MOUSE EMBRYONIC STEM CELLS EXPRESS FUNCTIONAL TOLL LIKE

RECEPTOR 2

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fulfillment of the requirements for the degree of Doctor of Philosophy.

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DEDICATION

I would like to dedicate this to my father Emmitt Monroe Taylor, my uncle James Ivory, and friend L.C. Tennin III. I know you are the angels watching over me each day.

ACKNOWLEDGEMENTS

ABSTRACT

Tammi M. Taylor

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Embryonic stem cells (ESCs) are unique in that they have potential to give rise to every cell type of the body. Little is known about stimuli that promote mouse (m)ESC differentiation and proliferation. Therefore the purpose of this study was to determine the role of Toll Like Receptor (TLR) ligands in mESCs proliferation, survival, and differentiation in the presence of Leukemia Inhibitory Factor (LIF). We hypothesized that TLRs are expressed and functional, and when activated by their ligand will induce survival, proliferation, and prevent differentiation. In this study, mESC line E14 was used to determine the expression of TLRs at the mRNA level and three mESC lines, R1, CGR8, and E14, were used to determine cell surface protein levels. We found expression of TLRs 1, 2, 3, 5, and 6 at the mRNA, level but no expression of TLRs 4, 7, 8 and 9 in the E14 mESC line. We confirmed the presence of TLR-2 but not of TLR-4, protein on the cell surface using flow cytometric analysis for all three cell lines. We focused our studies mainly on TLR-2 using the E14 cell line. Pam_3Cys, is a synthetic triacyl lipoprotein and a TLR-2 ligand, which induced a significant increase in mESC proliferation on Days 3, 4, and 5 and enhanced survival of mESC in a dose dependent manner in the context of delayed addition of serum. All the latter experiments were

performed in triplicate and student T-test was performed to establish significant differences. Next, we demonstrated functionality of TLR-2 via the MyD88/IKK pathway, where MyD88 was expressed and IKKα/β phosphorylation was enhanced. This was associated with increased NF-κB nuclear translocation upon activation by Pam_3Cys. Finally, we showed that there were no changes in expression of mESCs markers Oct-4, KLF-4, Sox-2, and SSEA-1, thus illustrating that the mESCs may have remained in a pluripotent state after activation with the TLR-2 ligand in the presence of LIF. These results demonstrate that mESCs can respond to microbial products, such as Pam_3Cys, and can induce proliferation and survival of the mESCs. This finding expands the role of TLRs and has some implications in understanding embryonic stem cell biology.

Hal E. Broxmeyer, PhD, Chair

TABLE OF CONTENTS

the presence of TLR-2 activation in the presence of LIF
Pam3Cys enhances and LPS decreases mES cell survival
upon delayed addition of serum

TLR-2 and TLR-4 are expressed in Embryoid Bodies
In the absence of LIF, TLR-2 activation caused an increase
in expression of Oct-4 and Sox 2 but not KLF-4 and SSEA-1
Oct-4 levels, as assessed by Western blot, were maintained and
a decrease in p-ERK in Day 6 EBs compared to EBs not treated
with TLR-2 ligand

LIST OF TABLES

MATERIALS AND METHODS

RESULTS

LIST OF FIGURES

INTRODUCTION

RESULTS

DISCUSSION

LIST OF ABBREVIATIONS (in order as they appear)

PAMP- Pathogen Associated Microbial Patterns

TLR- Toll Like Receptors

NK- Natural Killer

T- Tumor derived

B- Bone marrow

ESC- Embryonic Stem Cells

LIF- Leukemia Inhibitory Factor

IL-6 Interleukin- 6

R-receptor

LIF-Rβ- Leukemia Inhibitory Factor Receptor Beta

JAK- Janus Kinase

STAT3- Signal Transducer and Activator of Transcription 3

SHP- Scr homology phosphatase

ERK- Extracellular Signal Regulated Kinase

DNA- deoxyribonucleic acid

MAPK- mitogen activated protein kinase

SH2- Scr Homology 2

HPC- hematopoietic progenitor cell

TNF- tumor necrosis factor

IL-11- interleukin 11

STAT1- Signal Transducer and Activator of Transcription 1

IL-3- Interleukin 3

SCF- Stem Cell Factor

Oct-4- Octomer- 4

Sox-2- Sex determining region y box 2

KLF- Kruppler Like Factor 4

SSEA-1- stage specific embryonic antigen-1

SDF-1-Stromal Derived Factor 1

MyD88- Myeloid differentiation primary response protein 88

TIR- Toll/Interleukin-1Receptor associated Kinase

IFN-γ- Interferon gamma

TRIF- TIR domain adaptor inducing interferon β

IRAK- Interleukin 1 receptor associated kinase

AP-1- Activating protein 1

NF-κB- Nuclear Factor Kappa B

TRAF 6- TNF Receptor Associated Factor 6

IRFs- Interferon Regulatory Factors

PI3K- phosphoinositide-3 kinase

BMP- bone morphogenic protein

HSC- hematopoietic stem cells

MSC- mesenchymal stem cells

IKB- inhibitor of kinase B

INTRODUCTION

Survival of an organism depends on how prompt its innate immune system responds to an outside pathogen. The innate immune system is the first line of defense and is made up of myeloid cells. Myeloid cells include macrophages, neutrophils, and dendritic cells. Pathogen Associated Molecular Particles (PAMPs) are recognized by a Toll Like Receptor (TLR) on the surface of lymphoid cells and induce maturation and differentiation of myeloid cells (Nagai, Garrett et al. 2006).

Previous studies have shown TLRs to couple adaptive and innate immunity via lymphoid cells (Iwasaki and Medzhitov 2004). Lymphoid cells, include bone marrow derived B, thymus derived T, and Natural Killer (NK) cells. These cells are replenished by hematopoietic stem cells in the bone marrow (Kondo, Weissman et al. 1997). Stem cells are unique cells in the body formed during gestation, and are capable of self renewal. Embryonic (ES), fetal or adult stem cells preserve this unique quality. Stem cells can also give rise to multipotent progenitors that help replenish the hematopoietic and immune system, and other cell types of the body. They are products of ex-vivo culture of the blastocyst.

Embryonic Stem Cells

Embryonic Stem (ES) cells have the unique ability to self renew and differentiate into derivatives of all three germ layers *in vivo* and *in vitro* (Friel, van der Sar et al. 2005). The three germ layers are ectoderm, mesoderm, and endoderm (Figure 1). ES cells are derived from the inner cell mass of the blastocyst (Evans and Kaufman 1981; Martin 1981; Axelrod 1984; Wobus, Holzhausen et al. 1984; Doetschman, Williams et al. 1988; Smith 2001). The blastocyst consists of an outer cell layer called the trophoblast that give rise to the placenta, and an inner cell layer called the inner cell mass that gives rise to all tissues of the body (Friel, van der Sar et al. 2005). Scientists isolated cells from this region in the mouse blastocyst and were able to maintain these cells in culture on gelatinized plates with feeder layers (Mintz and Illmensee 1975). A factor found in the feeder layer was identified that prevented differentiation and promoted stem cell self renewal of mouse ES cells (Martin and Evans 1974; Evans and Kaufman 1981; Martin 1981). Today this factor is known as Leukemia Inhibitory Factor (LIF). LIF allows murine ES cells to grow in the absence of a feeder layer (Smith, Heath et al. 1988; Williams, Hilton et al. 1988; Friel, van der Sar et al. 2005). Human ES cells do not respond to LIF. Important unknowns are finding the factors that induce and maintain self renewal of human ES cells.

Figure 1. Stem Cell Hierarchy adapted from (Wobus and Boheler 2005). mES cells come

from the inner cell mass of the blastocyst and can give rise to every cell type of the body.
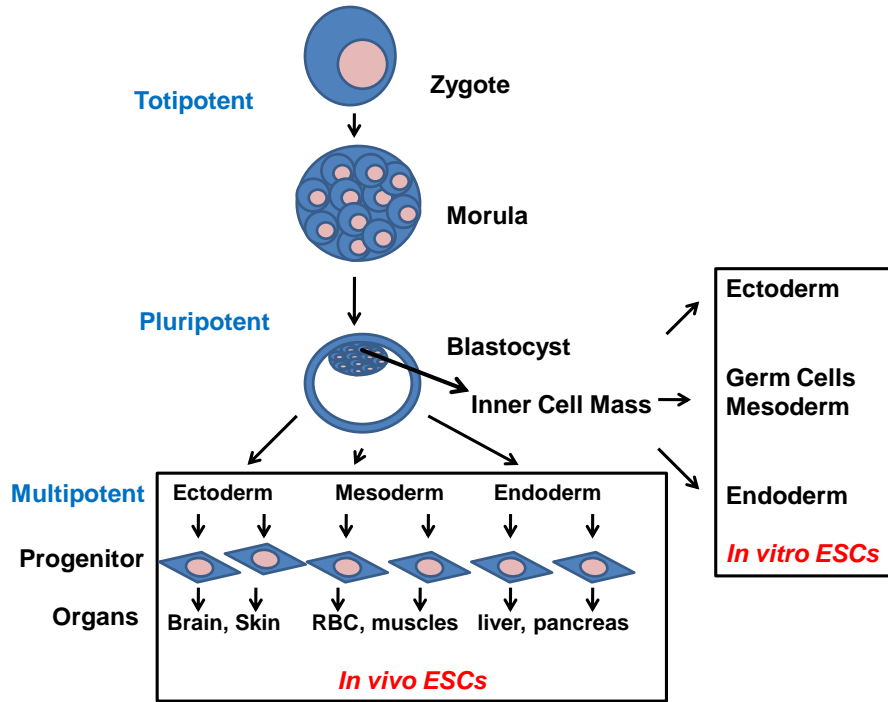
Figure 1

Leukemia Inhibitory Factor

LIF is a member of the Interleukin (IL)-6 family of cytokines. The LIF receptor (R) consists of LIF-Rβ and the receptor gp130 (Figure 2). The binding of LIF to its receptor on mES cells activates two major pathways, the Janus tyrosine associated kinase (JAK) - signal transducer activator of transcription 3 (STAT-3) Pathway and the SHP2-ERK Pathway (Figure 2) (Burdon, Chambers et al. 1999; Burdon, Stracey et al. 1999). LIF binding to LIF-Rβ activates the JAKs which phosphorylate the Src Homology 2 (SH-2) domain of its downstream target, STAT-3. Once STAT-3 is phosphorylated it undergoes autophosphorylation and forms a STAT-3 homodimer. The STAT-3 dimer then translocates to the nucleus and binds to DNA binding sites of genes which control murine ES cell self -renewal (Friel, van der Sar et al. 2005).

Another pathway that is activated by LIF is the SHP2-ERK pathway via the gp130 receptor. The LIF-gp130R activates the RAS/MAPK pathway. SHP2 associates with Gab1, which causes the activation of Ras which induces the transactivation of MAPKs and ultimately ERK phosphorylation. This pathway is thought to lead to differentiation of murine ES cells (Burdon, Stracey et al. 1999; Burdon, Smith et al. 2002).

A recent report has shown that mesenchymal stem cells which give rise to bone cartilage, adipose tissue, and chondrocytes can remain in an undifferentiated/ proliferating state when Pam_3Cys is bound to its receptor TLR-2 in these cells (Pevsner-Fischer, Morad et al. 2007). This is an important finding because in murine ES cells the activation of the MAPK/ERK pathway is thought to promote differentiation.

Figure 2. LIF/STAT3 Paradigm for ES cell Self Renewal. Some parts of the Figure were adapted from (Friel, van der Sar et al. 2005). LIF activates the JAKs which then activates STAT3 and forms a homodimer which activates expression of Nanog and Oct-4 genes. TLRs may activate the Gab1-Shp2 MEK/ERK pathways.

Current LIF/STAT3 Paradigm for ES cell Self Renewal
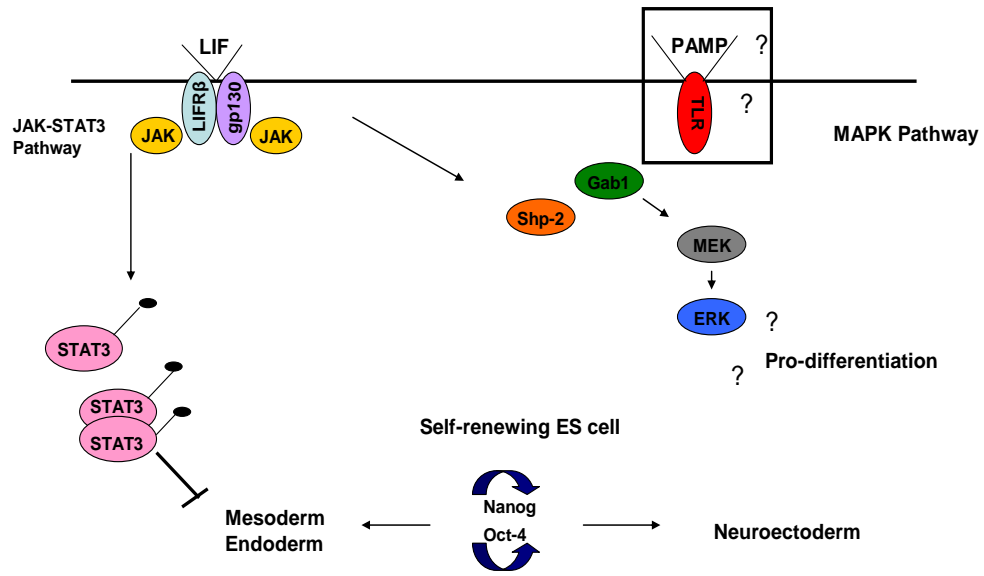
Figure 2

Previous studies have shown that activation of gp130 Rβ can elicit production of IL-6, TNF-α, and IL-11 (Hilton, Hilton et al. 1994; Friel, van der Sar et al. 2005; Jenkins, Roberts et al. 2005), which are cytokines produced in response to activation by TLRs. The IL-6 cytokine family has a role in the biological activities of multiple hematopoietic lineages and helps to maintain pluripotency of mouse (m) ES cells (Burdon, Chambers et al. 1999). For example, they can stimulate the production of immature hematopoietic progenitor cells (HPC) when used in synergy with stem cell factor (SCF) and interleukin (IL)-3 (Dexter, Allen et al. 1978; Jenkins, Roberts et al. 2005) and directly stimulate megakayocytes to become functionally mature. The binding of IL-6 to the gp130 subunit induces gp130 homodimerization which leads to activation of JAKs (Heinrich, Behrmann et al. 1998). This ultimately leads to the phosphorylation of STAT-1 or STAT-3 (Gerhartz, Heesel et al. 1996).

Studies have shown TLR2 activation can lead to an increased production of IL-6 and TNF-α in macrophage cells. This cytokine production will be used as one means to determine if TLR-2 is functional in mES cells in our studies.

Self Renewal Markers

There are several mouse/human ES cell self renewal markers that are distinct in undifferentiated ES cells. They include Nanog, Oct 4, and Sox 2 (Niwa, Miyazaki et al. 2000; Avilion, Nicolis et al. 2003; Sato, Meijer et al. 2004; Friel, van der Sar et al. 2005). Mouse ES cells in un-differentiated states also express distinctive markers. These include an isozyme of alkaline phosphatase and SSEA-1. (Solter and Knowles 1978; Wobus,

Holzhausen et al. 1984; Thomson, Itskovitz-Eldor et al. 1998; Reubinoff, Pera et al. 2000; Reubinoff, Pera et al. 2001; Xu, Inokuma et al. 2001; Henderson, Draper et al. 2002; Friel, van der Sar et al. 2005). Oct-4 and Sox-2 but not Nanog are thought to be regulated by the transcription factor STAT-3 (Niwa, Burdon et al. 1998; Thomson, Itskovitz-Eldor et al. 1998; Pesce, Anastassiadis et al. 1999; Friel, van der Sar et al. 2005). Nanog is downstream of the transcription factor Oct-4, and they both play a critical role in murine ES cell self renewal (Niwa, Miyazaki et al. 2000; Friel, van der Sar et al. 2005). Although the expression of these markers are considered one way to determine a non-differentiated state, the best way to determine non-differentiated state is putting the ES cells back into a blastocyt to develop into a mouse, or place the cells into a mouse to for teratomas.

STAT-3 activation is not involved in TLR activation pathway in macrophages therefore this pathway was not studied in ES cells (Hu, Chen et al. 2007). The regulation of STAT-3 is critical in the homeostasis of hematopoiesis because STAT-3 when hyper-activated can act as an oncogene (Bromberg, Wrzeszczynska et al. 1999). Previous data has shown that STAT-3 plays an important role in the homeostasis of the undifferentiated and differentiated states of murine ES cells by activating ES cell self renewal marker Oct-4 (Niwa, Burdon et al. 1998; Friel, van der Sar et al. 2005; Guo, Mantel et al. 2008).

Toll-Like Receptor Pathway

Toll-Like Receptors (TLRs) play a critical role in innate and adaptive immune responses against microbial pathogens (Akira, Takeda et al. 2001; Kambris, Hoffmann et

al. 2002; Beutler, Hoebe et al. 2003; Gangloff, Weber et al. 2003; Beutler, Hoebe et al. 2004; Hoebe, Janssen et al. 2004; Beutler, Jiang et al. 2006). Today there are ten TLRs reported in humans and 12 TLRs reported in mice (Takeda and Akira 2005). They include TLR-1-10 in humans and TLR1-9, RP105/11, MD1/12, and MD2/13 in the murine system. TLR ligands better known as PAMPs include lipopolysaccharides (LPS) of Gram negative bacteria such as *Salmonella or Escherichia coli* which activate TLR-4 (Figure 3) (Poltorak, He et al. 1998; Hoshino, Takeuchi et al. 1999). Lipoprotein, lipopeptides, and peptidoglycan of Gram positive bacteria are recognized by TLR-2. TLR-2 forms a TLR-1/2 and or 2/6 heterodimer which recognizes triacyl and diacyl lipoproteins respectively (Figure 3 and 4) (Takeuchi, Hoshino et al. 1999; Ozinsky, Underhill et al. 2000; Takeuchi, Kaufmann et al. 2000; Takeuchi, Sato et al. 2002). Pam_3Cys is a synthetic triacyl lipoprotein and known activator of the TLR-2/1 heterodimer (Shimizu, Kida et al. 2008).

