Immune modulation potential of ESC extracts on T cells

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ABSTRACT

Embryonic stem cells (ESCs) possess hypo-immunogenic properties and have the capacity to modulate allogeneic immune response. ESCs have been shown to reduce immune activation in response to third party antigen presenting cells (APCs) *in vitro* and have the capacity to promote allograft survival *in vivo*. Clinical use of live ESCs to treat immunological disorders, however, risks teratoma or ectopic tissue formation. Accordingly, the way lab is studying the immune modulatory potentials of ESC-derived factors and recently, found that dendritic cells (DCs) treated with human ESC extracts are poor stimulators of purified allogeneic T cells compared to those DCs treated with vehicle or fibroblast extracts. In the present study, I found that ESC-derived extracts directly inhibit T cell proliferation and suppress their activation without inducing cell death. Furthermore, ESC extracts are able to suppress Th1 polarization while increasing the numbers of Foxp3$^+$ CD4$^+$ CD25$^+$ regulatory T cells. Moreover, I found that a protein called Milk fat globule-EGF factor 8 (MFG-E8) appears to be highly expressed in ESCs. Importantly, neutralizing MFG-E8 substantially abrogated the immune suppressive effects of ESC extracts on T cell activation. These findings lead to future studies to further define specific immunomodulatory factors derived from ESCs for potential applications.

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LIST OF ABBREVIATIONS

7AAD	7 actinomycin-D
APC	Antigen presenting cell
BMT	Bone marrow transplantation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CD	Cluster of differentiation marker
CTLs	Cytotoxic T lymphocytes
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified eagle's Medium
ESC	Embryonic stem cell
EDTA	Ethylenediaminetetraacetic acid
E-value	Expect-value
Foxp3	Factor forkhead box P3
FITC	Fluorescein isothiocyanate
GVHD	Graft versus host disease
HLA	Human leukocyte antigen

iPS	Induced pluripotent stem cells
IFN-γ	Interferon-γ
IL-10	Interleukin-10
IL-12p40	Interleukin-12-p40
IL-17	Interleukin-17
IL-2	Interleukin-2
IL-4	Interleukin-4
ISSCR	International Society for Stem Cells Research
MHC I	Major histocompatibility class I
MHC II	Major histocompatibility class II
MSC	Mesenchymal stem cell
MFG-E8	Milk fat globule-EGF-factor 8
MLR	Mixed lymphocyte reaction
MEF	Mouse embryonic fibroblast cells
MS	Multiple sclerosis
NCBI	National Center for Biotechnology Information
NK cell	Natural killer cell
IκB-α	Nuclear factor kappa-light-chain-enhancer B cells inhibitor alpha
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells

PMA	Phorbol myristate acetate
PBS	Phosphate buffer saline
PKC-θ	Protein kinase C theta
QPCR	Quantative Polymerase Chain Reaction
Treg	Regulatory T cells
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SD	Standard deviation
TCR	T cell receptor
Th	T helper cell
TGF-β	Transforming growth factor-beta
TBST	Tris-buffered saline and tween 20
TNF-α	Tumor necrosis factor-alpha

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Chapter 1: INTRODUCTION

1.1 Overview of the Immune System

1.1.1 Innate and Adaptive Immunity

The human body is equipped with an immune system that encompasses natural and acquired immunity. The innate immune system represents the body's first line of defense which extends from physical barriers to specialized cells that are ready on the go with prompt responses to foreign elements without the need for priming or memory. Adaptive immunity, in contrast, requires antigen priming and immune memory development to fulfill highly specialized immune reactions and more comprehensive defenses with the help of lymphocytes and antigen presenting cells (APCs).

1.1.2 T Lymphocytes

T cells play an essential role in initiating, maintaining and modulating diverse immune responses when they become activated. These cells were first classified by Mosmann *et al.*, in 1986 into distinct CD4$^+$ T helper (Th) subsets based on the cytokines they produce upon stimulation (1). Th1 cells secrete IL-2, IFN-γ and other Th1 cytokines that elicit a cytotoxic response involved in clearance of viral infections. Th2 signature cytokines IL-4, IL-5, IL-10 and IL-13 enhance a humoral response over a Th1 cell-mediated cytotoxic response (1). In general, a Th1 cytokine profile appears to promote allograft rejection, whereas the Th2 cytokine profile inhibits Th1 responses and promotes allograft tolerance. The more recently discovered Th17 cells, which were once thought to be a Th1 subset, secrete IL-17. The Th17 lineage has been implicated in autoimmunity and has been shown to provide protection against extracellular pathogens (2, 3). CD8$^+$ cytotoxic T lymphocytes (CTLs) secret IFN-γ and mediate direct cell killing via perforin and granzyme B to eliminate intracellular pathogen-infected cells and tumor cells (4-7).

On the other hand, T regulatory cells (Treg) are identified by intracellular expression of the transcription factor forkhead box P3 (Foxp3) and high surface expression of the transmembrane IL-2 receptor alpha chain (IL-2Rα), CD25 (8). TGF-β can induce Treg cell development in the periphery while natural occurring Treg cells arise in the thymus. Regulatory T cells are capable of suppressing other effector responses allowing a state of immunological tolerance (9, 10). Many stimuli influence CD4$^+$ T cell polarization, apoptosis, activation, and functions. Recently, embryonic stem cells (ESCs) have been found to possess specific immune modulatory properties.

Full activation of naive CD4$^+$ T cells occur when the T cell receptor (TCR) and costimulatory molecules on the T cell interact with antigen presenting cell (APC) MHC class II, signal 1, and its costimulatory molecules B7.1/CD80 and B7.2/CD86, signal 2 (11, 12). Similarly, CD8$^+$ T cell activation requires simultaneous delivery of both signals wherein, in signal 1, TCR complex on CD8$^+$ CTLs recognize MHC class I, expressed on almost all nucleated cells (13). Upon receiving both signals, CD4$^+$ T cells become successfully activated and subsequently adopt distinct phenotypes with specialized effector function. Indeed, the process of Th polarization is also governed by cytokines provided by the innate immune cells in the surrounding milieu. Several subsets of CD4$^+$ helper and suppressive T cells, including Th1, Th2, Th17 and Treg, each with distinct cytokine profiles and functions are shown in **Figure 1**.

1.1.3 T Lymphocyte Function

The pivotal role of T cells as a major component of the effector arm of the adaptive immune system is reflected in their contribution in clearing infection and eliminating cancerous cells. However, T cells also play a crucial role in autoimmune diseases (as seen in rheumatoid arthritis (RA), ulcerative colitis, multiple sclerosis (MS), Type I diabetes, systemic lupus erythematosus (SLE), Scleroderma, Graves disease, Sjogren's syndrome, Guillain-Barre syndrome, Celiac disease, Addison's disease, Psoriasis and Crohn's disease) and transplantation rejection.

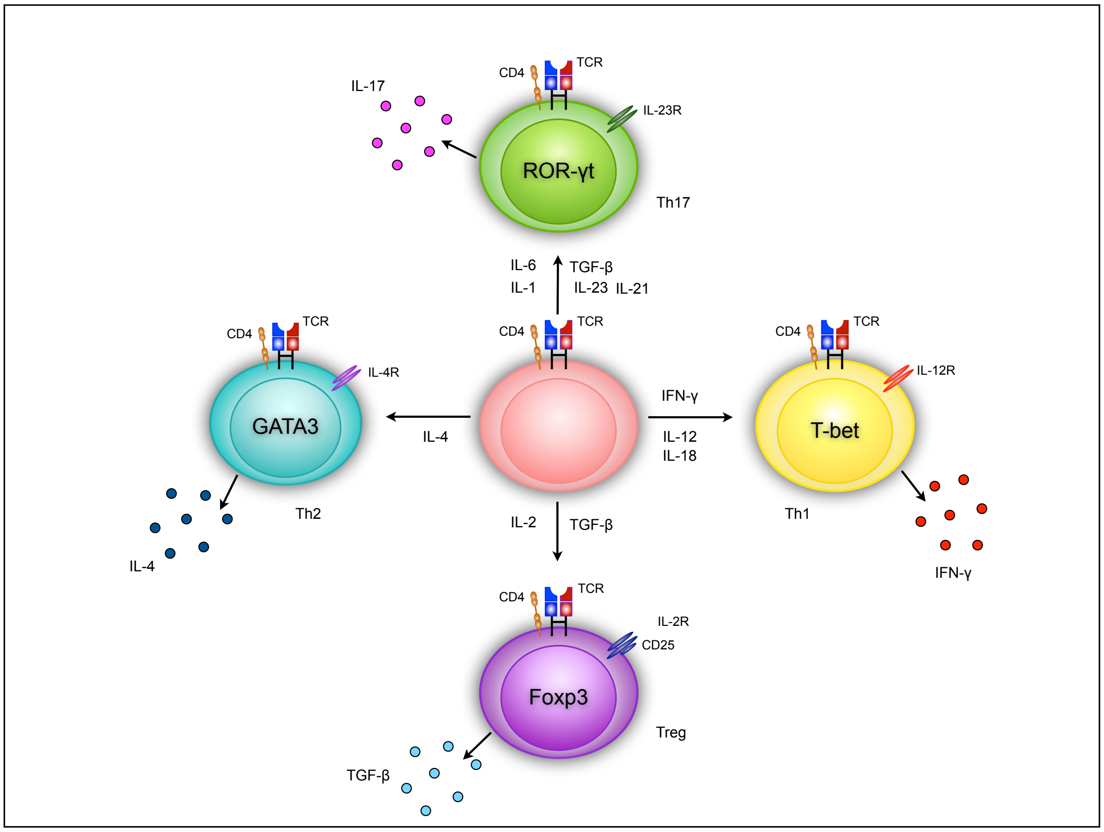
In autoimmune diseases auto-reactive effector CD4$^+$ T cells that secret IFN-γ or IL-17 promote autoimmunity by influencing the pathology and clinical course of the disease (2, 14-17). The contribution of CD4$^+$ T cells in the pathological mechanism of autoimmune diseases is attributed to their directed specific effector function and the potential in activating and interacting with other cells such as macrophages and B cells (18-20). T cells provoke inflammatory autoimmune responses either indirectly through secreted cytokines, or via direct cell-cell interaction mechanisms. Consequently, this direct interaction activates APCs to secret pro-inflammatory cytokines, chemokines, and matrix-degrading enzymes to the inflamed milieu. Regulatory

T cells which balance self-tolerance and autoimmunity have been shown to suppress autoimmune diseases (21). Disrupted balance between tissue-destructive Th17 and tissue-protective Treg cells has been reported in the pathology of autoimmune diseases such as RA and MS (22, 23). Auto-reactive $CD8^+$ T cells are also reported in various autoimmune conditions (24, 25).

