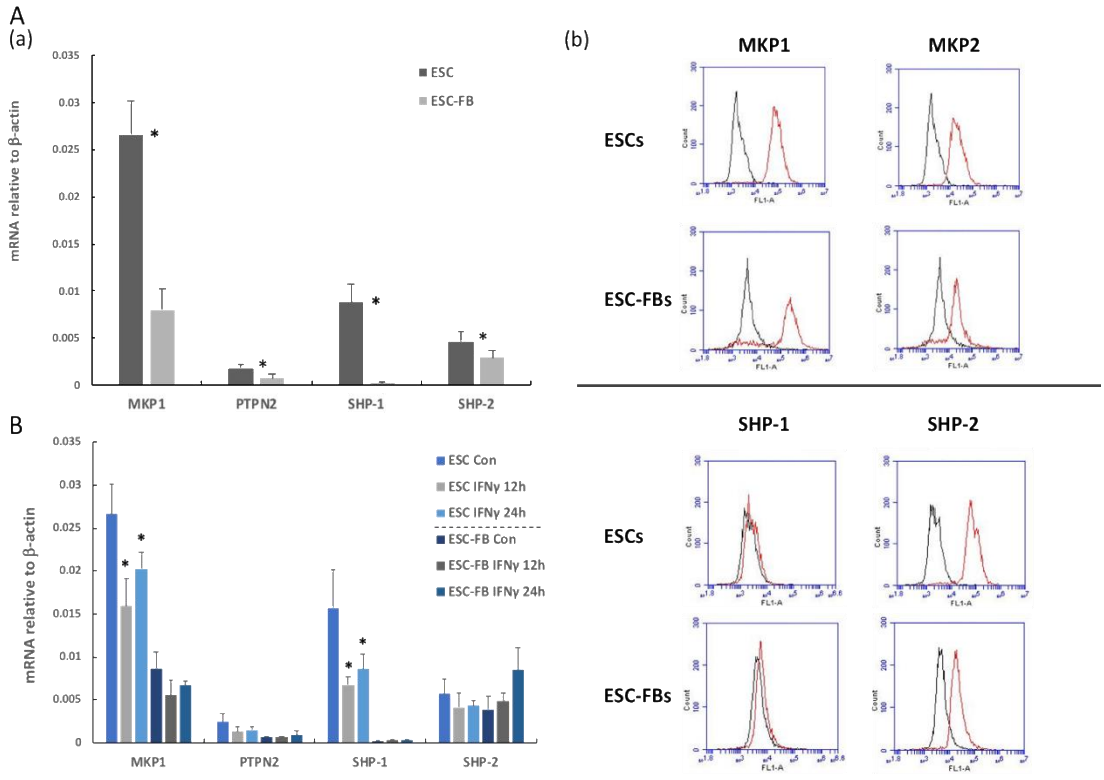

Figure 11. Relative expression levels of protein phosphatases that regulate IFNγ signaling and their induction by IFNγ.

(*A*) Relative basal mRNA level of protein phosphatases in ESCs and ESC-FBs was compared after normalization to *β*-actin mRNA in each cell type (a). The basal protein level of various protein phosphatase was determined by flow cytometry (Red curve) (b). (*B*) ESCs or ESC-FBs were treated with IFNγ (20ng/ml) for 12 h or 24 h. Effects of IFNγ on the mRNA expression of protein phosphatases in ESCs and ESC-FBs were determined by RT-qPCR. RT-qPCR data are mean ± SD of representative experiments from three independent experiments. *$p < 0.05$. Flow cytometry data are derived from a representative experiment that was performed three times yielding similar results.

Effects of PTP Inhibitor on the Responses of mESCs to IFNγ

Based on the above results, the relatively high expression of protein phosphatases in mESCs was speculated as a potential factor that causes mESCs' attenuated responses to IFNγ. To test this, I measured the mRNA level of major IFNγ signaling molecule after pre-treating mESCs with the general PTP inhibitor vanadate (Vn) (Huyer et al., 1997) for 30min, followed by IFNγ treatment for 3 h. IRF-1 mRNA expression was then analyzed

by RT-qPCR. As compared with each control from designated groups, IRF-1 was significantly upregulated by IFNγ in Vn-pretreated mESCs, with higher concentrations of Vn can induceing higher induction of IRF-1 in the presence of IFNγ (Fig. 12). The above results suggest that PTPs and DSPs contribute to the attenuated mESCs' responses to IFNγ.

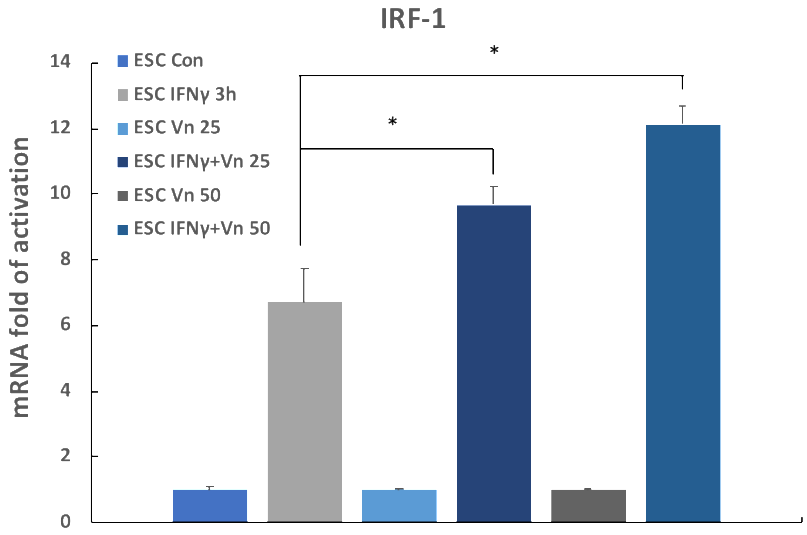

Figure 12. Vn upregulates the response of mESCs to IFNγ.