Figure 3. Toll Like Receptor Pathway. Some parts of this figure were adapted from (Naumann 2000; Kawai and Akira 2005). TLR-1 and TLR-2 form a heterodimer which is triggered by lipoproteins such as Pam_3Cys, while TLR-6 and TLR-2 form a heterodimer that is activated by diacyl lipoproteins. LPS is the PAMP that triggers TLR-4 to illicit an immune response.

Toll Like Receptor Pathways
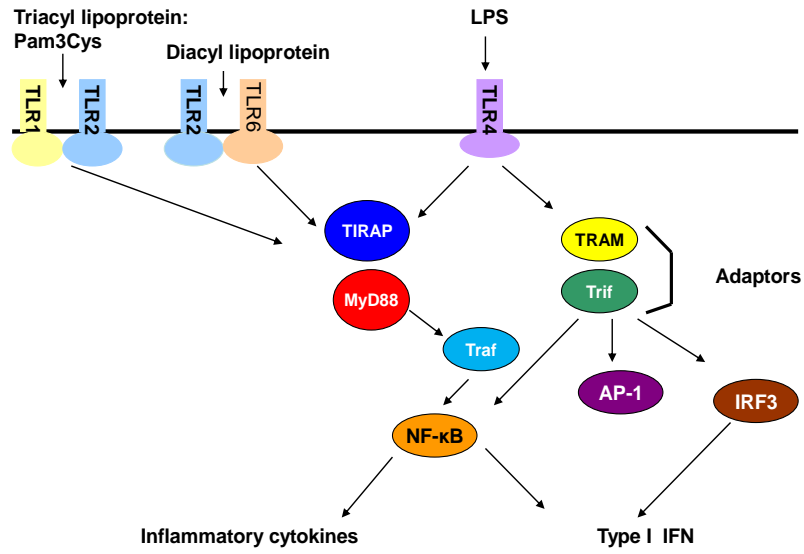
Figure 3

Figure 4. TLR-2 mediated signaling pathways adapted from (Arbibe, Mira et al. 2000; Naumann 2000; Kawai and Akira 2005; Kawai and Akira 2006). Ligands bind to heterodimers TLR-1/2, and TLR-2/6. Triacyl lipoproteins bind TLR-1/2 and Diacyl lipoproteins bind to TLR-2/6. They both mediate their activities through the adaptor molecules MyD88 and TIRAP, and recruit IKK which phosphorylates NF-κB and cause it to translocate to the nucleus.
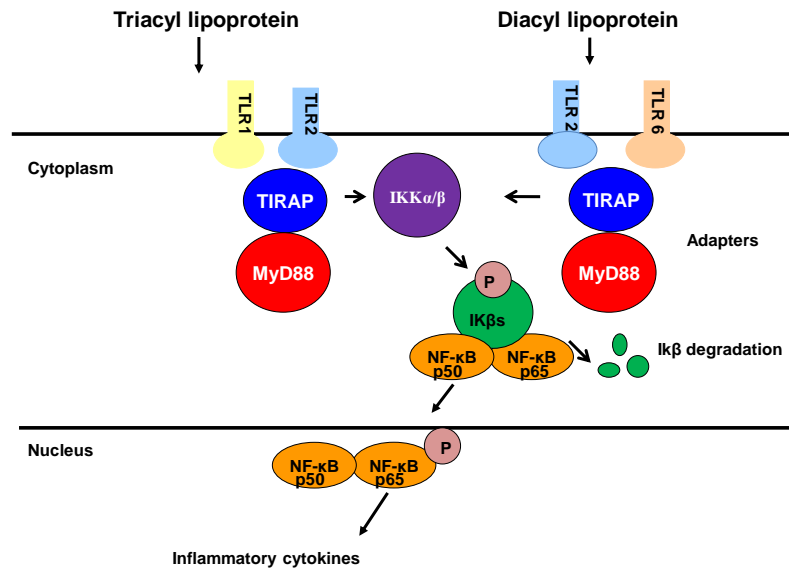
Figure 4

TLR stimulation by PAMPs trigger signaling cascades that require intracellular adaptive proteins such as myeloid differentiation primary response protein 88 (MyD88), Toll Interleukin-1 receptor (TIR) domain-containing adaptor inducing interferon-beta (IFN-β) also known as TRIF, and Interleukin 1 receptor associated kinase (IRAK) (Figure 3 and 4) (Ferrandon, Imler et al. 2004; Takeda and Akira 2005). Activation of most TLRs induce the production of cytokines via the MyD88 pathway, excluding TLR-3 which uses a MyD88-independent/TRIF pathway (Oshiumi, Matsumoto et al. 2003; Yamamoto, Sato et al. 2003). This leads to the activation of transcription factors such as activating protein 1 (AP-1), nuclear factor kappa-B (NF-κB), and interferon regulatory factors (IRFs) (Figure 4).

Nuclear Factor of Kappa light polypeptide gene enhancer in B cells

Nuclear factor kappa B (NF-κB) is a Rel family transcription factor composed of homo and heterodimers such as p50 and p65 subunits (Arbibe, Mira et al. 2000) (Figure 4). NF-κB plays an important role in mammalian immunity and is activated by TLRs (Rosetto, Engstrom et al. 1995). TLRs initiate this pathway leading to NF-κB activation via signals through MyD88, IRAK, and TNF receptor associated factor -6 (TRAF6) adaptor protein molecules (Arbibe, Mira et al. 2000; Bowie and O'Neill 2000; Irie, Muta et al. 2000) (Figure 4). The inactive form of NF-κB is a cytoplasmic heterodimer that consist of p50 and p65 subunits (Arbibe, Mira et al. 2000). Upon TLRs activation, IκB kinases (IKKs) are activated. IκB, the NF-κB inhibitor is degraded thus allowing NF-κB to translocate to the nucleus to mediate transcriptional gene activation (Mercurio, Murray

et al. 1999; Zandi and Karin 1999; Arbibe, Mira et al. 2000; Arsura, Mercurio et al. 2000) (Figure 4).

Previous studies have shown NF-κB can induce tissue repair genes, inflammation, and neutrophil-specific chemokines in mouse embryonic fibroblasts and macrophage cells that are in a necrotic state (Li, Carpio et al. 2001). Therefore, it is thought that the triggering of NF-κB by TLRs may play a role in survival of the ES cells. Thus, we performed survival assays to determine if TLR activation can enhance survival of mES cells.

Previous studies have shown TLRs 2 and 4 to be expressed in human hematopoietic progenitor cells and when stimulated with their ligands, to help replenish cells of the innate immune system *in vitro* (Banchereau and Steinman 1998; Nagai, Garrett et al. 2006). Previous studies have also shown that mesenchymal stem cells (MSC) express all TLRs except 9 and when stimulated with their ligands prevent differentiation of MSCs into chondrocytes, adipose tissue, and cartilage, thus causing an increase in MSC proliferation in their non-differentiated state (Pevsner-Fischer, Morad et al. 2007). It was this information that interested us in studying a role for TLRs and their ligands in ES cell function. Activation of TLRs can promote the production of multiple cytokines and chemokines at a transcriptional level thus influencing the adaptive immune response (Figure 4). Some cytokines and chemokines whose secretion is induced by TLRs, are not produced or expressed only at low levels in the conditioned media of undifferentiated E14 ES cells. This includes TNF-α, and IL-6. (Guo, Graham-Evans et al. 2006). Therefore in the current studies, the expression of these cytokines and

16

transcription factors via the IKK-αβ/NF-κB signaling pathway upon TLR stimulation was performed to determine the functionality of specific TLRs in mES cells

Cytokines IL-6 and TNF-α

IL-6 is a member of the IL-6 cytokine family and elicits a response via the gp130 receptor-beta (Rβ) subunit. Once IL-6 binds to its soluble IL-6 receptor-alpha (Rα) subunit, gp130 homodimerizes and activates the JAKs. JAKs then activate STAT-1 or STAT-3 (Gerhartz, Heesel et al. 1996). Previous studies have shown that homeostasis of STAT-3 activation plays a role in mediating the cellular production of immature or committed hematopoietic progenitors, and is regulated by IL-6 production (Jenkins, Roberts et al. 2005; Chung, Park et al. 2006; Jenkins, Roberts et al. 2007). Previous reports have shown that in the absence of IL-6 signaling there is a decrease in STAT3 hyper-activation and abnormal lymphopoiesis. Previous reports also have shown that if IL-6 is deleted in mice there are abnormalities of both immature and committed progenitors from multiple lineages from the spleen and bone marrow (Dexter, Allen et al. 1978; Zipori 1989; Zipori 1990; Zipori 1992; Bernad, Kopf et al. 1994). IL-6 plays a role in mediating pathological hematopoietic and lymphoid responses by STAT-3 hyper-activation (Jenkins, Roberts et al. 2007).

The over expression of IL-6 could lead to abnormalities i.e. tumorgenesis and human lymphoproliferative and myeloproliferative diseases, including multiple myeloma (MM), non-Hodgkin's lymphoma (NHL), and acute myeloid leukemia (AML) which all display STAT3 deregulated activation (Jenkins, Roberts et al. 2007). We considered it

important to determine the role of TLRs in mESC differentiation, survival/proliferation and self renewal. Some TLRs are capable of inducing the production of inflammatory cytokines, such as IL-6, by antigen presenting cells. To date, IL-6 hasn't been shown to be produced in mESCs (Guo, Graham-Evans et al. 2006).

TNF-α is a cytokine produced by TLR-2/4 signaling, and is required for dendritic cell maturation and migration. (Kurt-Jones, Sandor et al. 2004; Adema, de Vries et al. 2005). It is a major player in anti-tumor immunity and has been shown to reduce the activation of integrin αVβ3, an adhesion receptor that plays a key role in tumor angiogenesis, and causes a decrease in endothelial cell adhesion and survival (Ruegg, Yilmaz et al. 1998).

The cytokines whose production are signaled via TLRs play a role in cell survival and replenishment of the innate immune system and we sought to determine if they play a role in the proliferation and survival of undifferentiated mES cells. ES cells are in a highly regulated environment and respond to various growth factors, chemokines, and cytokines differently. Studies have shown that bone morphogenic protein-4 (BMP-4), an anti neural factor in embryos in conjunction with LIF helps to maintain ES cells in a pluripotent state. Yet when ES cells are in BMP media alone, they differentiate into mesoderm and hematopoietic cells (Hilton, Hilton et al. 1994; Johansson and Wiles 1995; Ying, Stavridis et al. 2003). BMP interacts with serine threonine receptor heterodimers, while TLRs interact with MyD88 which activates serine threronine IRAK kinases IRAK1 and IRAK4 (Hilton, Hilton et al. 1994; Ying, Stavridis et al. 2003). BMP has no direct

effect on the STAT-3 pathway nor does it inhibit the MAPK/ERK pathway, suggesting that it acts in parallel with LIF/STAT-3 (Hilton, Hilton et al. 1994).

The cytokines produced by undifferentiated ES cells may be important in maintaining their stable environment and increase their survival and proliferation. When this niche is disrupted (i.e. when the ES cells are irradiated), there is an increase in levels of several cytokines whose production is induced upon TLR activation in other cell types. Therefore, we propose that these cytokines may play a role in ES cell survival because these cytokines may normally be used to maintain the hematopoietic stem cell niche (Hackney, Charbord et al. 2002; Zhang, Niu et al. 2003). The levels of these cytokines were at lower levels or not detected in normal mES cell conditioned media (Guo, Graham-Evans et al. 2006). The cytokines that were at higher levels under apoptotic conditions include IL-6 and TNFα. Production of the latter cytokines mentioned, are induced when either TLR-2 or TLR-4 are activated on other cell types such as MSC (Pevsner-Fischer, Morad et al. 2007).

AIMS

AIM 1: Determine if murine Embryonic Stem (ES) cells express functional Toll Like Receptors (TLR), and if their ligands can induce or modulate murine ES cell proliferation/survival, self renewal and/or differentiation in the presence of Leukemia Inhibitory Factor (LIF).

AIM 2: Clarify the roles of TLR ligands and their receptors on ES cell function under differentiating conditions in the absence of LIF.

MATERIALS AND METHODS

Part I

Do murine ES cells express Toll Like Receptors?

Cell Cultures

Wild-type ESC lines, E14, R1, CGR8 were cultured on gelatinized plates in Dulbecco's modified Eagle's medium (DMEM) with 15% ESC qualified fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY), 5.5x 10^{-2} mM β-mercaptoethanol (Gibco-BRL, Grand Island, NY) and 10^3 U/mL of LIF; (Chemicon, Temecula, CA). Raw264.71, a mouse macrophage cell line, was purchased from ATCC (Manassas, VA) and cultured in DMEM (Gibco, Grands Island, NY) with 15% FBS.

Primers

RT-PCR primers were designed and optimized as previously reported (Derbigny, Hong et al. 2007) (Table 1). Primers were purchased from Invitrogen (Carlsbad, CA).

Table 1. Primers used for RT-PCR

Product	Sense Primer	Anti Sense Primer	Product Size(bp)
TLR1	5'-GTGAATGCAGTTGGTGAAGAAC-3'	5'-GCTCATTGTGGGACAAATCCAA-3'	450
TLR2	5'-CTTGTTTCTGAGTGTAGGGGCT-3'	5'-CGAACCAGGAGGAAGATAAACT-3'	483
TLR3	5'-ACCCTTTCAAAAACCAGAAGAATC-3'	5'-GGACAGACGCTGTATATTGTTG-3'	521
TLR4	5'- TCAACCCCTTGAAGATCTTAAA-3'	5'-CAATTGGGTTCAAAGACATGTC-3'	459
TLR5	5'-CAGTATCAGCTGATGAGACATGAG-3'	5'-GACAGTACCGCAATAGGGATGG-3'	463
TLR6	5'- TACGGAGCCTTGATTTCCATGT-3'	5'-TGGACCTCTGGTGAGTTCTGAT-3'	485
TLR7	5'-AACCACATACCAAGCATCTCTC-3'	5'-AAATTAGGTGGCAAAGTGGTGG-3'	458
TLR8	5'-CAGAGTTGGATGTTAAGAGAGA-3'	5'-GTATATAACTGGTTGTCTTCCA-3'	459
TLR9	5'-GCCTGAGCCACACCAACATCCT-3'	5'-CCAGACCTTGGAACCAGGAAGA-3'	477

Adapted from (Derbigny, Hong et al. 2007)

RNA Extraction

5×10^5 E14 mES cells were seeded in 60 mm culture dishes and grown to confluency. Total cellular RNA was extracted using the QIAGEN RNeasy Kit[TM] according to manufacturer's instructions (Qiagen Inc., Valencia, CA, USA). RNA was stored in RNAse- free water at -80°C.

DNase Treatment

RNA samples were DNase treated using QIAGEN DNase free[TM] according to manufacturer's instructions (Qiagen Inc., Valencia, CA, USA).

Reverse transcriptase-polymerase chain reaction

Expression of TLRs 1 -9 and GAPDH were measured using a semi quantitative RT-PCR one-step AccessQuick [TM] RT-PCR system. (Promega, Madison, WI). The oligonucleotide primers used for TLR-1 –TLR-9 and GAPDH have been reported (Derbigny, Hong et al. 2007). Total RNA was isolated from the E14 mESC line and Raw 264.71 mouse macrophage cells using RNeasy minicolumns (QIAGEN, Valencia, CA). All RNA samples were treated with RNasefree DNase I (QIAGEN, Valencia, CA) to remove genomic-DNA contamination and were quantified by spectrophotometric analysis. RNA integrity was confirmed by agarose gel electrophoresis. Using 1μg of total RNA as the template for each reaction, RT-PCR was accomplished by using a polymerase kit (Access RT-PCR; Promega, Madison, WI). Cycling conditions were as follows: 1 min and 30 s of initial denaturation at 95°C, followed by eight cycles of 30 s at 95°C, 15 s at 60°C, and

30 s at 72°C. After the initial 8 cycles, the 30-s 72°C extension cycle was increased 3 s per cycle for 25 cycles. During the 40th cycle, the 72°C extension was 3 min to complete the RT-PCR. Reactions were also amplified in the absence of reverse transcriptase as negative controls. PCR products were electrophoresed on 1.5% agarose gels. Each DNA band was visualized by staining with ethidium bromide. Experiments were done in triplicate.

Primary antibodies and TLR ligands

Primary antibodies were: isotope-control PE rat IgG2a (eBioscience, San Diego, CA; 17-4331), isotype-control APC rat IgG2b (eBioscience, San Diego, CA;12-4321), TLR2 /CD282 anti-mouse clone 6C2 (eBioscience, San Diego, CA ;17-9021) and anti-mouse TLR-4/MD2 (eBioscience , San Diego, CA; 12-9924), p-IKKα/β (Cell Signaling, Denver, MA; 2697S), myeloid derived factor 88 (MyD88) (Abcam, Cambridge, MA; ab 2068), total IKKα/β (Santa Cruz, Santa Monica, CA; sc7607), NF-κB p65 (Upstate Cell Signaling Solutions, Temecula, CA; 0701049995), PARP (Cell Signaling, Denver, MA; 9542), and blocking anti-TLR-2 antibody, T2.5 (Biolegend, San Diego, CA; 121802), and ERK1/2 (Cell Signaling, Denver, MA; 9102). TLR ligands included: TLR-2 agonist, Pam$_3$Cys, and TLR-3 agonist, Poly I:C purchased from (Invitrogen, Carlsbad, CA). TLR4 agonist, LPS from Salmonella was from Sigma Aldrich, St. Louis, MO.