As organ transplantation treats end-stage organ failure, short-term survival of allograft is achieved with immunosuppressive drugs while long-term success remains a challenge. Hence, the major goal in transplant immunology is the induction of immune tolerance. In solid organ transplantation, activated and expanded host allo-reactive $CD4^+$ T cells direct destructive activity against the engrafted organ. Evidently, infiltration of T cells is correlated with increased rejection incidence and severity in various transplants (26-28). Chronic leukemia, Hodgkin's lymphoma, aplastic anemia and severe combined immune deficiency are immune and blood disorders that can be treated with bone marrow transplantation (BMT) (29-32). Yet 40% of donor-related BMT patients still develop graft versus host disease (GVHD) (33). GVHD develops when allo-activated donor dendritic cells and T cells attack host tissue (34). Notably, a beneficial shift in Th17/Treg homeostasis towards dominance of Tregs over Th17 might facilitate transplant tolerance (35).

To deal with these unwanted effects, a series of approaches including induction of anergy, dominant suppression by Treg, and peripheral deletion of self-reactive T cells have been considered. $CD4^+$ T cells play an indispensable role in ongoing tolerance induction and immune modulation research focused on amelioration of T cell-mediated alloimmune and autoimmune responses.

Figure 1. Overview of CD4$^+$ T cell polarization. T cells are activated upon antigen recognition and subsequent stimulation signaling, as well as by the surrounding priming cytokines. Activated downstream transcription factors in turn instruct naive CD4$^+$ T cells to differentiate into Th1, Th2, Th17, or Treg lineages. Each subset produces its signature cytokines with distinct immune functions.


CD4
TCR
IL-17
IL-23R
ROR-γt
Th17
IL-6
IL-1
TGF-β
IL-23
IL-21
CD4
TCR
IL-4R
GATA3
IL-4
Th2
IL-4
CD4
TCR
IFN-γ
IL-12
IL-18
CD4
TCR
IL-12R
T-bet
Th1
IFN-γ
IL-2
TGF-β
CD4
TCR
IL-2R
CD25
Foxp3
Treg
TGF-β

1.2 Embryonic Stem Cells (ESCs)

1.2.1 What are ESCs?

ESCs are derived from the inner cell mass of a 3-5 day blastocyst **(Figure 2)**. ESCs can proliferate indefinitely *in vitro* without losing their embryonic characteristics (termed self-renewal). They also have the ability to differentiate into all three germ layers (i.e. mesoderm, endoderm and ectoderm), capable of giving rise to almost any type of cell or body tissue (termed pluripotency) (36-39). As such, ESCs not only provide a unique model for the study of early embryogenesis but also have strong application potential in regenerative medicine.

1.2.2 Maternal-FetusTolerance

Given the origin of ESCs and some shared properties with the early embryo, ESCs may provide a unique tool to better understand maternal-fetus tolerance. The fetus survives and is accepted by the maternal immune system during pregnancy even though it expresses paternal alloantigens. Maternal-fetal tolerance sets a naturally occurring model of true human immune tolerance. The root cause of this tolerance is not fully understood but several factors have been elucidated. Nonclassical MHC molecule HLA-G (40, 41), fetal alloantigen shedding (42), T regulatory cells (9, 43-45), programed death ligand (PDL)-I (46, 47) are among factors that have been identified to promote successful engraftment of semi-allogeneic embryonic tissue in the uterus throughout gestation. In accordance with maternal-fetal tolerance, autoimmune disease remissions sustained throughout pregnancy followed by postpartum relapse is observed in autoimmune conditions such as RA (48, 49) and MS (50, 51). Furthermore, the report by Aluvihare *et al.* has shed light on how systemic expansion of maternal T regulatory cells during pregnancy protects against fetal rejection and suppresses autoimmune responses (9). Nevertheless, the existence of fetal antigens is a key to maternal-fetal tolerance. Thus, study of ESC immune modulation potential may provide an alternative approach to understand this unique immune process.

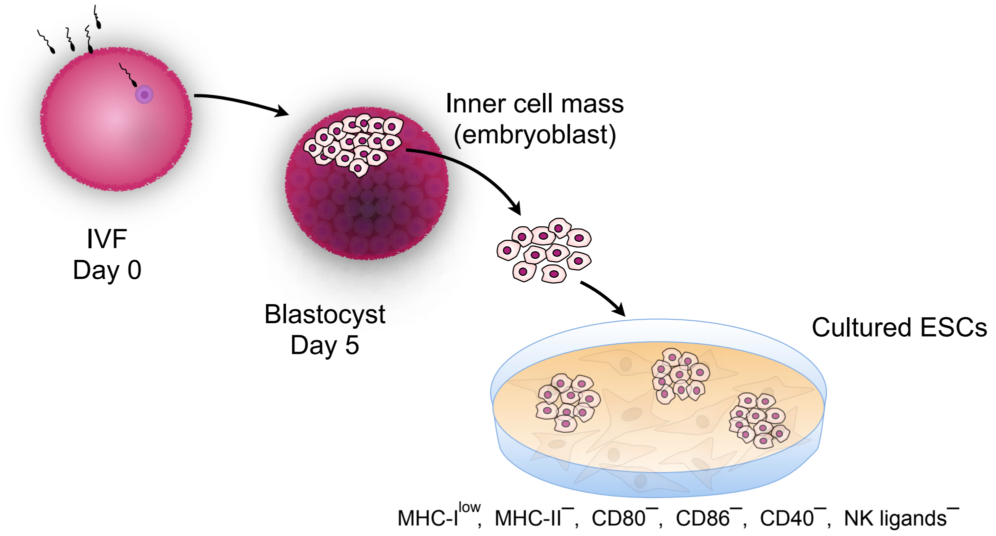
1.2.3 ESCs in Cellular Therapy and Regenerative Medicine

ESCs have gained increasing attention in the field of regenerative medicine and cellular therapy since the first generation of a human ESC line in 1998 by James Thomson (37). Human ESCs can be used a source of precursor stem cells as they can be propagated without limit *in vitro* (38). By use of specific *in vitro* differentiation protocols, human ESCs can be differentiated into a limitless variety of cell types, such as neuronal progenitor cells, hematopoietic cells, hepatocytes, keratinocytes and cardiomyocytes (52-57). Thus, human ESCs may provide promising therapeutic potential for a large variety of degenerative and genetic diseases (54, 56-59).

1.2.4 ESC Immunological Properties

ESCs have been shown to possess immune privileged properties similar to fetal tissue during pregnancy. Recently, several groups have reported that ESCs could survive across both allogeneic and xenogeneic barriers without evoking an immune response (60-68). The ability of ESCs to evade the immune system has been attributed to their unique immunological phenotype. Under undifferentiated conditions, ESCs show very low levels of MHC I expression and no MHC II expression (60-65). In addition, ESCs lack expression of co-stimulatory molecules CD80, CD86 and CD40 that contribute to activating immune effector cells (60-65). Differentiation of ESCs and treatment with inflammatory cytokines such as IFN-γ, however, results in robust MHC I expression and immune recognition (61, 65). Notably, these properties are found to be consistent across human, mouse and rat ESCs (61, 62, 64).

Figure 2. Derivation of ESCs from IVF. An *in vitro* fertilized egg starts to divide forming the so-called blastomere. By day 3 (mouse) or 5 (human) blastocysts containing inner cell masses are developed. Isolation of these inner cell mass cells and their subculture on feeder cells in a petri dish will lead to the generation of ESCs with immune privilege properties. Figure adapted from Menendez *et al.* 2005 **(69)**.


IVF
Day 0
Blastocyst
Day 5
Inner cell mass
(embryoblast)
Cultured ESCs
MHC-I^{low}, MHC-II^{-}, CD80^{-}, CD86^{-}, CD40^{-}, NK ligands^{-}

1.2.5 ESCs and Immune Modulation

In addition to evading the immune system, ESCs also have the capacity to actively modulate the immune system towards a tolerant state. In mixed lymphocyte reaction assays, ESCs suppress immune activation and proliferation in response to third party antigen presenting cells (APCs) (63, 65). Over the past decade, it has become apparent that ESCs have the ability to influence APCs (61, 63, 65, 70). It has also been demonstrated that both human and mouse ESCs are able to directly inhibit T cell and NK cell activity (61, 71-73).