ESCs were treated with IFNγ (20ng/ml) alone or IFNγ plus Vn (25 or 50nm) for 3 h. mRNA induction of IRF-1 was determined after normalization to control mRNA in each group. Data are mean ± SD of representative experiments from three independent experiments. *$p < 0.05$.

CHAPTER V – DISCUSSION

The finding of the underdeveloped innate immunity in both mouse and human ESCs has attracted increasing attention due to the fact that the innate immune response is an important concerns for their applications in tissue engineering and regenerative medicine (L.-L. Chen et al., 2010; D'Angelo et al., 2017; Földes et al., 2010; Wang et al., 2013). While several studies have revealed some possible explanations for the lacking or low responses to several infectious or inflammatory stimuli in mESCs, innate immune responses are a complicated subject that needs to be further investigated. The biological implications of this special property of mESCs remain to be elucidated.

In this study, I first demonstrated the insensitivity of mESCs to the cytotoxic effects of several different inflammatory conditions, including TNFα, LPS-CM, HKE, and their synergistic effects with IFNγ, as compared with the differentiated cell model mESC-FBs. This insensitivity correlates with the attenuated signaling pathway activity and lack of induction of inflammatory molecules after the designated treatments in mESCs. Like a double-edged sword, the inflammatory responses can inhibit invasion of various pathogens, but they can also cause collateral cell damage including cell cycle arrest and eventually cell death (Hertzog et al., 1994; Kotredes & Gamero, 2013; Sedger & McDermott, 2014). Thus, these results suggest that the attenuated innate immune responses in mESCs could help them evade the toxic effects from inflammatory conditions and maintain their viability as well as the pluripotent capability. However, most of the molecular mechanisms behind their attenuated responses are still not clear.

IFNγ is robustly produced by the maternal uterine natural killer cells during the early stage of the embryogenesis (Ashkar et al., 2000; Platt & Hunt, 1998) and it is also

one of the most important inflammatory cytokines that would be induced during different types of inflammation (Boehm et al., 1997; Stark, Kerr, Williams, Silverman, & Schreiber, 1998). In this study, I further investigated the potential molecular mechanisms behind mESCs' attenuated responses. I found that STAT1, a major transcription factor induced by IFNγ signaling, is not activated in mESCs, although they express IFNγ receptors and associated factors at mRNA and protein levels similar to mESC-FBs. I next found that siRNA-mediated knockdown of SOCS1, one of the negative regulators of IFNγ pathway, did not increase responsiveness of mESCs to IFNγ, indicating that SOCS1 is unlikely the critical repressor in this context. However, several PTPs were found to have higher basal mRNA levels in mESCs than mESC-FBs, though at the protein level, only SHP-2 was more highly expressed in mESCs. Pre-treating mESCs with the general PTP inhibitor Vn was shown to upregulate the mRNA induction level of IRF-1 by IFNγ, which indicates an elevated responsiveness to IFNγ. Thus, the higher basal expression of protein phosphatases could, at least in part, explain the attenuated responses of mESCs to IFNγ.

It is interesting to note that studies of trophoblast stem cells and trophoblasts, which compose the outer layer of the blastocyst that surrounds ESCs, showed that those cells also have selective attenuated responses to IFNγ (Albieri et al., 2005; J. C. Choi, Holtz, Petroff, Alfaidy, & Murphy, 2007; Jason C. Choi, Holtz, & Murphy, 2009) and are resistant to apoptosis induced by IFNγ, which are believed to be important for maintaining the integrity of the placenta and successful conception (Sun, Peng, & Xia, 2006; Yui, Garcia-Lloret, Wegmann, & Guilbert, 1994). While embryogenesis involves intricate interactions between the ICM and trophectoderm, it would be physically more

relevant to explore the immune properties of the blastocyst to unveil the development of the innate immune system during the early stages of embryogenesis. Recent studies that have generated in vitro models of blastocyst development will provide more opportunities for a deeper understanding of the early stages of organismal development and answering the fundamental questions in developmental and stem cell biology (Harrison, Sozen, Christodoulou, Kyprianou, & Zernicka-Goetz, 2017; Rivron et al., 2018).

In summary, this study demonstrated that the resistance of mESCs to the cytotoxic effects from different infections or inflammatory conditions, which is due to their attenuated responses to inflammation cytokines and bacterial cytokines, could be a self-protective mechanism. High expression levels of protein phosphatases in mESCs, especially SHP-2, could be the molecular basis that partially explains their attenuated responses to IFNγ. Together with the lacking response of ESCs to TNFα demonstrated in our previous studies, the data presented in this thesis provide additional evidence that underdeveloped innate immunity is an intrinsic property of ESCs making them less vulnerable to cytotoxicity associated with inflammatory responses and infection.

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