Flow Cytometric Analysis for TLR-2 and TLR-4

An aliquot of 1x 10^6 cells was washed in PBS containing 1% BSA (PBS-1% BSA) three times. 100µl of staining buffer (PBS containing 1% BSA and .5% EDTA) was added to the cell pellet along with 5µL of TLR-1-PE, TLR4-PE, or TLR-2-APC antibody for 1hr in the dark at 4°C. Cells were washed three times with wash buffer (PBS containing 1% BSA) and 300µL of wash buffer was added to cells and cells for analysis by flow cytometry. IgG2a was used as an isotype control for TLR1-PE, and TLR4-PE and IgG2B was used as an isotype control for TLR-2-APC

Flow Cytometric Analysis for Oct-4, SSEA-1, SOX 2, and KLF-4

Wild-type ESC lines E14, R1, and CGR8 were cultured with and without TLR-2, TLR-3, and TLR-4 agonists. Cells were collected after days 1, 2, 3, 4, and 5 for proliferation assay and after 30 minutes, 1 hr, and 4 hrs of TLR-agonist treatment. An aliquot of 1x 10^6 cells was washed in PBS containing 1% BSA (PBS-1% BSA) and incubated with anti-mouse CD16/CD32 receptor monoclonal antibody at 1µg/100µL (Pharmigen, San Diego, CA) to block non-specific binding of immunoglobulin to mouse FcIII/II receptors, and cells were used for SSEA-1 antibody staining. Cells analyzed for SSEA-1 expression were incubated with a 1:20 dilution of monoclonal anti-SSEA-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at 4°C.

Cells were then washed and incubated with a 1:100 dilution of FITC: goat anti-mouse IgM antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and analyzed. Remaining cells were fixed and permeabilized with Cytoperm/Cytofix (BD Biosciences,

San Jose, CA) and stained with a 1:100 dilution rabbit-mouse Oct-3/4 polyclonal antibody (Chemicon, Temecula, CA) and KLF-4 and Sox-2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 1hr at 4°C in the dark. Cells were washed 3 times with 1mL of 1x Perm/Wash Buffer, followed by staining with 1:100 dilution of FITC: goat anti-mouse IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Finally, cells were washed 3 times with 1x Perm/Wash Buffer and resuspended in 300µL of 1x Perm Wash Buffer for FACScan analysis (Becton Dickinson, Sunnyvale, CA).

Western Blot p-IKK, Total IKK, and MyD88

Cells were lysed with 100µL of MPER (Pierce, Rockland, IL) with proteinase and phosphatase inhibitors added (40µL of proteinase and phosphatase inhibitors added to 4mL of MPER). The 1.5mL centrifuge tube was vortexed to resuspend the pellet into solution. Total protein in supernatant was collected.

After protein was collected, we determined protein concentration using BCA analysis reagents (Pierce, Rockland, IL). Standards were made using 200µL of BCA reagents and 20µL of each standard (125µg/mL-2000µg/mL). Samples were plated at a 1:10 dilution. All sample and standards were performed in triplicate. The plate was incubated at room temperature for 2 hours and analyzed using a spectrophotometer with a plate reader at 540nm.

After protein concentration was determined, 40µg/40µL of each protein sample was used to perform Western blotting analysis of E14 cells. Protein was electrophoresed on a 4-12% gel (Invitrogen, Carlsbad, CA) in the X Cell II Sure Lock Apparatus

(Invitrogen, Carlsbad, CA) at 120V for 1 hr. Next the gel was transferred to the PVDF membrane (Millipore, Temecula, CA) in the Cell II Blot Module (Invitrogen, Carlsbad, CA) in the X cell II Sure Lock apparatus (Invitrogen, Carlsbad, CA) at 25 V at 4°C for 2hrs. The MyD88 anti-rabbit (Chemicon, Temecula, CA), p-IKK anti -rabbit, Total- IKK, anti-rabbit, and β-actin anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used. β-actin anti-mouse antibody was used as a loading control.

Enzyme Linked Immunosorbent Assay (ELISA) for Protein

Cytokine release was assessed by enzyme linked immunosorbent assay (ELISA) with 5×10^5 ESCs seeded in 24 well plates. Twenty-four hours later, the media was replaced with DMEM with or without the ligands for TLR-2 or TLR-4 respectively. IL-6, TNF-α, and IFN-β protein amounts were determined by ELISA (Ready-SET-G0! ELISA kit, (eBioscience San Diego, CA) according to manufacturer's instruction. Standard curves were established using mouse recombinant IL-6, TNF-α and IFN-β growth factors respectively. The assay detection limit was 4 pg/mL protein.

IL-6, TNF-α and IFN-γ mRNA and protein expression

Total RNA was isolated by TRIZOL preparation followed by phenol chloroform/isoamyl alcohol extraction and ethanol precipitation. Changes in IL-6, TNF-α, and IFN-γ RNA levels in mES cells were analyzed by quantitative real time PCR polymerase chain reaction. Primers used for this assay were obtained from the Dent laboratory and are shown in Table 2. Relative changes in IL-6, TNF-α, and IFN-γ were determined using the

27

$2^{-\Delta\Delta Ct}$ method. Data are expressed as fold change. To test our q-RT-PCR primers we analyzed cytokine expression in Raw264.71 mouse macrophage-like cells stimulated with TLR-2 and TLR-4 ligand.

Table 2. Primers for qRT-PCR

Product	Sense	Anti-Sense
TNF-α	5'-CACAAGATGCTGGGACAGTGA-3'	5'-TCCTTGATGGTGTGCATGA-3'
IFN-γ	5'-TCAAGTGGCATAGATGTGGAAGAA-3'	5'-TGGCTCTGCAGGATTTTCATG-3'
IL-6	5'-CCAGAAACCGCTATGAAGTTCCT-3'	5'-CACCAGCATCAGTCCCACGA-3'
β-tubulin	5'-CTGGGAGGTGATAAGCGATGA-3'	5'-CGCTGTCACCGTGGTAGGT-3'

Obtained from Dent laboratory

Proliferation Assay

We cultured E14 ES cells in the presence of LIF and the TLR-2 activator Pam$_3$Cys in our experiments for cell suspension proliferation assays. ES cells under undifferentiated conditions without Pam$_3$Cys stimulation served as a control. One hundred thousand E14 mESCs were stimulated with 0.1μg/mL, 0.5μg/mL, and 1.0μg/mL of Pam$_3$Cys in 5 mL of undifferentiated media. Incomplete Media includes DMEM for undifferentiated ES cells (Hyclone, Waltham, MA), 15% FBS, 1% Penicillin/Streptomycin (Gibco, Grand Island, NY), 1% Pyruvate (Gibco, Grand Island, NY), 1% MEM Essential Vitamins (Gibco, Grand Island, NY), and 1% L-Glutamine (Gibco, Grand Island, NY). Complete Media was made with 50mL of incomplete media plus LIF at [1:10000] and β2-mercapthoethanol (2ME) at [1:1000] which were added fresh. Next the ES cells were incubated in a 5% CO_2 37°C incubator for 5 days.

To harvest cells for counting each day cells were washed with 3mL of PBS, trypsinized with 1mL of trypsin-EDTA (Gibco, Grand Island, NY), and placed in the incubator for 5 minutes to remove cells from the bottom of plates. ES cells were washed with 5mL of incomplete media and spun down for 5 minutes at 12,000 rpm. Supernatant was removed and 1mL of incomplete media was added to the cells. Next 50μL of the cells were stained with 50μl of Trypan Blue (Gibco, Grand Island, NY) and 10μL of the cells was counted on the hemacytometer. This was done for samples harvested each day for the cells treated with or without Pam$_3$Cys ligand.

Survival Assay for ESCs in the Presence of LIF

ESC growth depends on serum. After withdrawal of serum from plates <25% confluency, 95% of ESCs die within 96 hours. To determine the effects of TLR-2 ligand, Pam_3Cys on ESC survival, studies evaluated control medium, 200ng/mL SDF-1/CXCL12, 0.1μg/mL, 1.0μg/mL, and 10μg/mL of Pam_3Cys, and 0.1μg/mL, 1.0μg/mL, and 10μg/mL of LPS. Reagents were added at the beginning of the experiments, and mES cell cultures were initiated without serum in 1% methylcellulose-based DMEM ($5.5x\ 10^{-2}$mM 2-ME and 10^3U/mL LIF) (Chemicon, Temecula, CA) at 2000 cells/mL. Serum was added at 0, 24, 48, or 96 hours to each group and colonies scored 7 days after addition of serum. The undifferentiated status of the cells was checked by staining of the cells with anti-mouse Oct-4, Sox-2, KLF, and SSEA-1 antibody.

Apoptosis Assay

To analyze mESC's undergoing apoptosis, cell cultures were subjected to serum withdrawal in the presence of LIF. Reagents were added at the beginning of cultures as followed: Control medium, TLR-2 ligand, Pam_3Cys (10μg/mL), TLR-4 ligand, LPS (10μg/mL), and SDF-1/CXL12 (200ng/mL). Cells were collected at days 1, 2, 3, and 4 after serum withdrawal and were stained with Annexin V (BD Biosciences, San Jose, CA). After withdrawal of serum for 4 days, mESCs were stained with undifferentiated markers Oct4, KLF4, SSEA1, and Sox2, respectively to determine if the cells remained undifferentiated.

Day 4 and Day 6 EBs in Suspension

We plated 1000 E14 mES cells/mL with or without 10μg/mL or 20μg/mL of Pam$_3$Cys in IMDM media in the absence of LIF and mixed cells well in a 50mL conical tube before they were plated into a 35mm tissue culture plate. EB's are formed from ES cells in the absence of LIF. We plated cells in the absence of LIF for 4 and 6 days.

Flow Cytometry of Day 4 and Day 6 EBs

 EB's were accessed by flow cytometry for TLR-2 and TLR-4 and for non-differentiation markers like SSEA-1, Oct-4, Sox-2, and KLF-4 to determine if TLR-2 ligand will prevent the ES cells from differentiating to EB's, therefore we only checked undifferentiated markers and not for increases in lineage markers or cell types.

Western Blot of Oct-4, phosphorylated-ERK and Total ERK

We performed Western blot analysis to determine if the TLR-2 ligand in the absence of LIF increased differentiation or helped to maintain stemness of ES cells, by looking at the expression of Oct-4 in the EBs treated with increasing doses of TLR-2 ligand as compared to EB's not treated with ligand, and its control, mES cells cultured in the presence of LIF. Cells were lysed with 100μL of MPER (Pierce, Rockland, IL) with proteinase and phoshotase inhibitors added (40μL of proteinase and phosphotase

inhibitors added to 4mL of MPER). The 1.5mL centrifuge tube was vortexed to resusspend pellet into solution and spun at 4°C for 10 minutes.

After protein was collected, we detected the protein concentration using BCA analysis reagents (Pierce, Rockland, IL). Standards were made using 200µL of BCA reagents and 20µL of each standard (125µg/mL-2000µg/mL), and samples were plated at 1:10 dilution. All samples and standards were done in triplicate. The plate was incubated at room temperature for 2 hours and analyzed using a spectrophotometer with a plate reader at 540nm.

After protein concentration was determined, 40µg/40µL of each protein sample was used to perform Western blotting analysis of E14 Day 4 and Day 6 EBs. Protein was electrophoresed on a 4-12% gel (Invitrogen, Carlsbad, CA) in the X Cell II Sure Lock Apparatus (Invitrogen, Carlsbad, CA) at 120 V for 1 hr. Next the gel was transferred to the PVDF membrane (Millipore, Temecula, CA) in the Cell II Blot Module (Invitrogen, Carlsbad, CA) in the X cell II Sure Lock apparatus (Invitrogen, Carlsbad, CA) at 25 V at 4°C for 2hrs. The Oct-3/4 (Chemicon, Temecula, CA), p-ERK anti -rabbit, Total ERK anti-rabbit, and β-actin (Santa Cruz Biotechnologies, Santa Cruz, CA) anti-mouse antibody was used to determine if the undifferentiated ES cells marker was expressed in differentiating conditions in the presence of TLR-2 ligand. β-actin anti-mouse antibody was used as a loading control.

RESULTS

<u>Part I</u>

mES Cells effects in the presence of LIF

TLR2 is expressed at the message level and on the surface of mES cells

Previous reports have shown that TLRs when activated on HSCs can cause HSCs to differentiate into B cells and dendritic cells thus helping in the replenishment of the immune system (Nagai, Garrett et al. 2006). Mesenchymal Stem Cells (MSCs) express functional TLRs and TLRs play a role in bone repair, proliferation of MSCs and prevent MSC differentiation into chondrocytes, adipocytes and osteoclasts (Fischer, et.al 2008). Little is known about TLRs and their role in mES cells, therefore we first determined if TLRs are expressed at the messenger RNA level using primers for TLRs 1-9 (Table 1). We found that mRNA for TLRs 1, 2, 3, 5, and 6 were expressed but not for 4, 7, 8, and 9 (Figure 5 A). Raw264.71, a mouse macrophage like cell line was used as a positive control expressing TLR-1-9 respectively (Figure 5 B and Table 2). There are antibodies available that detect TLR-2 and TLR-4 protein on the cell surface. Next, we determined if TLR-2 was expressed on the surface of mES cells and since mRNA for TLR4 was not expressed, we looked for protein expression of TLR-4, as a negative control. We found that TLR-2, but not TLR-4 was expressed on the surface of E14 mES cells (Figure 6) confirming the data shown at the gene level. Raw264.71 was used as a

positive control and expressed TLR-2 and TLR-4 on its surface. We also checked R1 and CGR8 mES cells for TLR-2 and TLR-4 expression. We show that these cell lines express TLR-2 but not TLR-4 (Figure 7 and 8). These data show that TLR-2 is expressed on three mES cells, so we then focused on determining if TLR-2 was functional on E14 mES cells.

Figure 5. A.) Murine embryonic stem cells express mRNA for TLRs 1, 2, 3, 5 and 6 obtained from wildtype, E14 C57/B6 cell line. mES cells were subjected to RT-PCR with primers specific for TLR-1, TLR-2, TLR-3, TLR-4, TLR-5, TLR-6, TLR-7, TLR-8, and TLR-9. PCR products were separated by 2% agarose gel electrophoresis and visualized with ethidium bromide, * marks appropriate size of RT-PCR products, -RT, control samples without reverse transcriptase were a negative control for all primers (not shown), +, GAPDH served as a positive control. B.) Raw264.71 was used as a positive control for all primers. These results are representative of the same findings in six separate experiments.

Toll Like Receptor:

M 1 2 3 4 5 6 7 8 9 +

A.

500bp--

E14
 Mouse ES cell line

B.

500bp

Raw264.71
Mouse macrophage
cell line
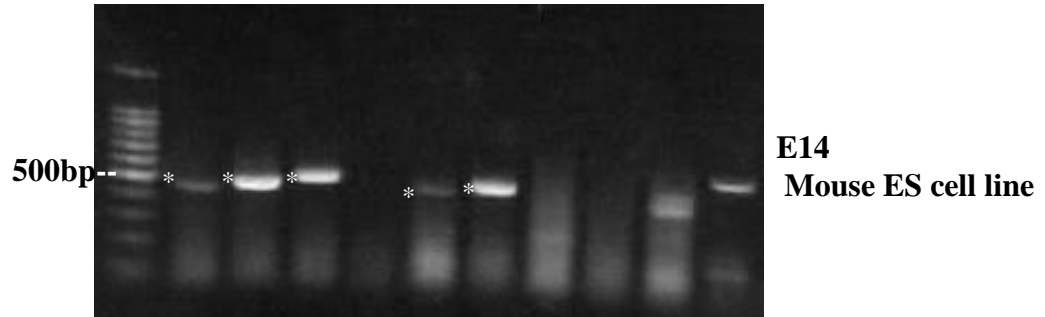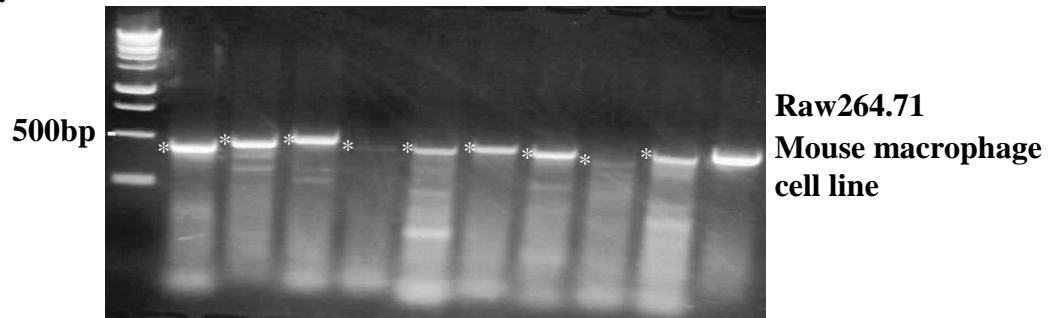
Figure 5

Table 3. TLR1-9 mRNA expression in cell lines

Cell line	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8	TLR9
E14	+	+	+	-	+	+	-	-	-
Raw264.71	+	+	+	+	+	+	+	+	+

TLR mRNA levels were determined by RT-PCR using mouse specific primers. Levels are noted as +, strong band, - band not detected.