Fandrich and his colleagues have shown that rat ESCs provide significant immune protection to allogeneic solid organ transplants (62). ESCs' ability to escape host immune surveillance is also indicated by tumor formation of ESC origin without evident immune rejection after infusion of ESCs in the host. Thus, besides immune modulatory properties that allow ESCs to enhance their own survival across allogeneic barriers, ESCs also suppress immune responses to third party APCs and provide protection to solid organ transplants. Translating these immune modulatory properties from bench to bedside may yield important applications in autoimmune conditions, allergy and transplantation. However, clinical use of ESCs to promote immune tolerance and reduce the severity of aberrant or unwanted immune activation is currently limited by potential serious adverse events.

1.2.6 Challenges for Potential Clinical Application of ESCs

In addition to ineffective generation of sufficient specific cell lineage from ESCs, ESC-derived teratoma tumor formation also represents a real danger to patients (37, 74-77). Critically, unlike mesenchymal stromal cells (MSCs), implanted donor ESCs are capable of forming teratoma (tumors) (**Figure 3**). In most studies it is evident that teratoma tumor formation is directly correlated with the infusion of a large dose of ESCs (66, 78). In addition to the risk of generating ectopic tumors, regulatory issues, along with high costs associated with the necessary personnel and specialized facility required for culture, storage and transplantation of live cells creates a formidable logistic barrier limiting the potential application of intact ESCs in immune modulation.

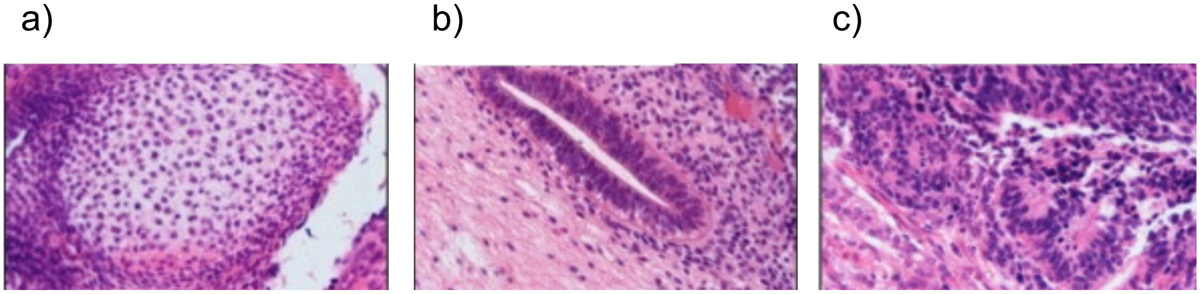
1.2.7 Alternative Approaches to Circumvent the Challenges of Using Intact ESCs

To overcome immunological barriers hindering ESC-based cell transplantation, researches have recently generated induced pluripotent stem cells (iPSCs) (79-81). Nonetheless, a recent report has shown that transplanted iPSCs induced CD4$^+$ T cell-mediated immune rejection in syngeneic recipients while ESCs were successfully implanted without evoking an immune response (82). Moreover, autologous cells, but not allogeneic ESC, are targeted by autoantibodies in patients with autoimmune diseases (83, 84). These observations suggest that the discovery of iPSC may not completely resolve the immune rejection hurdle and that immune inhibitory and stimulatory components in both ESCs and iPSCs need to be investigated. In addition to iPSC approach, current therapy available in clinic for immune modulation involves immunosuppressive drugs that risk multiple side-effects such as serious infection, organ toxicity and mortality.

Since clinical use of human ESCs to treat immunological disorders may risk teratoma or ectopic tissue formation, we have recently found that soluble cytoplasmic lysates of both human and mouse ESCs retain immune modulatory properties of intact cells and have the capacity to inhibit monocyte-derived DCs maturation (70). We have also demonstrated that ESC extracts prevented maturation of DCs in response to TNF-α by decreasing surface expression of CD80, MHC II and CD83 molecules. Accordingly, DCs treated with ESC extracts retained greater phagocytic ability, secreted low levels of IL-12p40 and were poor stimulators of allogeneic T cells (20). Interestingly, we observed that the extracts does not inhibit DC beyond maturation and the inhibition level of T cell activation by DCs could be further enhanced by the addition of ESC extracts in T cell activation assay. This suggests that ESC extracts may also affect T cell activation directly. Subsequently, we have recently demonstrated that ESC extracts have the capacity to directly modulate T cell function (85). Accordingly, this thesis will focus on the effects of soluble ESC extracts on T cell function and further identification of immune modulatory molecules from ESC extracts.

Figure 3. Histological analysis of teratoma formation by embryonic stem cells. Sections of different tumor tissues derived from three germ layers of ESC origin 8 weeks after injecting 1950 hESCs into SCID mice (Hentze *et al.* 2009) (86).

a) Cartilage; mesoderm.
b) Glandular epithelium; endoderm.
c) Neural rosettes; ectoderm.


a)
b)
c)

1.2.7 MFG-E8

In an attempt to identify specific immunomodulatory factors derived from ESCs, ESC proteomic database was extensively searched. I speculated that Milk fat globule EGF factor 8 (MFG-E8, also known as lactadherin/BA46 in human) in undifferentiated ESCs might be one of the immunomodulatory components. MFG-E8 is detected in ESCs and its expression decreases after differentiation (87-90). MFG-E8 is a soluble glycoprotein in nature that was first discovered in epithelial mammary glands in lactating mice (91). The protein is localized close to the plasma membrane contained within small-exosomes to be secreted through membrane-bound microvesicles to the extracellular milieu (92). Secretion occurs when microvessicles fuse to the plasma membrane and release MFG-E8 as a complex with exosomes.

MFG-E8 structure allows for unique bi-motif functions. First, towards the N-terminal site, following the signal peptide (SP), there are two epidermal growth factor (EGF) domains (**Figure 4**). A highly conserved arginine-glycine-aspartate (RGD) motif is contained within the second EGF domain (EGF-2), which signals through binding αvβ3/αvβ5 integrins expressed on the phagocyte. Second, the C-terminal factor VIII homologous (discoidin) domains

(C1 and C2) contain the second motif by which MFG-E8 recognizes phosphatidylserine (PS) exposed on the surface of apoptotic cells (**Figure 4**) (93-96). The C2 domain is thought to be responsible for the secretion process since mutation in C2 results in defective localization towards the cell membrane and in turn defective secretion (91). The EGF and C-domains are separated by proline/threonine (P/T) rich repeat(s). Lack of exon 4, encoding for P/T rich repeat(s) between the second EGF domain and the first discoidin domain, due to alternative splicing, results in a shorter isoform of MFG-E8 (SMFG-E8) with a molecular size of 50-56 kDa (95). While the variation in size is due to heavily glycosylated threonine residues in the P/T domain (91, 95), it is believed that P/T sequences increase the protein's binding affinity to PS and enhances extracellular secretion of MFG-E8 (95, 97).

MFG-E8 has been found to be expressed in mammary epithelial cells, ovaries, endometrial tissue, APCs, mature and immature DCs, keratinocytes and splenocytes (98-100). MFG-E8 is also detectable in macrophages and its expression deceases with maturation (101). Cell lines such as mammary epithelial cell line, human macrophage cell line P388D1 and mouse monocyte macrophage cell line RAW264.7 express MFG-E8 as well.

MFG-E8 is also reported to be expressed in tumour conditions and various tumour cell lines (102). Human breast carcinoma, for example, is associated with highly elevated levels of MFG-E8 expression, to the extent it has been considered a breast cancer marker (103-107). Elevated MFG-E8 is also reported in neoplastic skin tissue and malignant melanoma (90) and is found to augment tumorigenesis and metastasis in a melanoma mouse model (108). An independent study has reported that MFG-E8 blockade triggers destruction of a tumour microenvironment (96). Tumour cell lines positive for the protein include human and murine T cell lymphoma cell lines, RMA and EL-4 respectively.

Evaluation of MFG-E8 in human and animal models shows abnormally low levels of MFG-E8 in some pathological conditions such as AIDs, Alzheimer disease, atherosclerosis and sepsis. MFG-E8-null mice, for example, are reported to produce anti-dsDNA autoantibodies and develop SLE-like autoimmune disease in several independent studies (109-112). Yamaguchi *et al.* reported that intronic mutation during MFG-E8 gene expression in humans is considered a risk factor for SLE (109). In Taiwan, SLE patients tested positive for SNP of MFG-E8 mRNA in a case control study (113). Moreover, in blood, splenic and intestinal macrophages, MFG-E8 has been shown to be down regulated in sepsis (114, 115). However, to date it remains unclear whether MFG-E8 is expressed in ESCs and whether it contributes to the immune regulation of ESCs.