Figure 6. Expression of TLR-2, and TLR-4 was analyzed on the surface of E14 mES cells. A. TLR-2 was expressed on mES cells compared to IgG2a isotype control, B.TLR-4 was not expressed on the surface of mES cells compared to isotype control IgG2b, Raw264.71 mouse macrophage cell line was used as a positive control to confirm that the antibodies worked properly C.TLR-2-APC, and D.TLR-4-PE. TLR-2-APC and TLR-4-PE protein levels were analyzed by flow cytometry. IgG2a-APC and IgG2b-PE were used as the respective isotype controls for both cell lines in each separate experiment. These results are representatives of similar finding in 4 experiments mcf=mean cell fluorescence, a measure of the expression levels of protein on the cell surface.

A. **E14**

B. **Raw 264.71**

C. **E14**

D. **Raw 264.71**

Figure 6

40
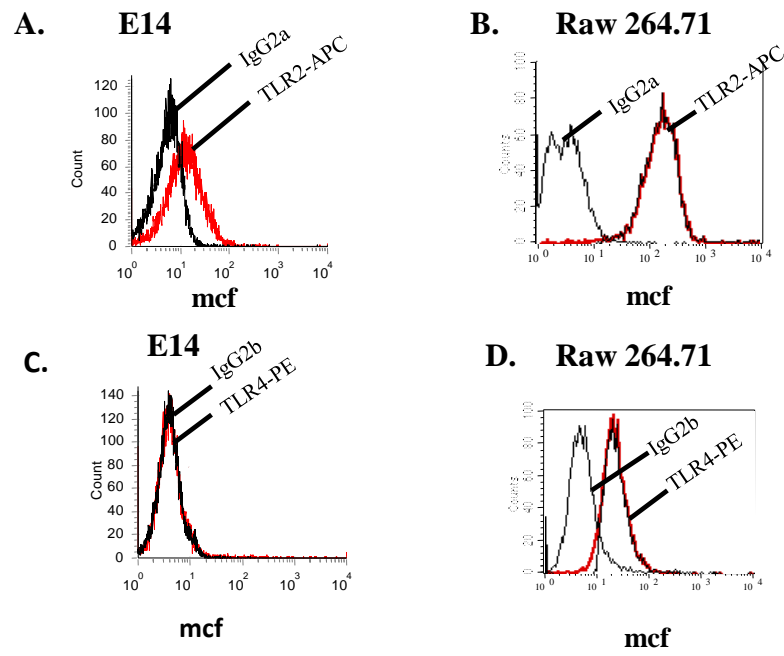
Figure 7. Expression of TLR-2, and TLR-4 was analyzed on the surface of CGR8 mES cells. A. TLR-2 was expressed on mES cells compared to IgG2a isotype control, B.TLR-4 was not expressed on the surface of mES cells compared to isotype control IgG2b, Raw264.71 mouse macrophage cell line was used as a positive control to confirm that the antibodies worked properly C.TLR-2-APC, and D.TLR-4-PE. TLR2-APC and TLR-4-PE protein levels were analyzed by flow cytometry. IgG2a-APC and IgG2b-PE were used as the respective isotype controls for both cell lines in each separate experiment. These results are representatives of similar findings in 4 experiments mcf=mean cell fluorescence, a measure of the expression levels of protein on the cell surface.
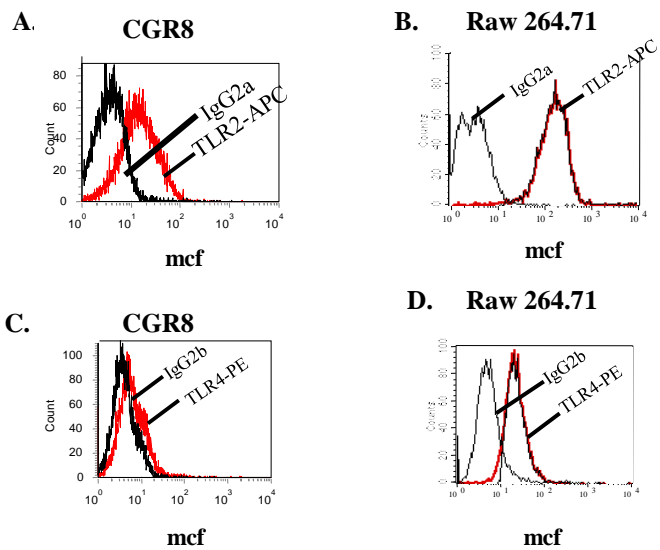
Figure 7

Figure 8. Expression of TLR-2, and TLR-4 was analyzed on the surface of R1 mES cells. A. TLR-2 was expressed on mES cells compared to IgG2a isotype control, B.TLR-4 was not expressed on the surface of mES cells compared to isotype control IgG2b, Raw264.71 mouse macrophage cell line was used as a positive control to confirm that the antibodies worked properly C.TLR-2-APC, and D.TLR-4-PE. TLR-2-APC and TLR-4-PE protein levels were analyzed by flow cytometry. IgG2a-APC and IgG2b-PE were used as the respective isotype controls for both cell lines in each separate experiment. These results are representatives of similar findings in 4 experiments mcf=mean cell fluorescence, a measure of the expression levels of protein on the cell surface.

A. **R1**

B. **Raw 264.71**

C. **R1**

D. **Raw 264.71**
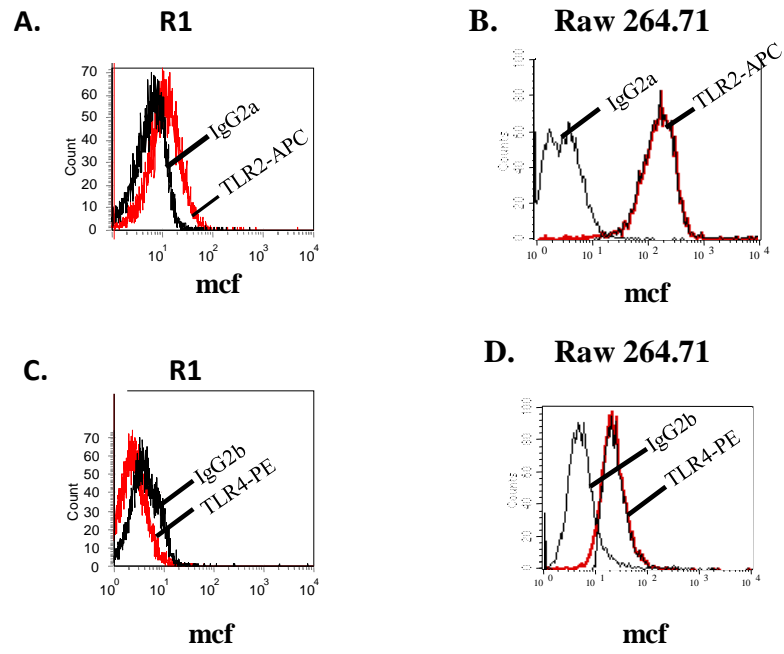
Figure 8

Treatment of mES cells with TLR-2 ligand Pam3Cys increases NF-κB nuclear translocation

mES cells were plated in the presence and absence of Pam$_3$Cys, a TLR-2 ligand, for 15 minutes to 1 hour in the presence of LIF. The concentration of TLR ligands were based on prior studies . Cells that were treated with 10μg/mL of Pam$_3$Cys, showed an increase in NF-κB nuclear expression compared to cells that were not treated. Cells were also treated with 10 μg/mL of bacterial lipopolysacchride (LPS), a TLR-4 ligand, but no enhancement in translocation was detected with LPS (Figure 9). We also checked for the expression of MyD88 and p-IKK in the E14 mESCs (Figure 10 and 11). We show that the E14 mESCs express MyD88, and after 15 minutes exposure to TLR-2 ligand Pam$_3$Cys or 30 minutes with Poly I:C, a ligand for TLR-3, the mES cells show an increase in phosphorylation of p-IKK demonstrating that TLR-2 and TLR-3 are functional likely by the MyD88/IKK pathway in the E14 mES cells (Figures 10 and 11).

TLR-2 Ligand Pam3Cys enhances E14 mES cell Proliferation

To determine if TLR-2 plays a role in mES cell proliferation, mES cells were treated with varying doses of the TLR-2 ligand, Pam$_3$Cys in presence of LIF. mES cells manifested a significant 2 fold increase in total cell number by day 3 at each dosage of Pam$_3$Cys (Figure 12 A). This increase was also noted at days 4 and 5 (Figure 12 A and B). In order to determine if the Pam$_3$Cys effects were mediated through TLR-2, we performed experiments in the presence and absence of a blocking antibody for TLR-2 (T2.5). The

antibody blocked enhanced proliferation on day 3 induced by Pam$_3$Cys (Figure 12 C and D).

Cytokine and chemokine expression levels upon stimulation with TLR-2 ligand

mESCs were treated with Pam$_3$Cys a ligand for TLR-2, Poly I: C, a ligand for TLR-3 and LPS, a ligand for TLR-4 in a 96 well plate for 18 hours. ELISA for IL-6, TNF-α and IFN-β were performed to determine if there would be an increase in cytokine release upon TLR challenge. mES cells showed no release of detectable IL-6, TNF-α, or IFN-β proteins (Figure 13).

In contrast, we did detect TLR-2-ligand enhancement of the mRNA expression for IL-6 (Figure 14 A), TNF-α (Figure 14 B.) and IFN-γ (Figure 14 C). Previous reports have shown that TNF-α, and IL-6 were not detected in normal media of the mES cell but were detected in mES cells that have undergone apoptosis (Guo, Graham-Evans et al. 2006). Next we treated the E14 mES cells with the ligands for TLR-2, TLR-3, and TLR-4 and we measured release of a number of cytokines and chemokines noted in Table 3. We did not detect enhanced release of cytokines or chemokines see e.g. Table 4. Samples were assessed for cytokine and chemokine production, and sensitivity for each are denoted at charlesriver.com. (Charles River Molecular Diagnostic Laboratory, Wilmington, MA). 100 µL of media from E14 mESCs in the presence and absence of TLR-2 ligands were sent Charles River Molecular Diagnostics Laboratory to measure 58 different cytokines and chemokines by ELISA (Table 3).

46

Next we wanted to determine if downstream targets to NF-κB would become activated. We looked for BCL-2 expression after 12 and 24 hours of TLR-2 stimulation (Figure 14 D). We saw an increase in BCL-2 expression after 24 hours in the presence of TLR-2 ligand, showing that NF-κB was functional in mES cells after 24 hours of ligand treatment. Expression of cytokines by the E14 mES cells showed minimal expression when compared to Raw264.71 macrophage- like cell line, the positive control after 12 hours (Figure 14 E, F, and G).

Figure 9. Pam$_3$Cys enhances NF-κB nuclear translocation in mES cells. 1x 10^6 mESCs were plated in DMEM media in the presence of LIF. Once the cells reached confluence, 10 μg/mL Pam$_3$Cys or 1μg/mL of LPS was added directly into the media. mES cells were harvested at 30 minutes or 1 hour and nuclear and cytoplasmic proteins were extracted. The cytoplasmic (A and C) and the nuclear (B and D) were quantified, run on SDS-Page gel and blotted with an anti NF-κB p65, PARP or total ERK1/2 antibodies, The autoradiographs were quantified by densitometry and shown the average results of 3 separate experiments (C and D).

Cytoplasmic Fraction   **Nuclear Fraction**
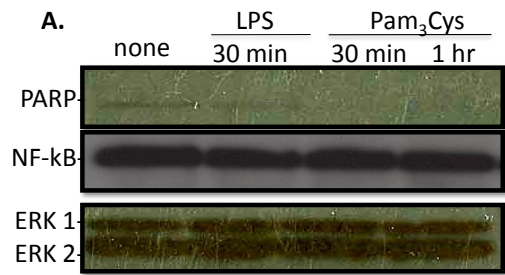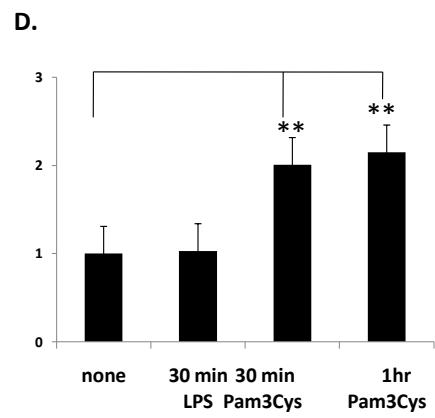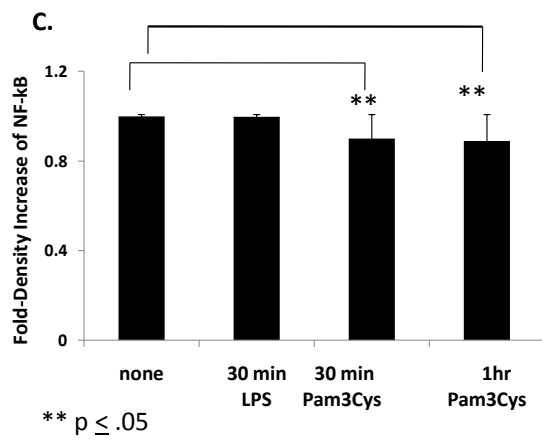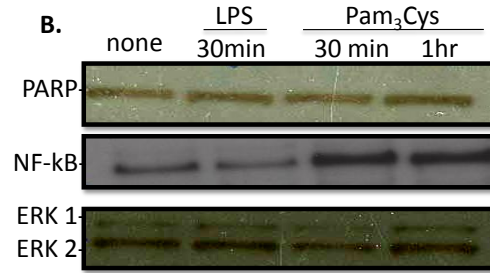
Figure 9

Figure 10. MyD88 is constituitvely expressed in mESCs with and without TLR-2 ligand

activation for 15 minutes to 4 hours. β-actin was used as a loading control. Results shown

are for one of two experiments with similar results.
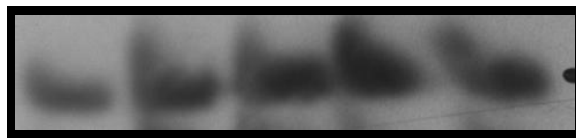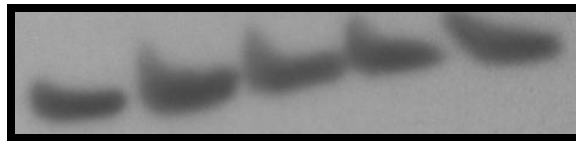
Figure 10

Figure 11. TLR-2- activation in mESC cause phosphorylation of IKK-α/β at 15 and 30 minutes with the ligand for TLR-2 and TLR-3. Total IKK and β-actin was used as a loading control for experiments. Shown are the results of 1 of two reproducible experiments.
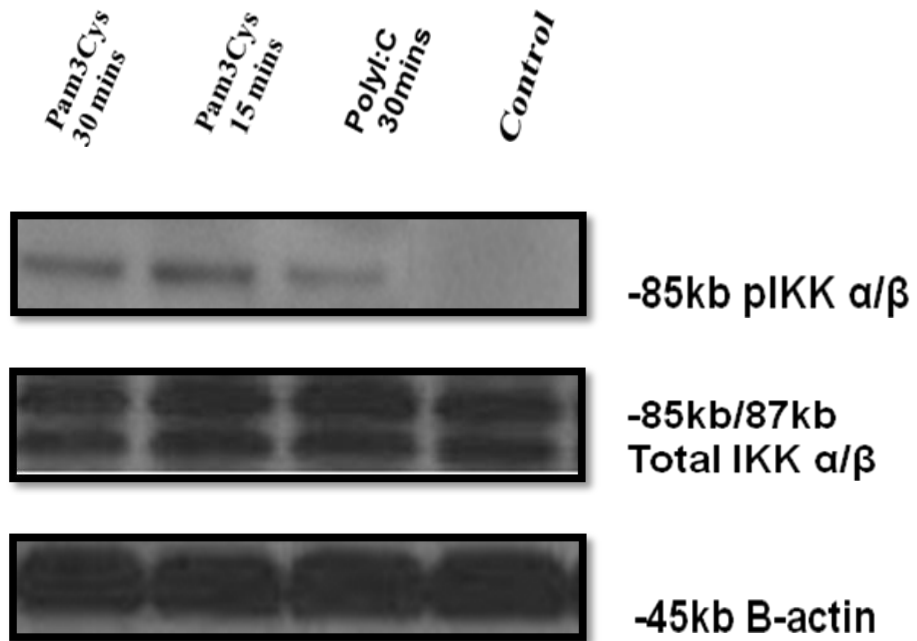
Figure 11

Figure 12. Pam$_3$Cys enhances ES cell numbers on Day 3, 4, and 5 in the presence of LIF. A. ES cells were treated with Pam$_3$Cys at varying doses for up to 96 hours. Significant changes are noted when compared to control for each day. B. Total cell number for each day with and without TLR-2 stimulation. C. Day 2 and Day 3 proliferation in the presence and absence of T.25 a blocking antibody for TLR-2 receptor with and without ligand. D. Day 3 proliferation assay in the presence and absence of TLR-2 ligand with and without blocking TLR-2 antibody (T.25). Results shown are the average of 3 experiments with each experiments performed in triplicate. p value $\leq$ .05.

A.
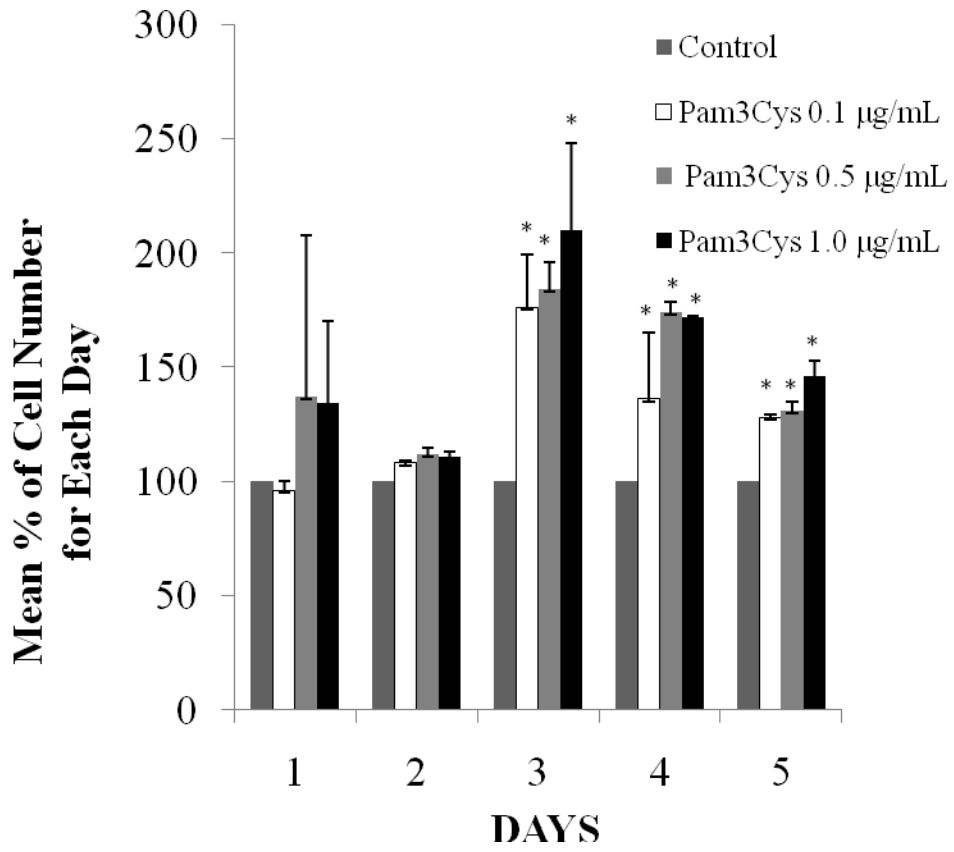
Figure 12

B.
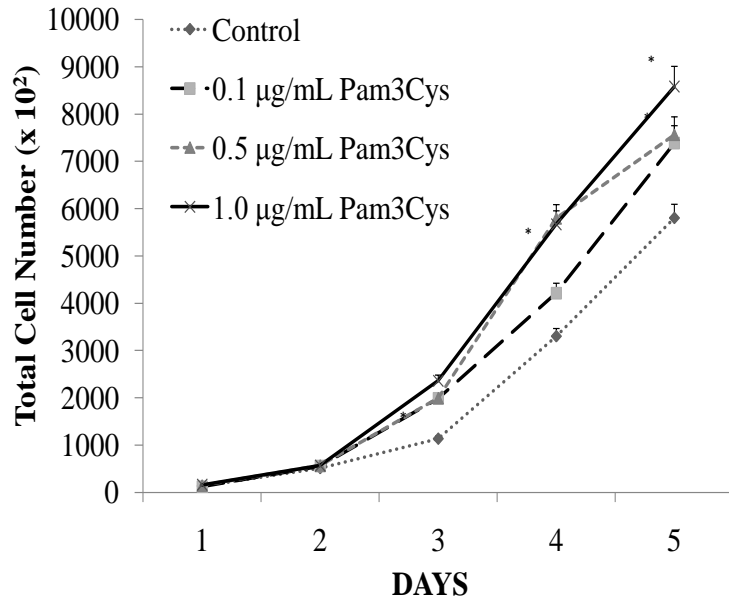
Figure 12

C.
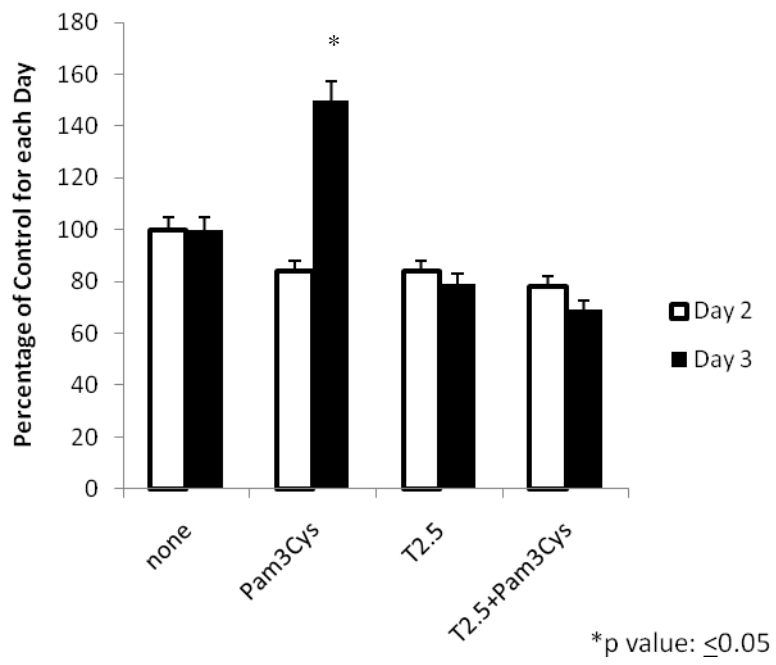
Figure 12

D.
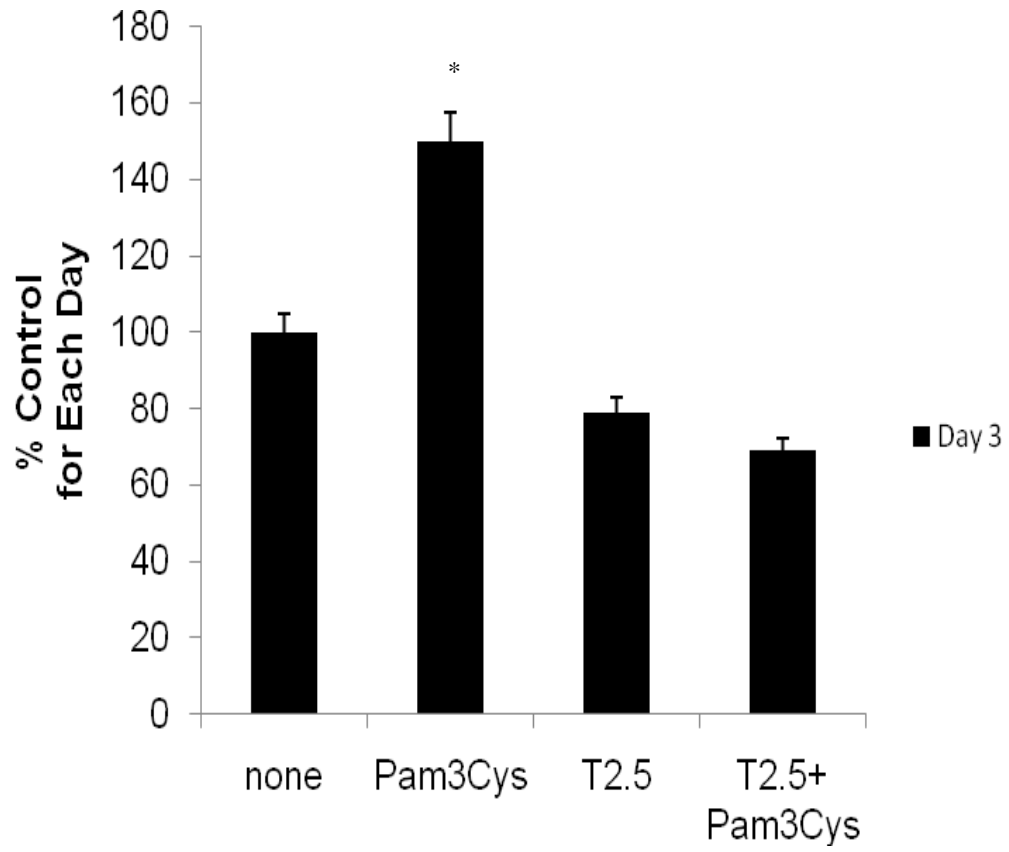
*p value $\leq$ 0.05

Figure 12

Figure 13. mESCs do not secrete various cytokines and chemokines following stimulation with TLR ligands, as assessed by ELISA. mES cells cultures were treated with various TLR ligands: 75μg/mL Poly (I:C), 10μg/mL LPS, or 10μg/mL Pam$_3$Cys, as noted, for 24 hours prior to harvesting the conditioned medium. While cytokine release in response to TLR ligands was detected in media conditioned by Raw264.71 cells, it was not detected (ND) in media conditioned by mES cell line E14. These are results each of one experiment performed in triplicate.

A. IFN-β

Figure 13

B. TNF-α
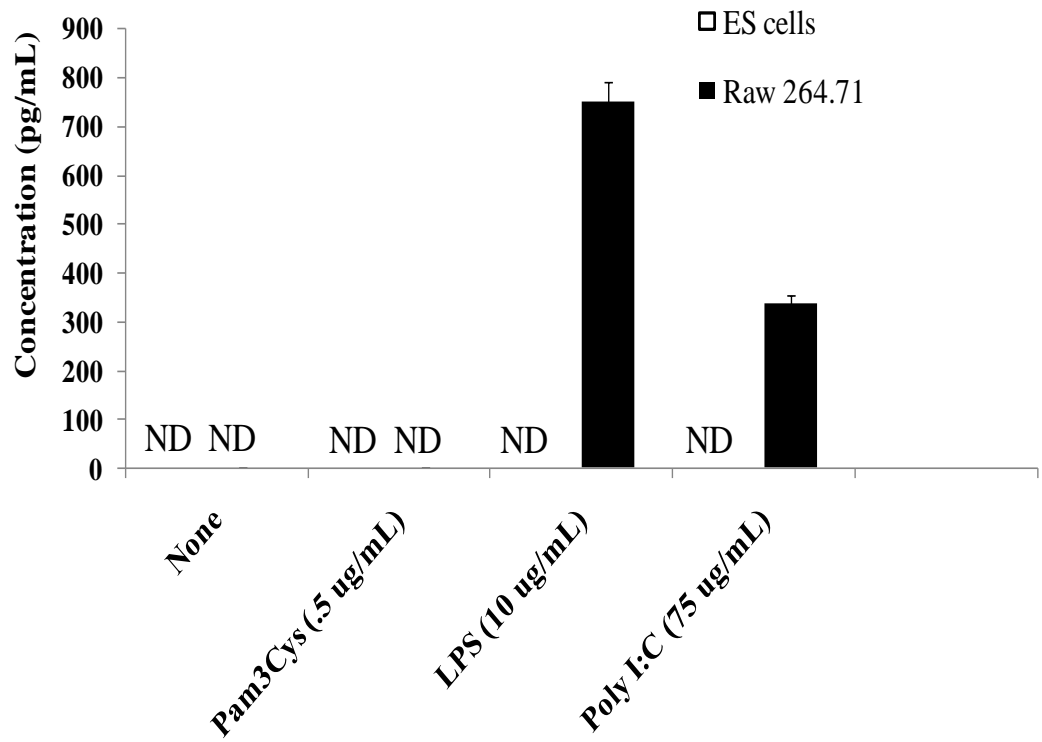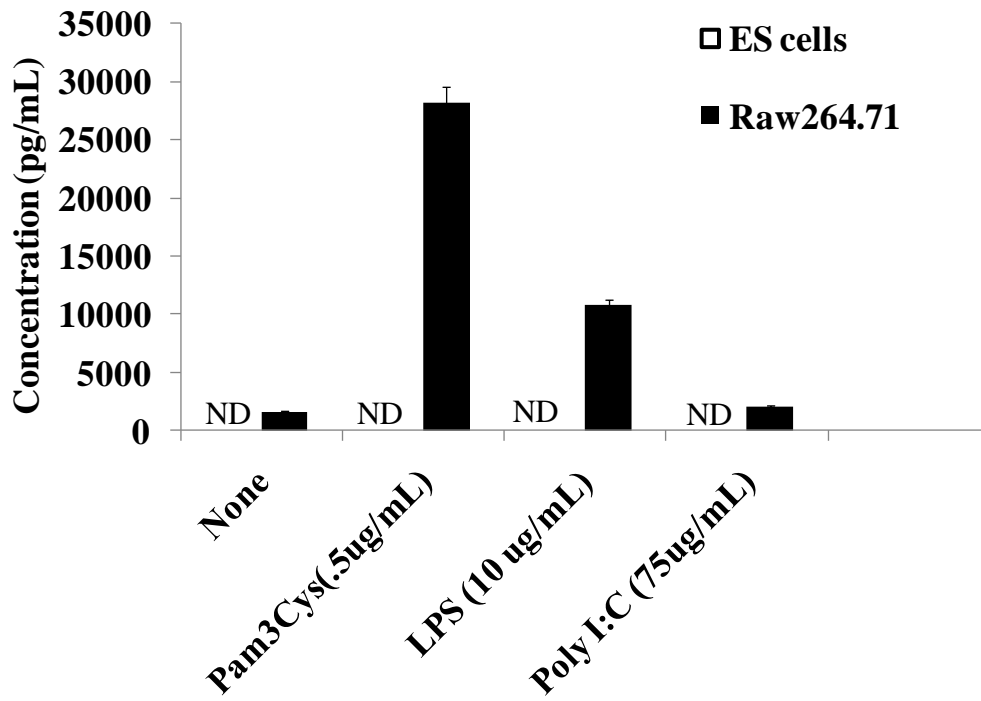
Figure 13

C. IL-6
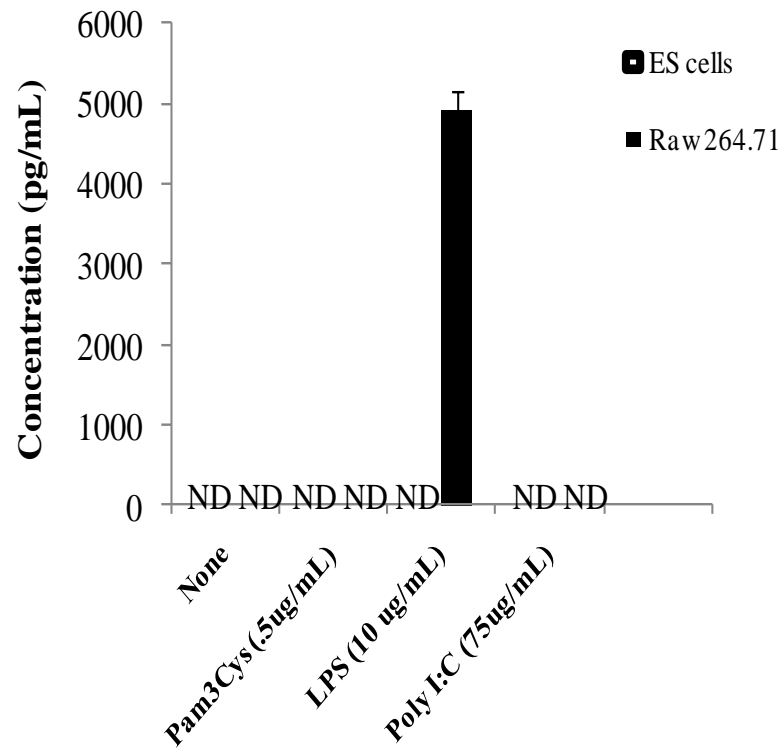
Figure 13

Figure 14. qRT-PCR for A. IL-6 B. TNF-α, and C. INF-γ at 12 and 24 hrs after TLR-2 and TLR-4 ligand stimulation on E14 mESCs. D. BCL-2 protein expression after TLR-2 activation for 24 hours. E. IL-6, F. TNF-α, and G. INF-γ at 12 hours after TLR-2 and TLR-4 ligand stimulation on Raw264.71 mouse macrophage like cells. These are results of two experiments. Each performed in triplicate.