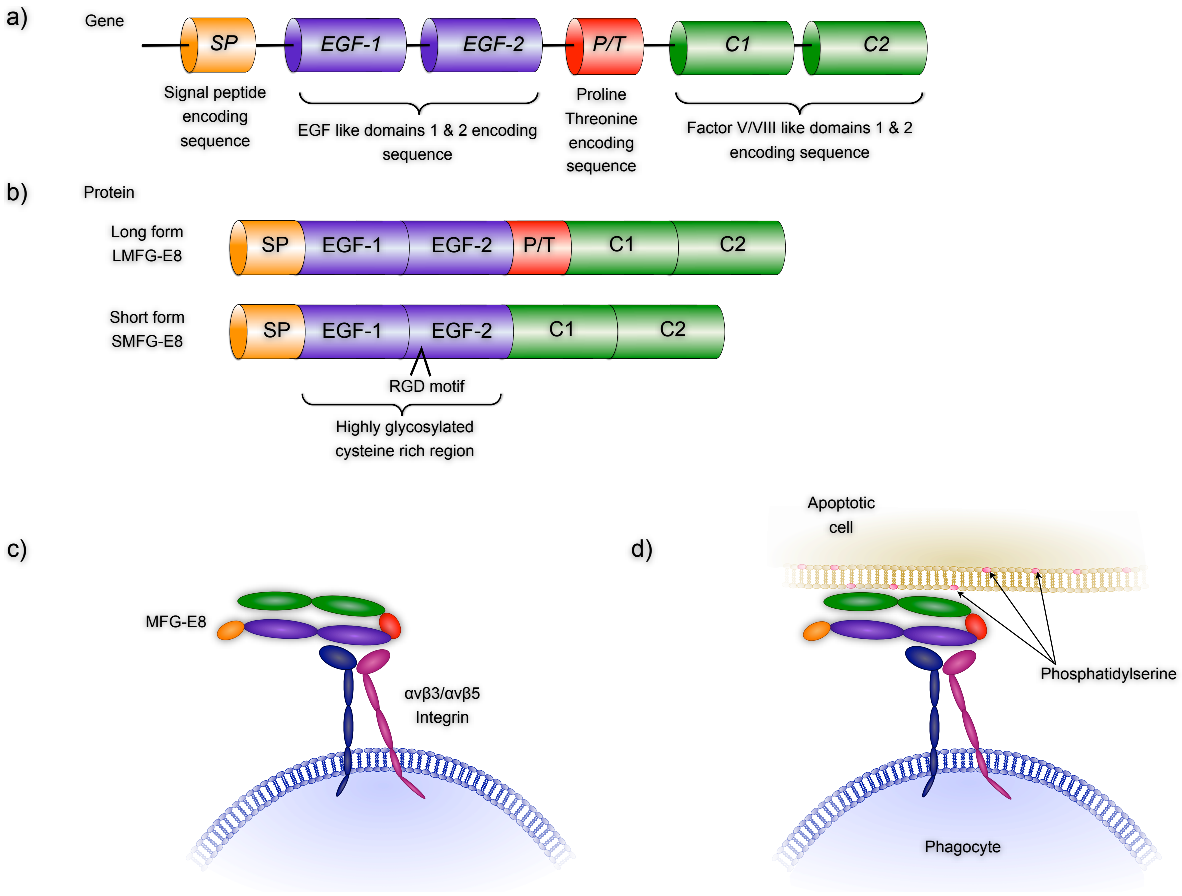
Figure 4. Function and structural motifs of MFG-E8.

a) MFG-E8 transcript.

b) Long form of MFG-E8 starts with N-terminal signal peptide (SP) followed by two epidermal growth factor (EGF-1 and EGF-2) domains with a highly glycosylated cysteine region. EGF-2 contains a highly conserved arginine-glycine-aspartate (RGD) motif. Proline/threonine (P/T) repeats are followed by two C-terminal factor VIII homologous (discoidin) domains C1 and C2. The short form of MFG-E8 lacks P/T repeats encoded by exon 4 as a result of alternative splicing.

c) The RGD motif on EGF-2 domain signals through binding αvβ3/αvβ5 integrins on cell surface.

d) MFG-E8 bridges phagocytes and apoptotic cells. C-terminal factor VIII homologous (discoidin) domains C1 and C2 contain the motif to recognize phosphatidylserine (PS) exposed on the surface of apoptotic cells while the RGD motif binds αvβ3/αvβ5 integrins on phagocytic cells.


a)
Gene
SP
EGF-1
EGF-2
P/T
C1
C2
Signal peptide encoding sequence
EGF like domains 1 & 2 encoding sequence
Proline Threonine encoding sequence
Factor V/VIII like domains 1 & 2 encoding sequence
b)
Protein
Long form LMFG-E8
SP
EGF-1
EGF-2
P/T
C1
C2
Short form SMFG-E8
SP
EGF-1
EGF-2
C1
C2
RGD motif
Highly glycosylated cysteine rich region
c)
MFG-E8
αvβ3/αvβ5 Integrin
d)
Apoptotic cell
Phosphatidylserine
Phagocyte

1.3 Rationale

Stemming from the fact that ESCs exhibit immune privilege, similar to fetal tissue, and in view of the reported increase in T regulatory cell numbers during pregnancy, ESC-derived factors may have a direct effect on T cell polarization (116). In addition, autoimmune diseases such as SLE and AIDs are T cell driven and MFG-E8-deficient mouse models show SLE-like autoimmunity and AIDs symptoms, thereby suggesting that MFG-E8 expressed by ESC might be a candidate factor, among others, to modulate T cell function. However, it remains unknown whether ESC extracts affect T cell polarization and which specific components in ESCs play important roles in the immune modulation of T cell activation. Undoubtedly, further studies will provide new insight into cellular and molecular mechanisms underlying immune regulation by ESCs and maternal-fetal tolerance. Moreover, identification of special immune components from ESCs may lead to future clinical applications.

1.4 Hypothesis

Based on the aforementioned findings I hypothesized that ESC extracts suppress T cell activation and function, regulate T cell polarization and that MFG-E8 might be one of the major players in ESC regulation of T cell activation.

1.6 Objectives

While previous findings provide a basis for pursuing further studies, it also raises several important questions that need to be addressed in advance. Thus, in order to explore ESC extract-mediated immune modulation on T cell activation and function, I sought to: 1) determine whether ESC extracts inhibit T cell activation or induce cell death; 2) examine whether ESC extracts are capable of modulating T helper function and/or polarization; 3) define MFG-E8 as a candidate factor that contributes to the immune modulation of ESC extracts. The three questions, therefore, are the focus of my MSc. studies and will be addressed in this thesis.

Chapter 2: MATERIALS AND METHODS

2.1 Mouse Strains

Mouse strains C57BL/6, B6C3F1, CD1 and Balb/c (10 to 16 weeks old) were obtained from the Charles River Laboratories (Montreal, QC, Canada). Animals were maintained at the Animal Care and Veterinary Services (ASVS) at Roger Guindon Hall (University of Ottawa, Ottawa, ON, Canada) in compliance with the Canadian Council on Animal Care guidelines under protocols approved by the Animal Use Subcommittee at The University of Ottawa. All animal were housed in a specific pathogen free environment.

2.2 Cell Lines

The mouse ESC C57BL/6 cell line was purchased from the American Type Culture Collection (ATCC). Mouse ESC D3 and J1 cells were kind gifts from Dr. Qiao Li and Dr. Michael Rudnicki, respectively (University of Ottawa, Ottawa, ON, Canada). These cell lines were grown in Dulbecco's modified eagle's Medium (DMEM) containing 4.0mM L-glutamine, 1.0% non-essential amino acids, 0.10μM 2-β-mercaptoethanol (2-βME), 100 units of Penicillin, 100 units of Streptomycin and 15% FBS (Invitrogen Canada Inc., Burlington, ON) supplemented with 1000 units/mL of leukemia inhibitory factor (LIF) (Millipore Canada Ltd., Etobicoke, ON) and incubated at 37°C with 5.0% CO_2. The RAW264.7 cell line was a kind gift from Dr. A. Makrigiannis (Department of Microbiology and Immunology, University of Ottawa, Ottawa, ON, Canada). The cells were grown in Dulbecco's modified eagle's Medium (DMEM) supplemented with 10% FBS and incubated at 37°C with 5.0% CO_2.

2.3 ESC Extraction

Mouse ES lines D3 and B6 were grown on MEFs that were mitotically inactivated with 10μg of mitomycin C for 2 hours at 37°C. To eliminate MEF cells, ESCs were subcultured on 0.1% gelatin coated plates for at least two passages. Subsequently, the cells were harvested upon reaching confluence by treatment with trypsin (Invitrogen Inc.) and dissociated to obtain a single-cell suspension. Afterwards, cells were washed twice with ice-cold PBS and centrifuged at 400g for 6 minutes at 4°C. After washing, the cells were resuspended in lysis buffer (50mM HEPES, 50mNaCl, 1.0mM EDTA, 1.0mM DTT, 50mM L-arginine, pH 8.2) which was supplemented with pan protease inhibitors (4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin, and aprotinin dissolved in DMSO) at 1:100 dilution according to manufacturer's instructions (Sigma Aldrich Canada Ltd, Oakvile, ON). Next, the cells were incubated on ice for 30 minutes with occasional flicking of the tube. At the end of incubation period, the cells were sonicated until complete lysis was achieved. The sonicated cells were centrifuged at 15000g for 15 minutes at 4°C to remove cell membranes, mitochondrial and nuclear fractions. Finally, the cell-free soluble fraction (supernatant) was separated from the insoluble fraction (pellet) and both were stored at -80°C. Protein concentration was determined using the Bio-Rad Protein assay kit (Bio-Rad Laboratories Canada Ltd., Mississauga, ON).

2.4 Mouse Splenocyte and CD3$^+$ T Cell Isolation

Male mice were sacrificed by cervical dislocation; spleens were removed aseptically, pooled and gently homogenized by grinding between the frosted ends of two sterile glass microscope slides and passed through a 45μm mesh filter to generate single cell suspensions. The cells were then washed twice with PBS. Red blood cells (RBCs) and dead cells were removed by Ficoll (lympholyte) centrifugation or ACK RBC lysis buffer (Cederlane Laboratories Canada Ltd., Burlington, ON). Afterwards, cells were washed twice with PBS and resuspended in complete RPMI medium. Purified CD3$^+$ T cells were obtained by negative selection using an immunomagnetic labeling kit (StemCell Technologies Inc.) according to manufacturer's instructions (purity was 97% for CD3 marker).

2.5 Mouse Splenocyte Activation and CFSE proliferation

Isolated splenocytes were suspended in serum free PBS at 1.0×10^6 cells/mL and stained with 0.01μM of carboxyfluorescein diacetate succinimidyl ester (CFSE) (Simga Aldrich Inc.) or 5.0μM of Violet Cell-Trace Cell Proliferation kit (Invitrogen Inc.) for 40 minutes at 37°C. Subsequently, the cells were washed twice with PBS. Splenocytes were plated at 1.0×10^5/well in 96 well plates in a total volume of 0.20ml of RPMI medium containing 10% FBS, 2mM L-glutamine, 100 U penicillin, 100 U streptomycin, 1.0mM Non-essential amino acids, 50μM 2-βME. Cells were stimulated with 1.2μg/mL of anti-CD3 and 0.50μg/mL of anti-CD28 (eBioscience Inc., San Diego, CA) in the presence or absence of 0.23mg/mL ESC extracts. The cells were allowed to proliferate for 2-3 days and CFSE dilution was analyzed by flow cytometry (CyAn, Beckman Coulter).

2.6 Cell Death Assays

Splenocytes were stimulated with anti-CD3 and anti-CD28 (eBioscience Inc.) in the presence or absence of 0.23mg/mL ESC extracts and allowed to proliferate for 3 days in 96 well plates as described above. Cells were harvested and washed twice with PBS. At this point, cells were resuspended in Annexin-V buffer and stained with 5.0µl of Annexin V-PE for 30 minutes (BD Biosciences Inc. Mississauga, ON, Canada). Cells also received 5.0µl of 7-amino-actinomycin D (7AAD) for the last 10 minutes of incubation. Cells were analyzed by flow cytometry (CyAn, Beckman Coulter).

2.7 T Cell Markers

T cell activation was examined using fluorochrome-conjugated antibodies specific to CD3, CD4, CD8, CD25, CD44 and CD69 (eBioscience Inc.). CD3$^+$ T cells were stimulated with plate-bound anti-CD3/anti-CD28 in the presence or absence of ESC extracts and allowed to proliferate for the indicated time-points. Cells were harvested and washed with PBS. Blocking was carried out with 10% rat serum on ice for 15 minutes. Antibodies were added to the cells according to manufacturer's recommendations and the cells were incubated for 30 minutes. At the end of the incubation period, cells were analyzed by flow cytometery (CyAn, Beckman Coulter). Data were analyzed by gating on CD3 positive cells followed by examination of activation markers CD25, CD44 and CD69 on $CD4^+$ and $CD8^+$ T cells separately.

2.8 Mixed Lymphocyte Reaction (MLR)

Splenocytes were isolated as described above. Prior to MLR culture, stimulator cells were pretreated with 50μg/mL of mitomycin C at 37°C for 40 minutes. One-way MLRs were carried out with either 1.0×10^5 splenocytes from both responder and stimulator cells in 96 well U-bottom plates or 1.0×10^6 splenocytes from both responder and stimulator cells in 48 well flat-bottom plates. The cells were allowed to proliferate for the specified time-points.

2.9 Quantitative Reverse transcription—polymerase chain reaction (RT/Q-PCR) analyses

A one-way mixed lymphocyte reaction was performed using mitomycin C-treated CD1 splenocytes as stimulator cells and C57BL/6 splenocytes as responder cells in the presence of 0.3mg/mL ESC extracts or vehicle (lysis buffer) control. Cells were harvested at the indicated time-points and total RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen Canada Inc., Mississauga, ON, Canada) according to the manufacturer's instructions. Immediately, isolated RNA was reverse transcribed into cDNA in a 20.0μl reaction volume using the Qiagen QuantiTect Reverse Transcription kit (Qiagen Canada Inc.) as follows; 500ng RNA was added to 2.0μl gDNA wipeout buffer and topped up to 14μl with RNase free water. The samples were then incubated at 42°C for 2 minutes and transferred immediately back on ice. Subsequently, 1.0μl of Reverse transcriptase, 4.0μl of Reverse transcriptase buffer and 1.0μl of RT primer mix were added to each tube from a master mix. The samples were incubated at 42°C for 15 minutes. Following cDNA synthesis, 0.4μl of cDNA from each sample,10.0μl iQ SYBR Green Supermix (Bio-Rad Laboratories Ltd.) and 9.6μl primer-mix was used to carry out QPCR with the iQ-iCycler (Bio-Rad Laboratories Inc.). The conditions for Q-PCR reactions were: one cycle at 94°C for 90 seconds, followed by 40 cycles at 94°C for 10 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Forward and reverse primers are as listed in **Table 1**. Gene expression levels were normalized to GAPDH and fold change was compared to relative gene expression with responder cells alone as a baseline through deltaC_t method.

Table 1. List of QPCR primer sequences.

Primer	Sequence
IL-2 forward	5’-CAGGATGGAGAATTACAGGAACCT-3’
IL-2 reverse	5’-TTTCAATTCTGTGGCCTGCTT-3’
IFN-γ forward	5’-GAAAATCCTGCAGAGCCAGA-3’
IFN-γ reverse	5’-TGAGCTCATTGAATGCTTGG-3’
T-bet forward	5’-GCCAGGGAACCGCTTATATG-3’
T-bet reverse	5’-GACGATCATCTGGGTCACATTGT-3’
TGF-β forward	5’-GTGCTCGCTTTGTACAACAGC-3’
TGF-β reverse	5’-TTACCAAGGTAACGCCAGG-3’
Foxp3 forward	5’-CGAAAGTGGCAGAGAGGTATTGA-3’
Foxp3 reverse	5’-ACTGTCTTCCAAGTCTCGTCTGAA-3’
MFG-E8 forward	5‘-ATATGGGTTTCATGGGCTTG-3’
MFG-E8 reverse	5’-GAGGCTGTAAGCCACCTTGA-3’

2.10 Intracellular Cytokine and Transcription Factor Staining

Two million splenocytes were pretreated with .023mg/mL ESC extracts or vehicle control overnight in 0.50ml of RPMI medium (containing 10% FBS, 2mM L-glutamine, 100 U penicillin, 100 U streptomycin, 1.0mM Non-essential amino acids, 50μM 2-βME) in 48 well plates. On the next day, cells were stimulated with either anti-CD3/CD28 or PMA/ionomycin for 6 hours. The cells were treated with protein transport inhibitor cocktail (eBioscience Inc.) after the first one to two hours of stimulation. At the end of the incubation period, cells were harvested and washed twice with FACS buffer. Subsequently, blocking was carried out with 10% rat serum on ice for 15 minutes. The cells were then stained with antibodies against CD4 and CD8 surface markers for 30 minutes followed by two washes with FACS buffer. At this point, cells were fixed and permeabilized using the Foxp3 Fixation/Permeabilization Concentrate and Diluent kit according to the manufacturer's instructions (eBioscience Inc.). Finally, intracellular staining was carried out with antibodies against IFN-γ and Foxp3 (eBioscience Inc.) following the manufacture's instructions and analyzed by flow cytometry (CyAn, Beckman Coulter).

2.11 Western Blot

Two million cells were harvested and lysed immediately in 100μl ice-cold lysis buffer (25mM Tris-HCl, 0.15M NaCl, 5.0mM $MgCl_2$, 1.0% NP-40, 1.0mM DTT, 5.0% glycerol, [pH 7.5]). The lysis buffer was supplemented with pan protease inhibitors (4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin, and aprotinin) at 1:100 dilution (Sigma), 1mM phenylmethane-sulfonylfluoride (PMSF) (serine protease inhibitor) and 1mM Sodium Orthovanadate (protein phosphatase inhibitor) dissolved in DMSO. After 20 minutes of incubation on ice, whole cell lysates were centrifuged at 15000g for 15 minutes at 4°C. Whole cell lysate supernatants were mixed with an equivalent volume of Laemmli Sample buffer containing 10% 2-βME (Bio-Rad Laboratories Ltd.). Samples were boiled for 3 minutes and electrophoresed on a 10% SDS-PAGE gel and transferred to Polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% powdered milk (w/v) or 5% BSA (w/v) in TBS-T for 1 hour at room temperature or overnight with gentle agitation at 4°C. Membranes were probed with rabbit anti-mouse MFG-E8 antibodies (Dr. Nagata, Kyoto University, Japan) at 1:500 dilution at 4°C overnight. Subsequently, membranes were washed 3 times with TBS-T for 15 minutes and were probed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody at 1:10000 dilution for 2 hours at room temperature. Again, membranes were washed 3 times with TBS-T for 10 minutes. At this point, the bands were visualized with Amersham ECL Plus western blot detection systems (GE Healthcare Biosciences Corp. Piscataway, NJ).

2.12 MFG-E8 Neutralization of ESC extract

Pan anti-MFG-E8 antibody was added to ESC extracts at a final concentration of 25 µg/mL. The extracts were incubated with the antibody for an hour at 4°C. Afterwards, extracts were used in T cell activation experiments as described above. Vehicle treated with anti-MFG-E8 and isotype antibody were used as controls.