A.
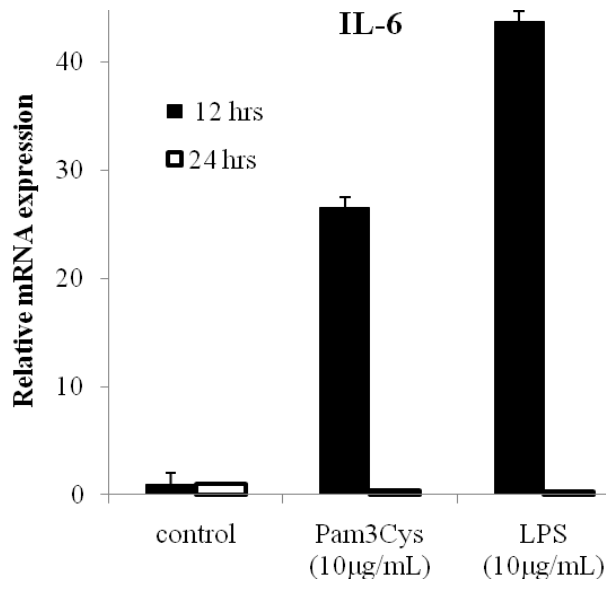
B.
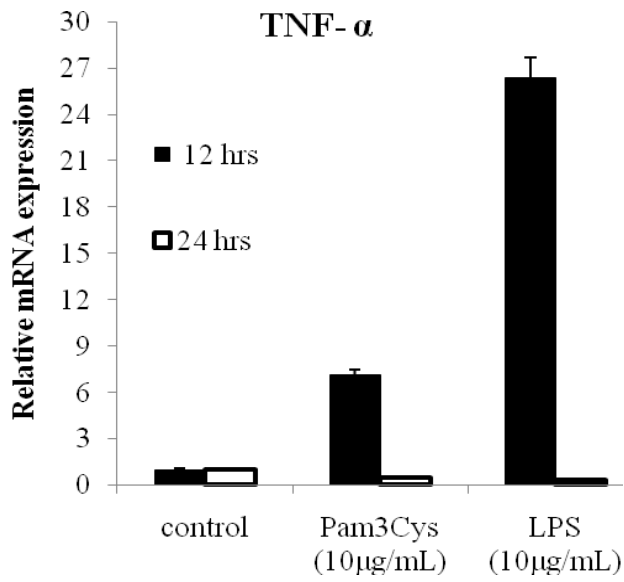
Figure 14

C.
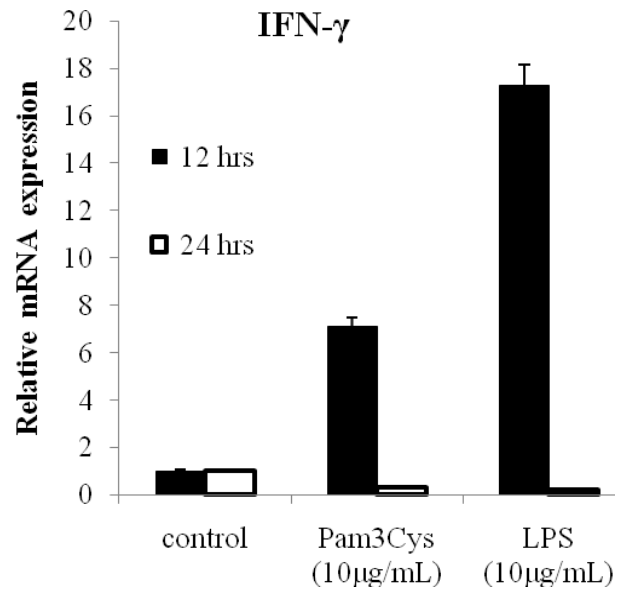
D.
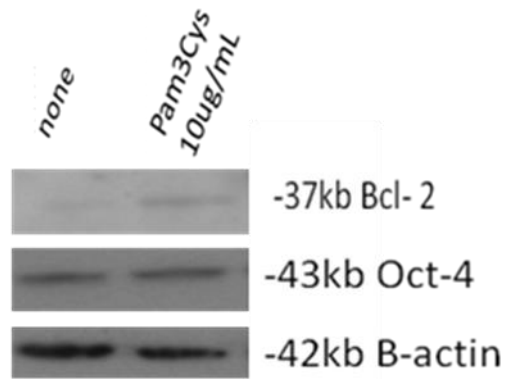
Figure 14

E.
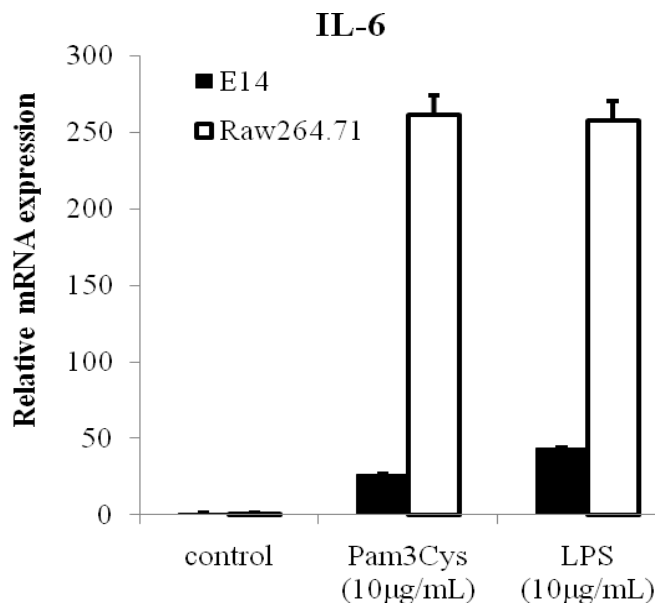
F.
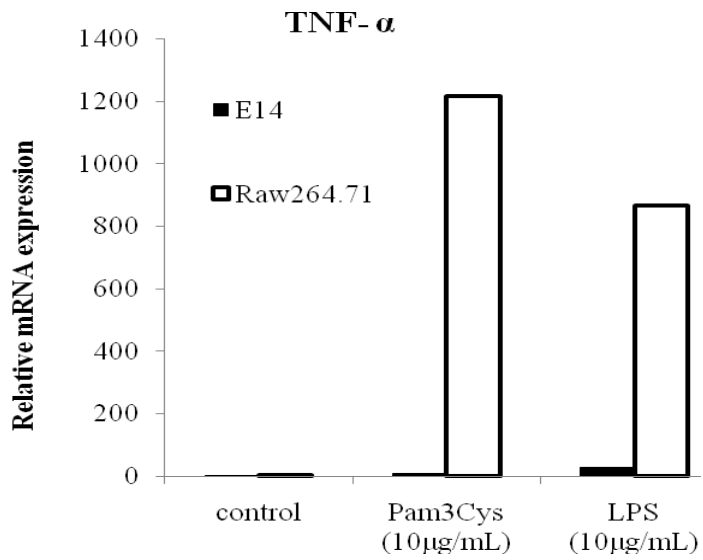
Figure 14

G.
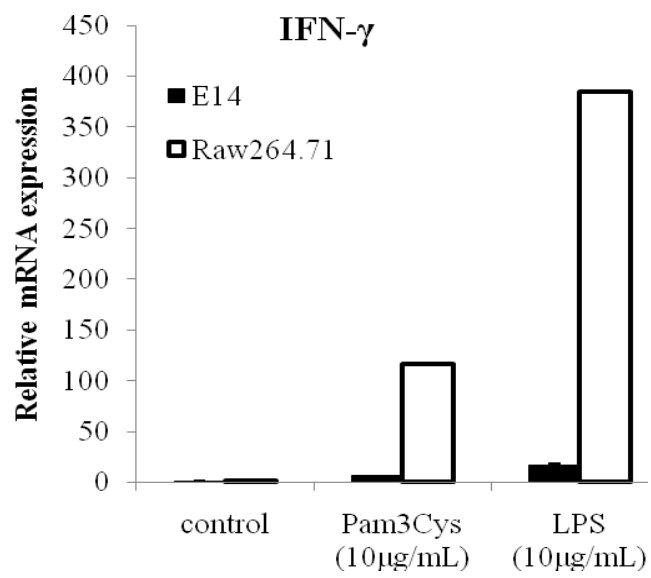
Figure 14

Table 4. Other Cytokines, Chemokines, and Growth Factors Assessed by ELISA

Apolipoprotein A1 (Apo A1)	Macrophage-colony stimulating factor (M-CSF)
C-Reactive Protein (CRP)	Macrophage derived cytokine (MDC)
CD40	Macrophage inflammatory protein 1 α (MIP-1alpha)
CD40 ligand	Macrophage inflammatory protein 1 β (MIP-1 beta)
Endothelin -1	Macrophage inflammatory protein 1 γ (MIP-1 gamma)
Eoxtaxin	(MIP-2)
Epidermal Growth Factor	Macrophage inflammatory protein 3 β (MIP-3 beta)
Factor VII	Matrix metalloproteinase-9 (MMP 9)
Fibrinogen	Monocyte chemoattractant protein -1(MCP-1)
Fibroblast growth factor basic (FGF-basic)	Monocyte chemoattractant protein -3(MCP-3)
Fibroblast growth factor -9 (FGF-9)	Monocyte chemoattractant protein- 5(MCP-5)
Granulocyte chemotactic protein 2 (GCP-2)	Myeloperoxidase
Granulocyte macrophage-colony stimulating factor (GM-CSF)	Myoglobin
Glutathione S-transferase α (GST-alpha)	Oncostatin M (OSM)
	Regulation upon activation, normal T-cell expressed and secreted (RANTES)
Haptoglobin	Serum Amyloid P
Immunoglobulin A (IgA)	Serum glutamic-oxaloacetic transaminase (SGOT)
Inducible Protein-10 (IP-10)	Stem Cell Factorn (SCF)
Insulin	Thrombopoietin (TPO)
Interferon-gamma (IFN-γ)	Tissue inhibitor of metalloproteinase type – 1 (TIMP1)
IL-1α (interleukin -1 alpha)	Tissue Factor
IL-1β (interleukin-1 beta)	Tumor Necrosis Factor-alpha (TNF-α)
IL-2 (interleukin-2)	vascular cell adhesion molecule -1 (VCAM-1)
IL-3 (interleukin-3)	Vascular endothelial cell growth factor (VEGF)
IL-4 (interleukin-4)	Von Willebrand Factor (vWF)
IL-5 (interleukin 5)	Leptin
IL-6 (interleukin 6)	Leukemia inhibitory factor (LIF)
IL-7 (interleukin 7)	Lymphotactin
IL-10 (interleukin 10)	
IL-11(interleukin 11)	
IL-12p70 (interleukin- 12p70)	
IL-17 (interleukin 17)	
Melanoma growth stimulatory activity protein (KC/GRO alpha)	

Table 5. Effects of Toll Like Receptor ligands on the capacity of E14 cells to release protein

Increased protein release after ligand stimulation	TLR2 challenge (pg/mL)	TLR3 challenge (pg/mL)	TLR4 challenge (pg/mL)	Conditioned Media (pg/mL)
CRP		2433±513		2300±265
EGF		5.1±0.30	5.1±2	4.7±0.81
Fibronogen	12000±1000		13000±1000	10500±1323
IL-17	2.0+0.1			1.5±0.25
IFN-β	3±1.1			2±0.4
Haptoglobin	223333±305 50		230000±4826	21667±57258
GM-CSF		0.23±0.06	0.24±0.06	0.19±0
GCP-2	1.8±0.83			1.7±0.4
IL-4	7.1±1.15			5.8±0
IP-10	15±8			13±8
MCP-1	4+0.8			2.4+0.8
MCP-3	6±0.6			4.2±0.2
KC/GRO-α	9.1±5			2.4±0.7
(SAP)		8933±3153		8867±2650
IL-6	0.7±0.6			0.06±0.5
IL-12p70	16±8			12±7
IL-17	2±0.1			1.5±0.25
Lymphotactin	4.13±0.5			3.93±1.5
MIP-1 alpha	81±12	88±0	81±12	76±29
SGOT	8000±13856	12333±21362	20000±3606	nd
MIP-2	2.4±0.1		2.2±0.1	1.9±0.5
VEGF	673±250		591±279	578±500
FGF-9	163±23		130±52	118±56
IgA		28333±90738		256667±150443
IL-1 alpha	13±2			12.6±2.5

Table shows average level of cytokine expression +/- SD (pg/mL).

mESCs maintain an un-differentiated state after TLR-2 activation in the presence of LIF

mESCs were treated with TLR-2 and TLR-4 ligand for up to 3 days. We observed that TLR-2 and TLR-4 ligands did not change the expression of undifferentiated markers Oct-4, SSEA-1, KLF, and Sox-2, when compared to cells that were not treated with TLR ligands (Figures 15-18). Thus, TLR2 ligand- TLR2 interactions in the presence of LIF do not appear to trigger differentiation of mES cells (Figures 15-18), although more rigourous methods of assessor the non-differentiated of ES cells, e.g. invivo/teratoma formation will he needed to condusively, determine this.

Pam3Cys enhances and LPS decreases mES cell survival upon delayed addition of serum

mES cells were plated in the presence of LIF, but with delayed addition of serum for 0, 1, 2 and 4 days and in the absence or presence of Pam$_3$Cys, the ligand for TLR-2, LPS, or SDF-1. SDF-1 was used as a positive control because our lab has shown that SDF-1 caused an increase in survival of mESCs in the presence of LIF with delayed addition of serum (Guo, Graham-Evans et al. 2006). As shown in Figure 19A, SDF-1 showed a significant increase in cell survival compared to cells that were not treated and also compared to cells treated for each day with 0.1µg/mL, 1.0 µg/mL, and 10 µg/mL of TLR-2 ligand. mES cells treated with the ligand for TLR-2 showed a dose-dependent increase in survival when compared to cells that were not treated for each day. Pam$_3$Cys resulted

in decreased apoptosis (Figure 19 B) interestingly, LPS triggered a decrease in survival

(Figure 19 A) and an increase in apoptosis (Figure 19 B).

Figure 15. Surface expression of stem cell marker SSEA-1 after TLR-2 and TLR-4 ligand stimulation in wild-type E14 mESCs in the presence of LIF. SSEA-1 protein levels were analyzed by flow analysis. These are one of three experiments showing the same expression levels.
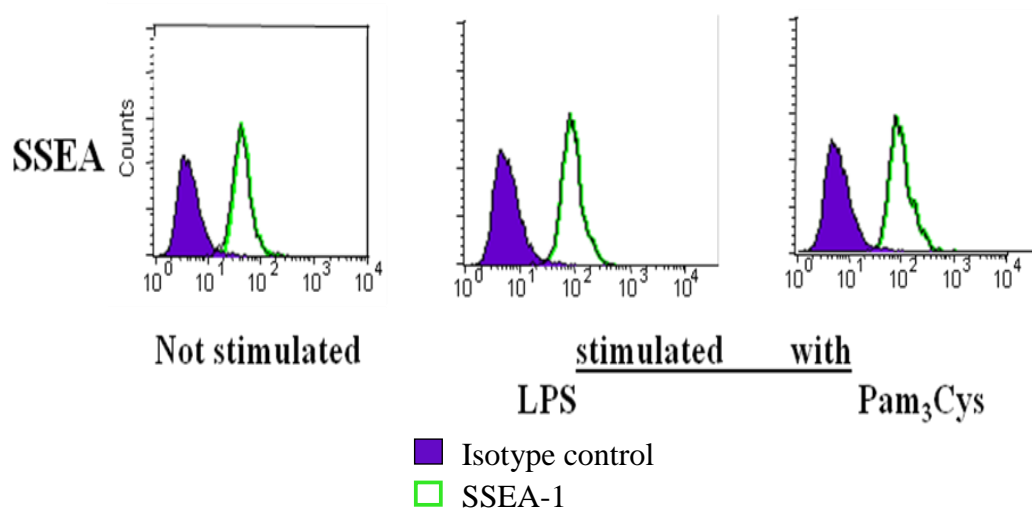
Figure 15

Figure 16. Intracellular expression of stem cell marker Oct 3/4 after TLR-2 and TLR-4 ligand stimulation in wild-type E14 mESCs in the presence of LIF. Oct 3/4 protein levels were analyzed by flow analysis. These are one of three experiments showing the same expression levels.

Oct 4

Not stimulated

stimulated with

LPS

Pam$_3$Cys

■ Isotype control
☐ Oct4
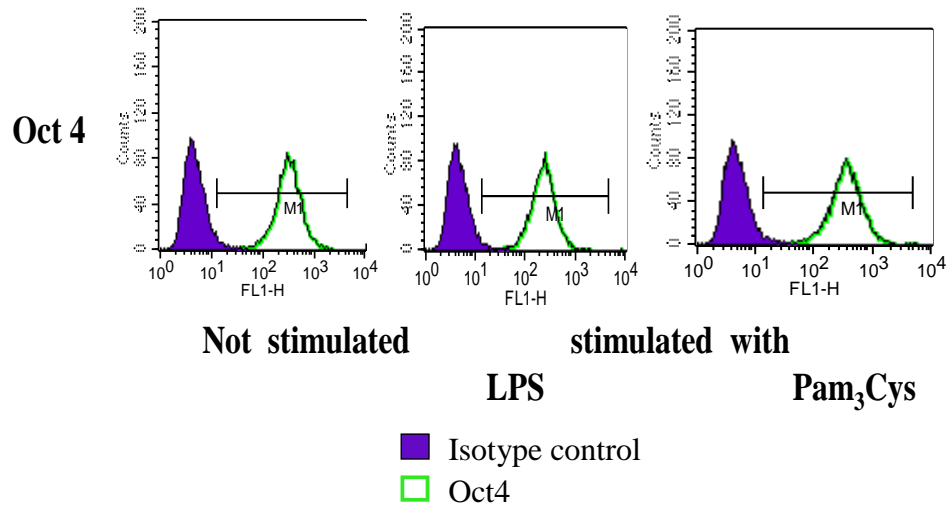
Figure 16

Figure 17. Intracellular expression of stem cell marker KLF-4 after TLR-2 and TLR-4 ligand stimulation in wild-type E14 mESCs in the presence of LIF. KLF-4 protein levels were analyzed by flow analysis. These are one of three experiments showing the same expression levels.

KLF-4

Not stimulated   **stimulated with**
                      LPS   **Pam$_3$Cys**

■ Isotype control
□ KLF-4
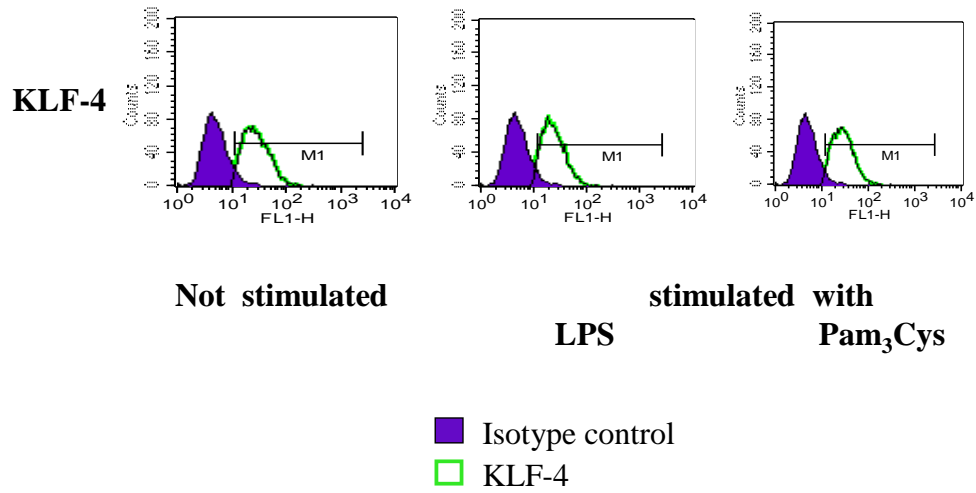
Figure 17

Figure 18. Intracellular expression of stem cell marker Sox 2 after TLR-2 and TLR-4 ligand stimulation for wild-type E14 mESCs in the presence of LIF. SOX-2 protein levels were analyzed by flow analysis. These are one of three experiments showing the same expression levels.