2.13 Statistical analysis

Statistical significance was determined using a Student’s *t*-test, ANOVA or *chi*-square test wherever applicable. Results were considered significant with a p value < 0.05.

Chapter 3: RESULTS

3.1 ESC-derived factors directly inhibit T cell proliferation and modulate their activation without inducing cell death and apoptosis.

3.1.1 T cell proliferation

Soluble C57BL/6 ESC-derived cellular extracts, of both mouse and human ESCs (hESCs), have been shown to retain the immune modulatory properties of the intact cells (70). ESCs have been shown to inhibit T cell proliferation in one-way allogeneic MLR assays (70, 78). However, it is unknown whether ESC extracts can directly modulate T cell proliferation in response to anti-CD3 and anti-CD28 stimulation. To answer this question, I stimulated CFSE-labeled splenocytes with anti-CD3 and anti-CD28 in presence of an increasing concentration of ESC extracts. Dilution of dye intensity, reflected by CFSE peaks, represents cell divisions in this proliferation assay. I found ESC extracts, at concentrations above 0.23 mg/ml, can directly inhibit anti-CD3 and anti-CD28 mediated T cell proliferation, in a dose dependent manner compared to vehicle controls (**Figure 5**). In addition, microscopic inspection of cells in MLR assays or after anti-CD3/anti-CD28 stimulation in both 96 and 48 well plates show reduced proliferation morphology under inverted microscopy in ESC extract-treated as compared to untreated and vehicle-treated wells (**Figure 6**). Similar results were obtained with T cells from different mouse strains, such as C57BL/6, B6C3F1, Balb/c or CD1, treated with either B6 or D3 ESC extracts (data not shown). These results suggest that ESC-extract-mediated immune modulation is not restricted to a given mouse strain or a specific ESC line.

3.1.2 Apoptosis and Cell Death

It is unclear whether ESC extracts inhibit T cell activation or induce T cells to undergo activation induced cell death (AICD). To answer this question, T cells were treated with ESC extracts or control extracts in one-way MLR for 3 to 4 days. T cell apoptosis was assessed by flow cytometry after staining with Annexin V-FITC, which binds phosphatidylserine expressed on apoptotic cell surfaces (117, 118). Necrosis was assessed with the membrane viability dye, 7AAD, which binds the DNA of dead/necrotic cells with permeable or distributed cell membranes (119-121). The stimulator cells were inactivated with mitomycin C and pre-labeled with a fluorescent dye (Violet Cell-Trace) to exclude them during FACS analysis. To reduce the background of dead cells after freeze-thaw I used Ficoll (Lympholyte) density gradient centrifugation prior to cell culture. Similarly, I also determined T cell death in anti-CD3 and anti-CD28 stimulation assays in the presence of ESC extracts. The results show that splenocytes treated with ESC extracts did not exhibit a significant increase in the number of dead CD3$^+$ T cells in both MLRs and after anti-CD3 and anti-CD28 stimulation (**Figure 7**), thereby suggesting that ESC extracts inhibit T cell proliferation without inducing T cell death following activation.

3.1.3 Activation markers

In order to explore the impact of ESC extracts on T cell activation, CD3$^+$ T cells were negatively selected form isolated mouse splenocytes and a small fraction was stained with FITC-conjugated anti-CD3 antibody to confirm purity by flow cytometery. CD3$^+$ T cell purity was determined to be 97% (**Figure 8a**). The cells were then stimulated with anti-CD3 and anti-CD28 antibodies for the indicated times and subsequently examined with flow cytometry for surface expression of T cell activation markers such as CD25, CD44 and CD69 (122-124). I found that ESC extracts have the capacity to noticeably reduce surface expression of CD25, CD44 and CD69 activation markers on both CD4$^+$ (**Figure 8b**) and CD8$^+$ (**Figure 9**) T cells. Therefore, ESC extracts delivered an inhibitory signal to CD3$^+$ T cells resulting in decreased proliferation and expression of important T effector activation markers that are necessary for proper activation, cytokine production and subsequent proliferation.

Figure 5. Cellular extracts from ESCs inhibit T cell proliferation in response to anti-CD3/anti-CD28 stimulation. ESCs were grown in feeder free cultures, harvested and lysed by sonication. Cell membranes, mitochondria and nuclei were removed by centrifuging the sonicate at 15000xg for 15 minutes. C57BL/6 splenocytes were pre-labeled with CFSE and activated with anti-CD3/anti-CD28 in complete RPMI media in presence of lysates from C2C12 cells (Control-extracts, panel 1), untreated (panel 2), extraction buffer alone (vehicle Treatment, panel 3), or increasing concentration of lysates from ESCs (ESC-extracts, panels 5-8). Unstimulated cells are presented in panel 4. After 48 hours of incubation, the cells were analyzed by flow cytometry for proliferation. Results are representative of 4 separate experiments.

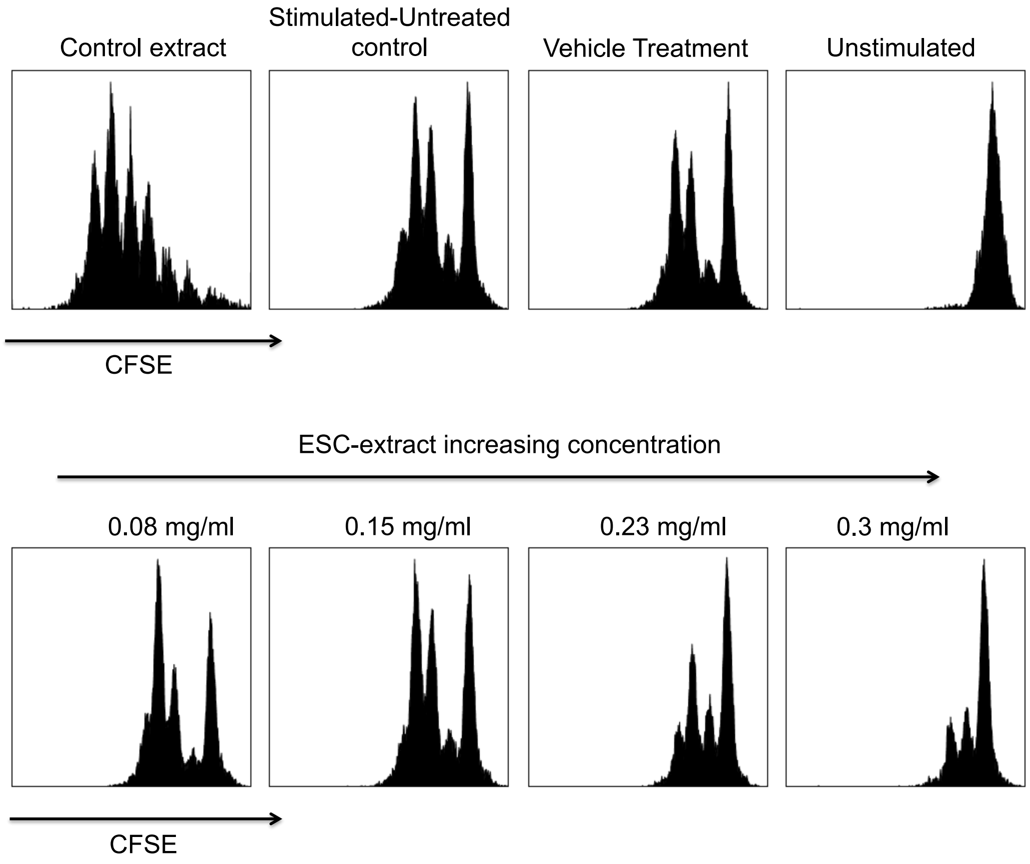

Control extract
Stimulated-Untreated control
Vehicle Treatment
Unstimulated
CFSE
ESC-extract increasing concentration
0.08 mg/ml
0.15 mg/ml
0.23 mg/ml
0.3 mg/ml
CFSE

Figure 6. ESC extracts inhibit T cell proliferation in a dose dependent manner. ESCs were grown in feeder free cultures, harvested and lysed by sonication. Cell membranes, mitochondria and nuclei were removed by centrifuging the sonicate at 15000xg for 15 minutes. C57BL/6 splenocytes were pre-labeled with CFSE and activated with anti-CD3/anti-CD28 in complete RPMI media and extraction buffer alone (vehicle Treatment, panel 1), without treatment (panel 2), unstimulated (panel 3) or increasing concentration of lysates from ESCs (ESC-extract, panels 4-7). Splenocytes were then plated at 1 x 10^5 cells/well in a 96-well plates. After 48 hours the cells were observed in an inverted light microscope and phase-contrast images were recorded. Results are representative of 4 separate experiments.


Vehicle treatment
Stimulated-Untreated
Unstimulated

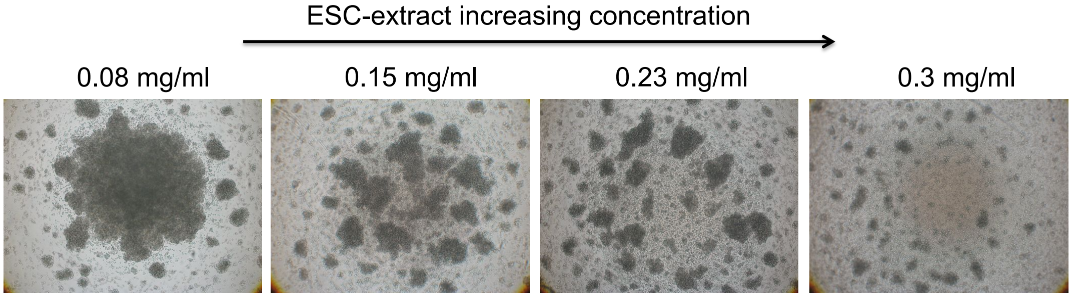

ESC-extract increasing concentration
0.08 mg/ml
0.15 mg/ml
0.23 mg/ml
0.3 mg/ml

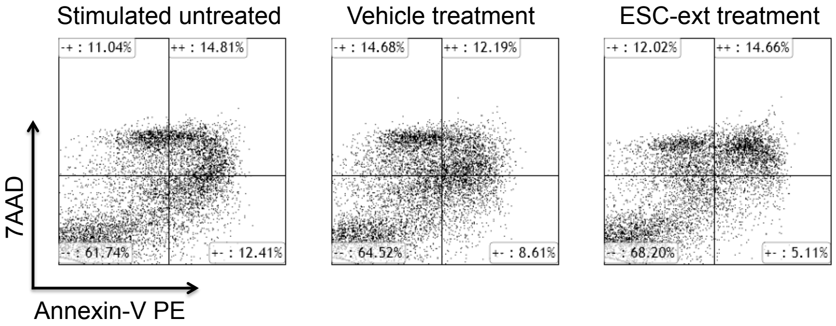
Figure 7. ESC-derived factors do not enhance T cell death.

a) C57BL/6 splenocytes were stimulated with anti-CD3/anti-CD28 antibodies in the presence of ESC extracts for 24 hours. The cells were harvested and washed with PBS and stained with anti-CD3 antibody, Annexin V-PE and 7AAD to examine T cell apoptosis and necrosis, respectively. Analysis was carried out by gating on $CD3^+$ cells followed by determination of Annexin V-PE and 7AAD frequencies.

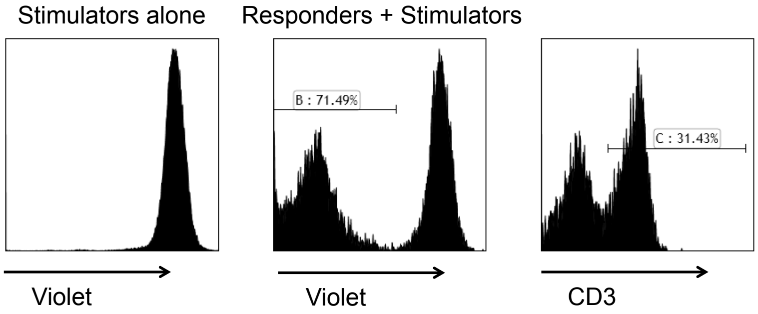
b) Responder C57BL/6 splenocytes were stimulated with CD1 splenocytes (pre-inactivated with mitomycin C and pre-labeled with violet cell-trace dye) in a one-way mixed lymphocyte reaction in the presence of ESC extracts or vehicle control. Cells were harvested and washed with PBS and stained with anti-CD3 antibody, Annexin V-PE and 7AAD to examine T cell apoptosis and necrosis, respectively. Analysis was carried out by gating on Violet negative cells and subsequently $CD3^+$ cells followed by determination of Annexin V-PE and 7AAD frequencies.

Results are representative of 4 separate experiments.

a)



b)



Sample	Absolute cell count-3days	% Viability	Viability	% Annexin-V	Annexin-V	% 7AA-D	7AA-D
Stimulated untreated	2.45 X 10^6	73.19	1.79 X 10^6	19.73	0.48 X 10^6	7.55	0.18 X 10^6
Vehicle treatment	2.21 X 10^6	72.43	1.6 X 10^6	17.27	0.38 X 10^6	10.89	0.24 X 10^6
ESC-ext treatment	2.45 X 10^6	77.88	1.9 X 10^6	14.4	0.35 X 10^6	8.01	0.19 X 10^6

Figure 8. ESC extracts inhibit up-regulation of CD25, CD44 and CD69 activation markers on CD4$^+$ T cells. Negatively isolated C57BL/6 CD3$^+$ T cells were stimulated with plate-bound anti-CD3/anti-CD28 antibodies in the presence of ESC extracts or vehicle control.

a) CD3 positive isolated T cells were stained with FITC-conjugated CD3 antibodies and examined for CD3 purity.
b) CD4 positive T cells were examined for CD25 and CD44 expression after 24 hours and CD69 expression after 6 hours of stimulation.

Results are representative of 3 separate experiments.

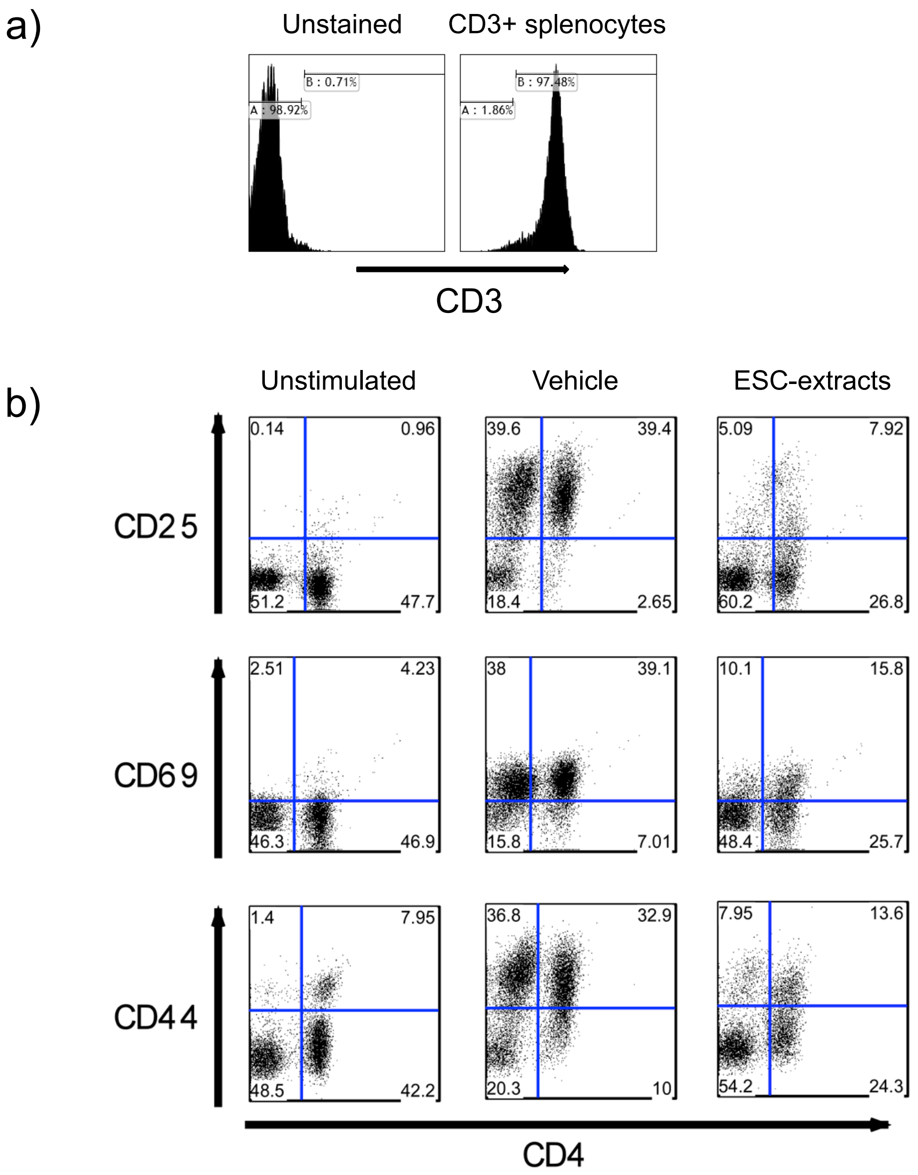
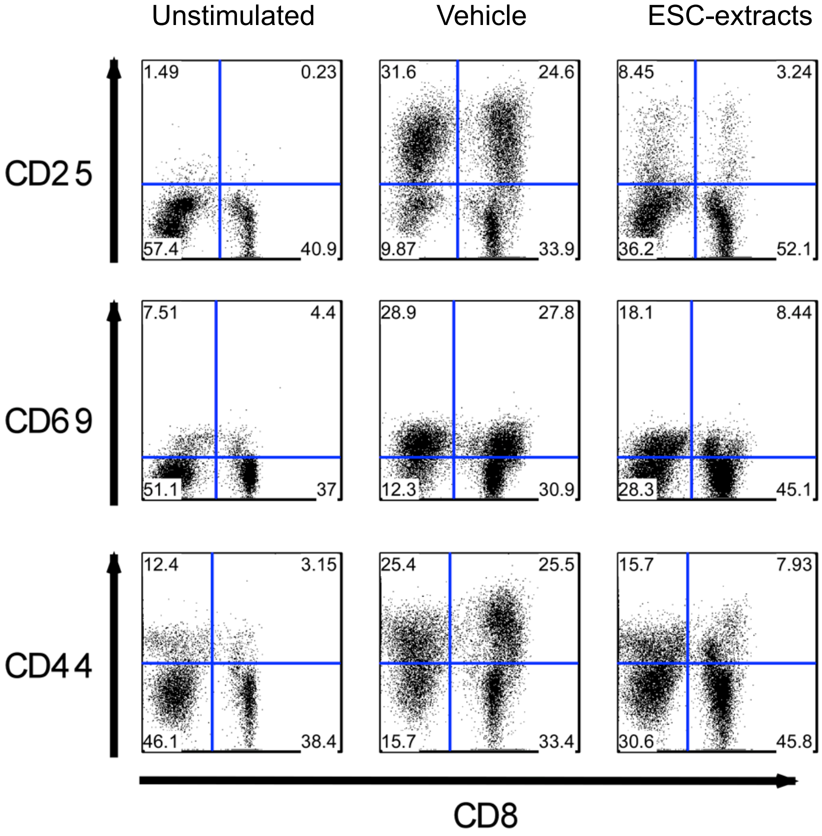

a)
Unstained
CD3+ splenocytes
B : 0.71%
A : 98.92%
B : 97.48%
A : 1.86%
CD3
b)
Unstimulated
Vehicle
ESC-extracts
CD25
0.14
0.96
51.2
47.7
39.6
39.4
18.4
2.65
5.09
7.92
60.2
26.8
CD69
2.51
4.23
46.3
46.9
38
39.1
15.8
7.01
10.1
15.8
48.4
25.7
CD44
1.4
7.95
48.5
42.2
36.8
32.9
20.3
10
7.95
13.6
54.2
24.3
CD4