Sox 2

Not stimulated

stimulated with

LPS

Pam$_3$Cys

Isotype control

Sox 2
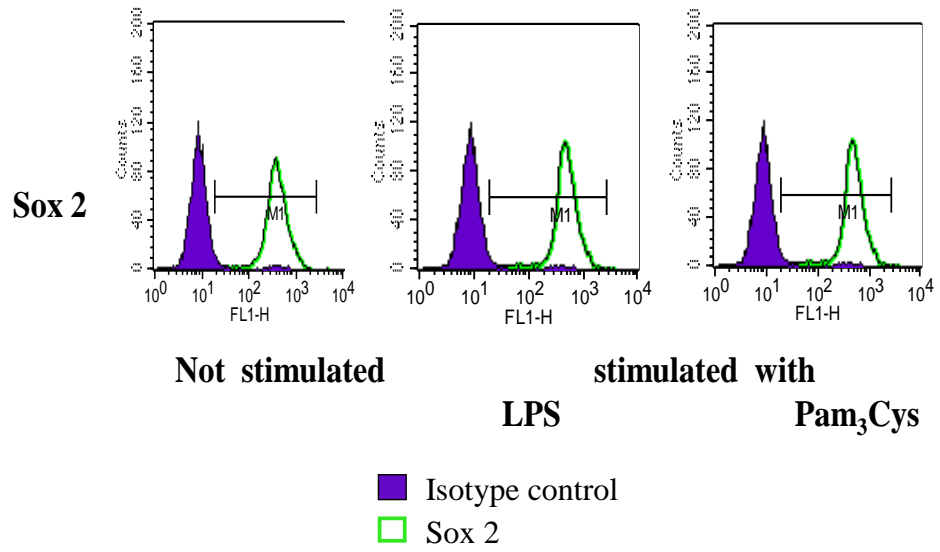
Figure 18

Figure 19. Influence of TLR-2 on survival of mES cell colony formation subjected to delayed addition of serum (A), and of apoptosis of mES cells in suspension culture as assessed by flow cytometry. The percent Annexin V-positive cells were compared to the controls of that specific day $*p \leq 0.05$. (B). mES cells were cultured without serum, and serum added at day 0, 1, 2, and 4 after the start of culture. Colonies formed by ESCs were counted 7 days after addition of serum. Results shown are the average of three experiments, each assessed in triplicate. Experimental points were compared with the time 0 of the control group: (*) $p \leq 0.05$.

A.
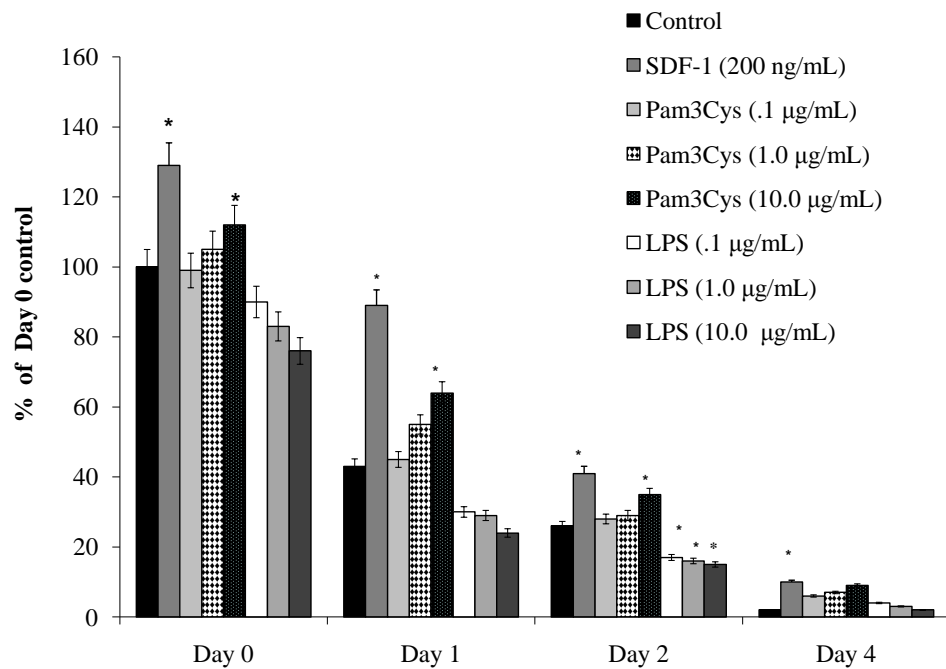
Figure 19

B.
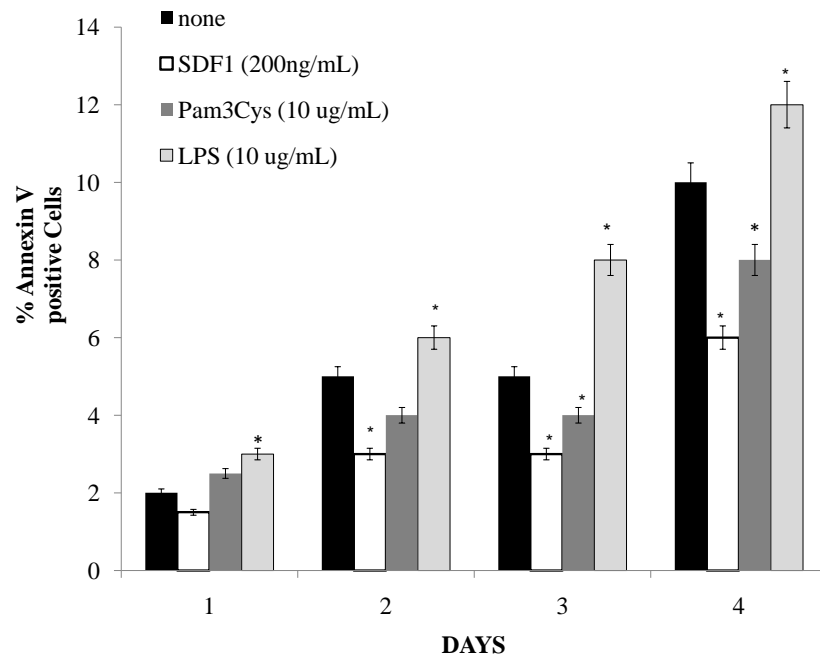
Figure 19

Part II

mES Cell Differentiation

TLR-2 and TLR-4 are expressed in Embryoid Bodies (EBs)

TLR-2 and TLR-4 protein expression was examined by flow analysis after removal of LIF. While we detected expression of TLR-2 and TLR-4 expression on the surface of mES cells after withdrawal of LIF, Pam$_3$Cys did not influence TLR-2 expression (Figure 20) and had only minimal effects on TLR-4 expression (Figure 21).

In the absence of LIF, TLR2 activation caused an increase in expression of Oct-4 and Sox-2 but not KLF-4 and SSEA-1 expression.

We determined if Day 4 and Day 6 EBs derived from mESCs in the absence of LIF but in the presence of TLR-2 ligand at 10 μg/mL expressed non-differentiated markers Oct-4, Sox-2, KLF-4, and SSEA-1. As shown in Figures 22-25 respectively, mESCs in the absence of LIF, do not express Sox-2, SSEA-1, KLF-4, and Oct-4. In the absence of LIF and the presence Pam$_3$Cys, there was a modest increase in expression of Sox 2 in Day 6 EBs (Figure 22), no effect on expression of cell surface marker SSEA-1 on Day 4 or Day 6 EBs (Figure 23), essentially no effect on expression of KLF in Day 4 or Day 6 EBs (Figure 24), and only a very modest increase in Oct 4 expression in day 6 EBs with 20

μg/mL Pam$_3$Cys (Figure 25). Thus from the flow data, there is little evidence other than for Sox-2 (Figure 22) and Oct-4 (Figure 25) expression that Pam$_3$Cys has effects on modifying differentiation of mES cells after removal of LIF.

Figure 20. Pam$_3$Cys does not modify expression of TLR-2 after removal of LIF. Flow

Cytometry results are shown for Day 6 EBs. These are one of three experiments showing

the same expression levels.

10ug/mL
Pam₃Cys

20ug/mL
Pam₃Cys

73% +/- 5.8%

76% +/- 8.76%

77.3% +/- 13.76%

Day 6 EBs

TLR-2

Isotype control
TLR-2
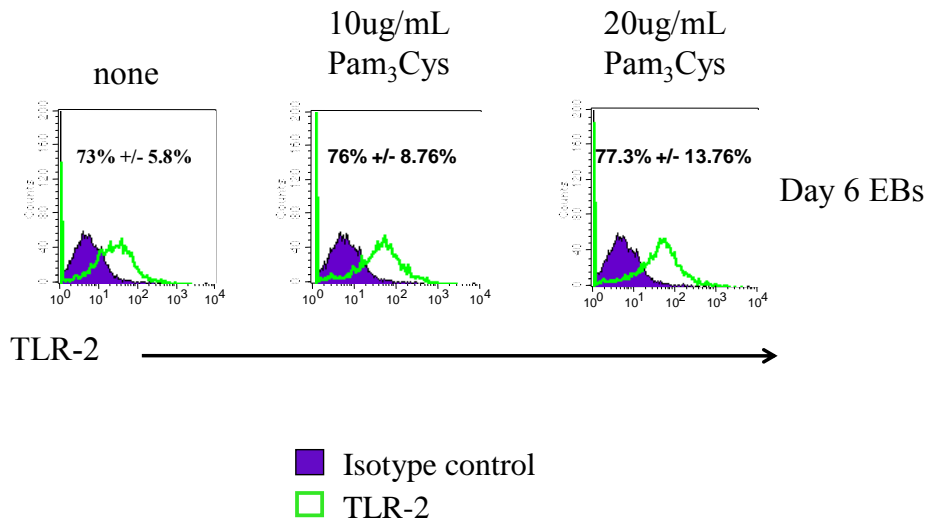
Figure 20

Figure 21. Pam$_3$Cys has minimal to no effects on expression of TLR-4 after removal of LIF. Flow Cytometry results are shown for day 4 and 6 EBs. These are one of three experiments showing the same expression levels.

Pam₃Cys

none   10ug/mL   20ug/mL

.53% +/- .30%   15.76% +/- .05%   26.76% +/- 15.9%

4 Day EBs

.76% +/- .43%   6.76% +/- .85%   10.76+/- .86%

6 Day EBs

TLR-4

■ Isotype control
□ TLR-4
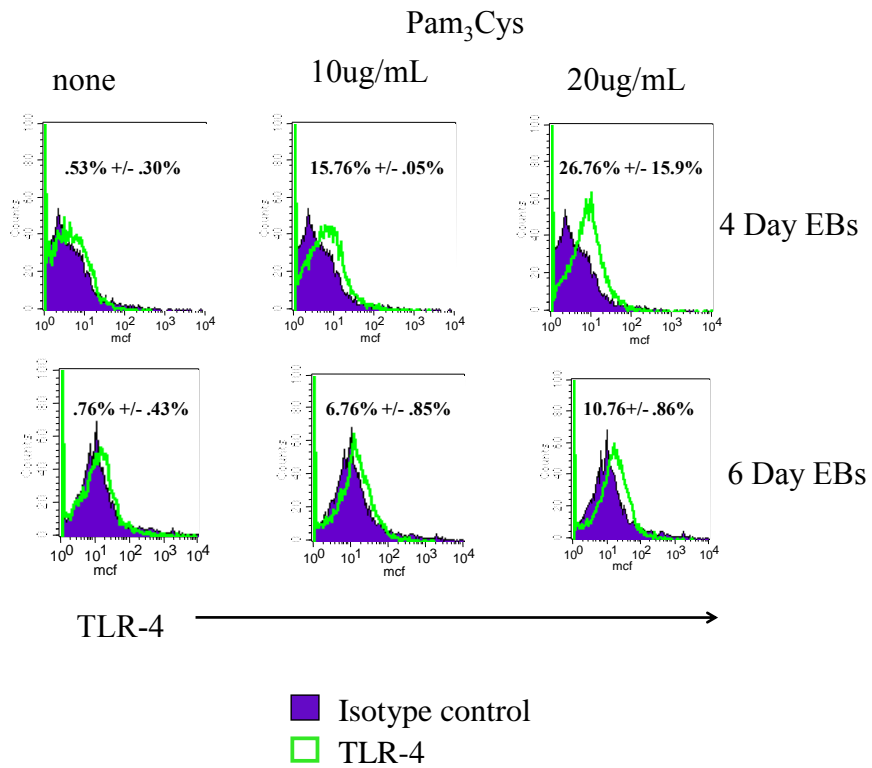
Figure 21

Figure 22. Flow analysis of Sox2 expression for Day 4 and Day 6 EBs in the absence of LIF but in the presence of Pam$_3$Cys for undifferentiated markers Sox 2. These are one of three experiments showing the same expression levels.
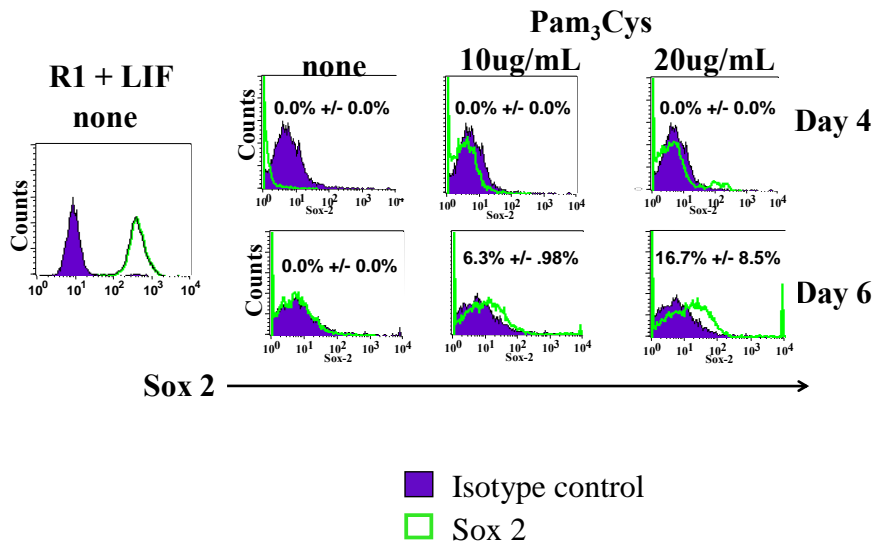
Figure 22

Figure 23. Flow analysis of SSEA-1 Expression for Day 4 and Day 6 EBs in the absence of LIF but in the presence Pam$_3$Cys. These are one of three experiments showing the same expression levels.
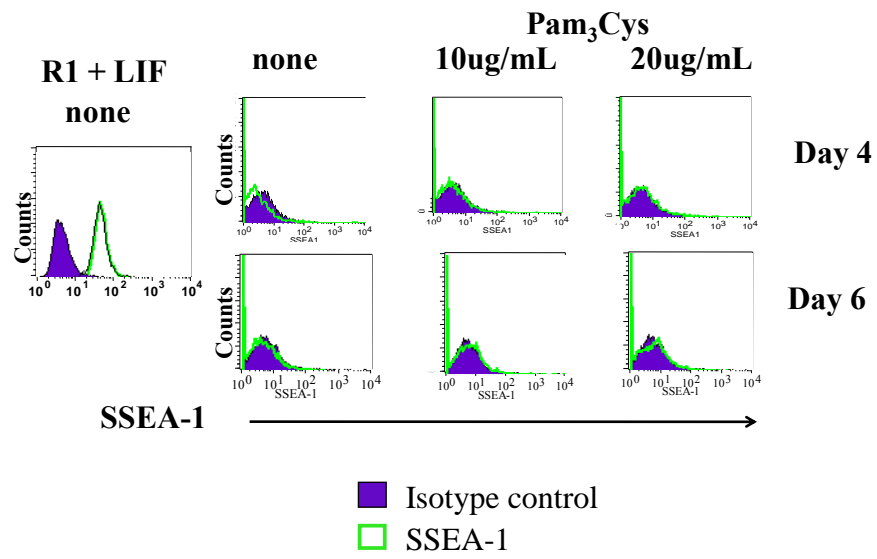
Figure 23

Figure 24. Flow analysis of KLF4 expression for Day 4 and Day 6 EBs in the absence of LIF but in the presence of Pam$_3$Cys. These are one of three experiments showing the same expression levels.
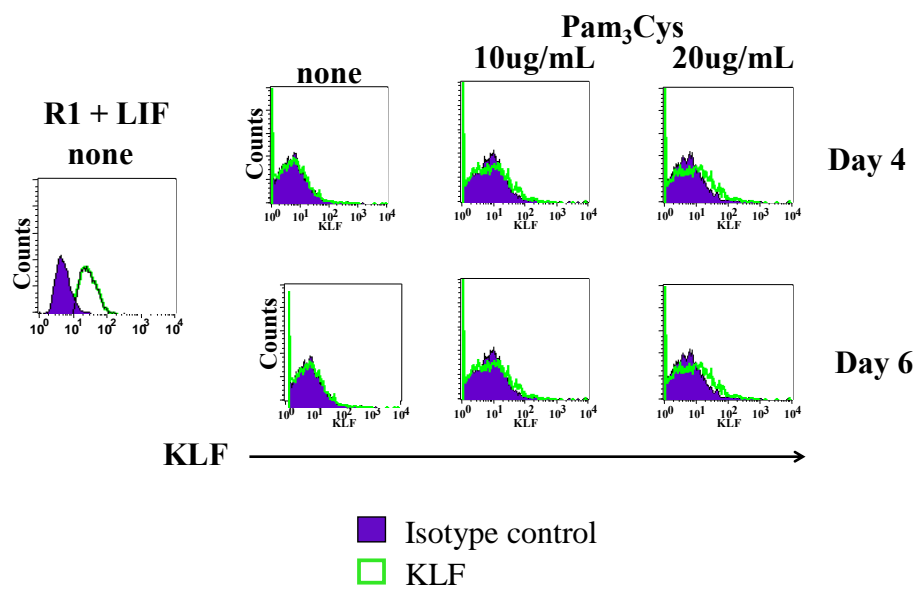
Figure 24

Figure 25. Flow analysis of Oct4 expression for Day 4 and Day 6 EBs in the absence of LIF but in the presence of Pam$_3$Cys. These are one of three experiments showing the same expression levels.
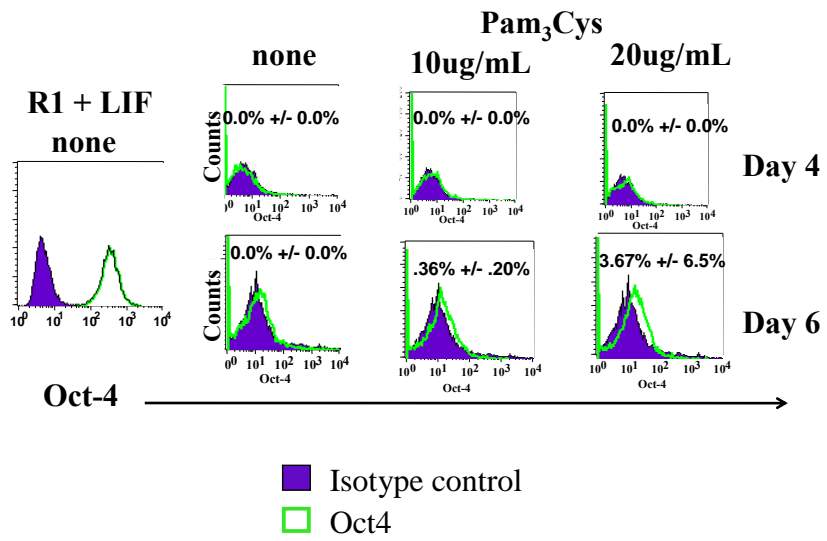
Figure 25

Oct-4 levels, as assessed by Western blot, were maintained and a decrease in p-ERK in Day 6 EBs compared to EBs not treated with TLR-2 ligand

mES cells were plated in the absence of LIF for 6 days, but in the presence of Pam_3Cys. As shown in Figure 26, Oct-4 and p-MEK were assessed by western blot analysis. Oct-4 was highly expressed in R1 cells in the presence of LIF. This was used as a positive control compared to cells cultured in the absence of LIF for 6 days. In contrast to the flow data showing little to no expression of undifferentiated cell marker Oct-4, in the presence of Pam_3Cys and absence of LIF we saw an increase in Oct-4 protein expression by Western blot analysis when compared to cells that were not treated with Pam_3Cys (Figure 26 A). Next, we checked for p-ERK expression in these cells (Figure 26 B). Removal of LIF resulted in enhanced expression of phosphorylated ERK, but this expression level was greatly decreased in the presence of Pam_3Cys. β-actin and total-ERK were used as loading controls (Figure 26 C and D).

Figure 26. mES cell line R1 treated with Pam$_3$Cys, in the absence of LIF show lessened down regulation of Oct 4 expression but decreased expression of phosphorylated ERK (42kb) compared to cells in the absence of Pam$_3$Cys. (A) Oct-4 (B) ERK phosphorylation. mES cells were grown to confluency and induced to differentiate by the removal of LIF. (C) Total ERK and (D) β-actin antibody to confirm equal loading. Representative data from two independent experiments are shown. These are one of three experiments showing the same expression levels.

Differentiation (without LIF)
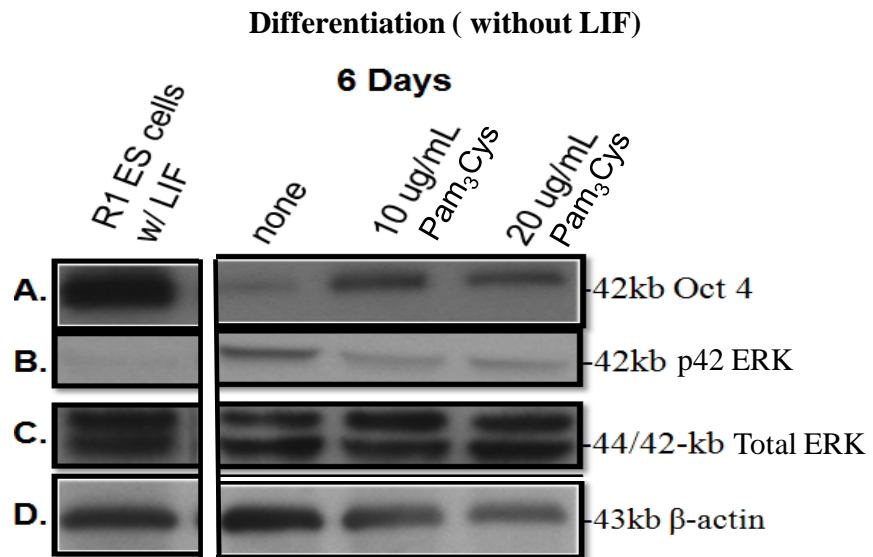
Figure 26

DISCUSSION

Experiments in the presence of LIF

TLRs are critical components of the adaptive immune response (Takeuchi, Hoshino et al. 1999; Underhill, Ozinsky et al. 1999; Hoebe, Du et al. 2003; Honda, Murao et al. 2003; Lund, Sato et al. 2003; Diebold, Kaisho et al. 2004; Heil, Hemmi et al. 2004; Krieg, Efler et al. 2004; Lebon, Adler et al. 2004). These receptors are expressed on hematopoietic stem and progenitor cells (Nagai, Garrett et al. 2006), as well as other pluripotent cell types (Rolls, Shechter et al. 2007) including mesenchymal stem/stromal cells (Pevsner-Fischer, Morad et al. 2007) and are selectively expressed on the mES cell line, D3 (Lee, Hong et al. 2009). While it is not entirely clear why mES cell lines would express TLRs, mES cell lines have been shown to express certain cytokine receptors, as well as produce cytokines that act on mES cell lines as well as on hematopoietic progenitor cells (Guo, Hangoc et al. 2005; Guo, Graham-Evans et al. 2006). Since our original studies on mES cell lines (Guo, Hangoc et al. 2005; Guo, Graham-Evans et al. 2006), we have been interested in a potential role for TLRs and their ligands in mESC line function. Our present study addresses our experiments on the expression of TLRs in the E14, CGR8, and R1 mES cells and the functional activities of Pam_3Cys, a ligand for TLR-2, and to a lesser extent, the activity of LPS on the E14 mES cell line. These studies significantly extend those of others (Lee, Hong et al. 2009) with regards to TLR expression in mES cell lines, and not unexpectedly point up some subtle differences in expression of TLRs in different mES cell lines.

We found that the E14 mES cell line growing in the presence of LIF expresses mRNA (as assessed by semi-quantitative RT-PCR) for TLRs 1, 2, 3, 5, and 6, but not TLRs 4, 7, 8, or 9. This profile of TLR expression differs from the D3 mES cell line by others (Lee, Hong et al. 2009), where RT-PCR analysis showed mRNA for TLRs 2-6, but not for TLR1 or 7-9, thus distinguishing differential expression of mRNA for TLR-1 and 4 between the D3 and our E14 cell line. Interestingly, we do not observe expression of cell surface protein for TLR4 on the E14, CGR8, and R1 mES cell lines, and we did not detect mRNA expression for TLR4 on the E14 mES cell line. These differences may be due to the cell line itself, or perhaps subtle differences in how the cell lines were maintained and grown. In this context, others (Zampetaki, Xiao et al. 2006) have demonstrated that expression of TLR-4 varies in the D3 mES cell line and is regulated by epigenetic modifications. The state of chromatin in pluripotent ES cells is different from that of lineage committed cells (Sha et al.2009). The basic subunit of chromatin is the nucleosome, which is made up of two copies of the four core histones; H2A, H2B, H3 and H4, wrapped around 147bp of DNA (Sha et al, 2009) and maybe different amongst different ES cell lines. The organization of the chromatin nucleosome can have local and global effects on DNA mediated processes including gene regulation (Figure 27). It has recently been shown that epigenetic changes can contribute to cell fate by reprogramming a somatic cell to an embryonic stem cell. Ectopic expression of key transcription factors: cMyc, Oct-4, Sox-2, and Nanog in differentiated cells has clearly demonstrated that the epigenome of a differentiated cell can be reprogrammed to support embryonic development (Martin 1981; Meissner, Wernig et al. 2007; Takahashi, Okita et al. 2007;

101

Takahashi, Tanabe et al. 2007; Aoi, Yae et al. 2008; Lowry, Richter et al. 2008; Nakagawa, Koyanagi et al. 2008; Park, Zhao et al. 2008; Wernig, Meissner et al. 2008; Yamanaka 2008).

Figure 27. The basic unit of chromatin organization includes the nucleosome, which is made up of 147bp of DNA wrapped around a histone protein. Figure adapted from (Sha et al, 2009)

Euchromatin "Active"   Heterochromatin "Silent"

147 bp DNA

Histone protein
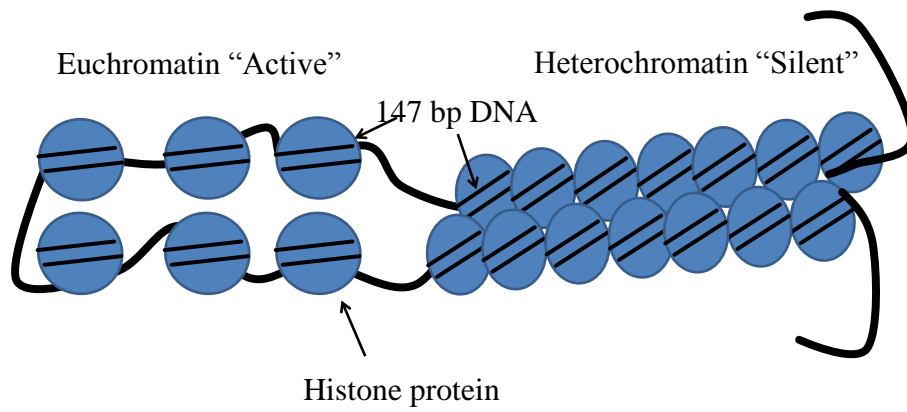
Figure 27

It is thought that TLR-2 and TLR-4 are important for differentiation and self renewal of other types of stem cells (Nagai, Garrett et al. 2006). A recent report shows MSCs that are set to undergo differentiation in the presence of TLR-2 ligand stimulation remained in a non-differentiated state (Pevsner-Fischer, Morad et al. 2007). Most importantly, we have defined a new role for TLR-2 in the E14 mES cell line, and its ligand Pam$_3$Cys on the E14 mES cell line. Pam$_3$Cys enhanced proliferation and cell survival, and decreased apoptosis in LIF-cultured cells, without apparent changes in the immature phenotype of the cells as assessed by cell morphology, and expression of SSEA-1, Oct-4, KLF-4, Sox-2 and alkaline phosphatase.

TLR-2 Pathway Activates NF-κB in mES cells

NF-κB nuclear translocation as well as increased proliferation did not result from TLR-4 ligand stimulation. We speculate that TLR-2 activation on mES cells may play a role in maintenance of the mES cell niche. Some of these effects may be, at least in part, mediated by translocation of NF-κB (Delhalle, Blasius et al. 2004; Liang, Zhou et al. 2004), and events upstream of NF-κB, including phosphorylation of IKK-α/β, as well as downstream reflects such as enhanced protein expression of BCL-2, as well as induced/enhanced expression of mRNA for TNF- α, IFN-γ, and IL-6. These cytokines have many functional activities, including effects on the hematopoietic system (Delhalle, Blasius et al. 2004). Although we did not detect, within the limits of our ELISA assay, TNF-α, IL-6 nor IFN-β protein released into mES cell line containing culture medium in the presence or absence of Pam$_3$Cys, if these three cytokines are having effects on

proliferation and/or survival of the E14 mES cell line, it would likely be through an autocrine-type interaction within the E14 cells, unless cytokine levels below that which we can detect are active, or working in synergy with each other and/or other released cytokines (Guo, Hangoc et al. 2005).

In contrast to the results of others (Lee, Hong et al. 2009) with the D3 mES cell line in which a large percentage of the cells expressed TLR-4, and LPS from E.coli modestly enhanced proliferation of these cells as detected by BrdU incorporation, our E14 mESC line showed no expression of TLR-4 on the cell surface, but responded to LPS from Salmonella with decreased cell survival and enhanced apoptosis. Whether this is mediated by TLR-4 or another TLR is not clear, but it did not appear to reflect nuclear translocation of NF-κB as noted in Figure 3 A-D. This does bring up caution in assuming that what occurs in one mES cell line will happen in another mES cell line. Again, this may reflect the mES cell itself, or how these cells are maintained in culture (Zampetaki, Xiao et al. 2006). It has recently been shown that "non-classical" LPS, such as that from bacteroides fragilis or any bacteria other than *Escherichia coli*, signals primarily through TLR-2 and not TLR-4 (Alhawi, Stewart et al. 2009). Classical LPS comes from *Escherichia coli* bacteria but in our studies we used LPS from Salmonella and this may be working through the TLR-2 receptor on the E14 mES cell line. Therefore, LPS maybe working through TLR-2 but functions to activate a pathway which causes apoptosis, where as Pam$_3$Cys functions through the TLR-2 receptor and activates a pathway which cause proliferation of ES cells. This requires further investigation. Lastly our results with poly I:C (Figure 3 F), an activator of TLR-3, suggest that TLR-3 is functionally active in

the E14 mES cell line, as it is enhancing cell proliferation in the D3 mES cell line (Lee, Hong et al. 2009). In our case, we showed that PolyI:C enhanced/induced phosphorylation of IKKα/β.

Differentiation Experiments

In our study, we found that TLR-2 activation, by its ligand Pam$_3$cys, may help to decrease differentiation of mES cells in the absence of LIF. However, these studies were not conclusive due to differing effects as assessed by Western blotting and flow cytometry.

Roles of TLRs on differentiation of HPCs have been reported in innate immune system replenishment by HPCs (Nagai, Garrett et al. 2006) and in regeneration of intestinal epithelia (Pull, Doherty et al. 2005). Further investigation of these processes will be needed to determine how signals delivered through the activation of TLRs influence stem cell differentiation.

In summary, we demonstrate that mES cells express functional TLR-2 and that the expression of TLR-2 may be involved in proliferation and survival of non-differentiated mES cells. It is possible, but as yet unproven that activation of TLR-2 may helps mES cells remain in an un-differentiated state under differentiation inducing conditions, such as with removal of LIF.

Murine ES cell lines have been and continue to be useful models to study stem cell function and responsiveness to cytokines/ligands. Overall, this work is in agreement with the statement of others (Lee, Hong et al. 2009), that although the biological

significance of functional TLRs in mES cell lines are not yet known, further investigation of these cells could shed important and new information on the self-renewing, pluripotent state of ES cells that may translate into useful information for other stem cell types, and their modulation.

FUTURE DIRECTIONS

Activation of Natural TLR-2 ligands on mESCs

There are many unanswered questions. For example, what would be the natural ligand for TLR-2 on ES cells? In our study we used one ligand, Pam_3Cys, but there are other ligands for the TLR-2/TLR-6 hetrodimer. Peptidoglycan is a natural TLR-2 ligand (Lien, Sellati et al. 1999; Asong, Wolfert et al. 2009; Shida, Kiyoshima-Shibata et al. 2009). It activates TLR-2 through the TLR-2/TLR-6 heterodimer and has also been shown to cause an induction of NF-κB nuclear translocation in other cells, and may also be functional in mES cells because we show expression of TLR-2 and TLR-6 at the mRNA level (Figure 5). We could also determine if TLR-6 is expressed on the surface of mES cells, when antibodies to TLR-6 become available.

Effects of TLR2 activation on mES cells differentiation

We did not perform self renewal colony assays on mESCs, and this can be done. Our attemps to determine if TLR-2 activation could block or prevent differentiation of the mES cells in the absence of LIF but in the presence of TLR-2 ligand was not conclusive. TLR-2 may be involved in helping to maintain the niche for ES cells self-renewal. We showed Oct-4 levels increase in the absence of LIF and that ERK phosphorylation decreased in mES cells in the absence of LIF but in the presence of TLR-2 ligand assessed by western blot, but our flow data showed little change in expression of Oct-4 levels in the absence or presence of Pam_3Cys but after removal of LIF (Figure 26). Since

109

ES cell differentiation to EBs results in the formation of a ball of cells, perhaps all the cells were not broken up in order to get an accurate readout of cellular and intracellular protein levels. In the future, we could also perform western blot experiments using other undifferentiated markers such as Nanog, Sox-2, and KLF-4 to determine if we see expression in Day6 EBs treated with Pam$_3$Cys.

Do other TLR-ligands influence differentiation of mES cells in the absence of LIF?

We can evaluate this by further differentiating the cells in the absence of LIF and determining if they form more differentiated cells, such as an hemangioblast. Next, we can check for differentiation markers such as Brachuary and FLK-1 expression in these EBs as compared to cells not treated with TLR ligands. We could also determine effects on differentiation down the hematopoietic and endothelial cell lineages that derive from hemangioblast.

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113

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Mediates Proliferation, Survival, NF-κβ Translocation, and Cytokine mRNA Expression
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