Figure 9. ESC extracts inhibit up-regulation of CD25, CD44 and CD69 activation markers on CD8$^+$ T cells. Negatively isolated C57BL/6 CD3$^+$ T cells were stimulated with plate-bound anti-CD3/anti-CD28 antibodies in the presence of ESC extracts or vehicle control. CD8 positive T cells were examined for CD25 and CD44 expression after 24 hours and CD69 expression after 6 hours of stimulation. Results are representative of 3 separate experiments.


Unstimulated
Vehicle
ESC-extracts
CD25
CD69
CD44
CD8
1.49
0.23
57.4
40.9
31.6
24.6
9.87
33.9
8.45
3.24
36.2
52.1
7.51
4.4
51.1
37
28.9
27.8
12.3
30.9
18.1
8.44
28.3
45.1
12.4
3.15
46.1
38.4
25.4
25.5
15.7
33.4
15.7
7.93
30.6
45.8

3.2 ESC-derived extracts affect T helper (Th) polarization by inducing CD4$^+$ Treg cells.

3.2.1 Affecting gene expression.

The ability of ESC extracts to affect proper T cell activation led me to ask whether ESC extracts influence T effector cell function and polarization. To answer this question I sought to detect major T effector cell cytokines and transcription factors expression after ESC extract treatment at both gene and protein levels. For quantitative RT-PCR analysis, I extensively searched the immunology literature for murine T cell cytokines and markers to choose the most specific primer sequences with high specificity and low random background noise, termed expect-value (E-value), based on analysis performed with NCBI primer-BLAST hits. Subsequently, I also searched the literature for peak mRNA expression of murine effector T cell markers and cytokines in response to allo-stimulation. Since some cytokines such as IL-2 and IFN-γ are expressed with earlier kinetics, while others such as IL-10, TGF-β and transcription factor Foxp3 are expressed later, I isolated RNA at different time-points following stimulation in MLRs (**Figure 10**) for quantitative RT-PCR analysis. I observed significant decreases in the expression levels of IL-2 and IFN-γ after 8 hours stimulation and a significant up-regulation of TGF-β expression levels by 24 hours in one-way MLRs treated with ESC extracts compared to vehicle-treated controls (**Figure 11a**,**b**,**d**). For transcription factors, ESC extract treatment significantly induced a 10-fold increase in the level of Foxp3 mRNA (**Figure 11e**), whereas Tbet expression remained unchanged (**Figure 11c**). From these results, it can be concluded that ESC extracts may skew T cells from a Th1 to a T regulatory subset in response to MHC-alloantigen stimulation.

3.2.1 Intracellular cytokines and transcription factors.

Quantitative RT-PCR analysis of mRNA levels in mixed lymphocyte reaction is a measure of steady-state transcript levels and does not distinguish between cell types producing specific transcripts. Hence, I sought to further confirm cell-specific induction of cytokine and transcription factors through intracellular IFN-γ and Foxp3 protein detection in T cell subsets. Mouse splenocytes treated with ESC extracts or controls were stimulated with anti-CD3 and anti-CD28 or PMA/Ionomycin. The cells were then harvested at different time-points and analyzed by flow cytometry. First, I observed that ESC extract treatment induced an increased frequency of $CD25^{+}$ $Foxp3^{+}$ Tregs in the $CD4^{+}$ T cell subset (**Figure 12**). Secondly, I found that ESC extract treatment reduced intracellular IFN-γ production in $CD8^{+}$ T cells compared to controls (**Figure 13**). These data support the results obtained from quantitative RT-PCR analysis of transcript levels described above (**Figure 11**). Hence, ESC extract treatment of splenocytes favours a Foxp3$^+$ Treg phenotype over a Th1 response.

Figure 10. One-way Mixed lymphocyte reaction (MLR). Splenocytes from two different mouse strains were isolated and separated using ACK lysis buffer or Ficoll (lympholyte) gradient centrifugation. Prior to MLR, one set (stimulators) was inactivated with 50μg/mL of mitomycin C for 40 minutes at 37°C. Subsequently, 1.0 x 10^6 splenocytes from both responder and stimulator cells were mixed together in 48 well flat-bottom plates or 96 U-bottom plates in 1:1 ratio and received either ESC extracts or vehicle control**.** The cells were allowed to proliferate for the time-points.

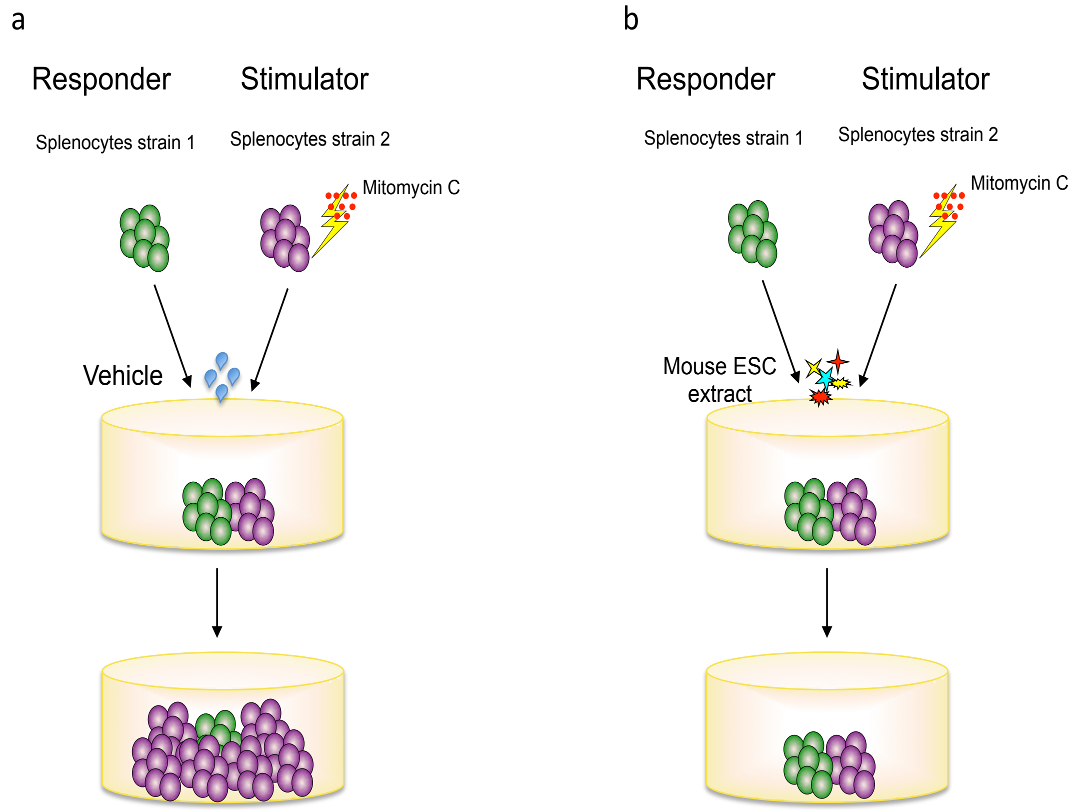

a
Responder
Stimulator
Splenocytes strain 1
Splenocytes strain 2
Mitomycin C
Vehicle
b
Responder
Stimulator
Splenocytes strain 1
Splenocytes strain 2
Mitomycin C
Mouse ESC extract

Figure 11. ESC extracts modulate T cell responses in allogeneic immune stimulation. Responder C57BL/6 mouse splenocytes were stimulated for 6, 8 and 24 h with CD1 mouse splenocytes in a one-way mixed lymphocyte reaction in the presence of ESC extracts or vehicle control. Cells were harvested and total cellular RNA was isolated and cDNA was synthesized. The expression of cytokines and master regulator transcription factors of T helper cells were measured by QPCR and normalized to GAPDH mRNA levels. mRNA levels in non-stimulated splenocytes (responders alone) was set at 1.0 (baseline) and all other normalized mRNA levels were plotted relative to that value. a) IL-2, b) IFN-γ, c) T-bet d) TGF-β and e) Foxp3 mRNA. Results are representative of 3 separate experiments. Data points represent mean +/- SD. * indicates *p* value <0.05. White bars represent results obtained from vehicle-treated MLRs and black bars indicate results obtained from ESC extract treated MLRs.

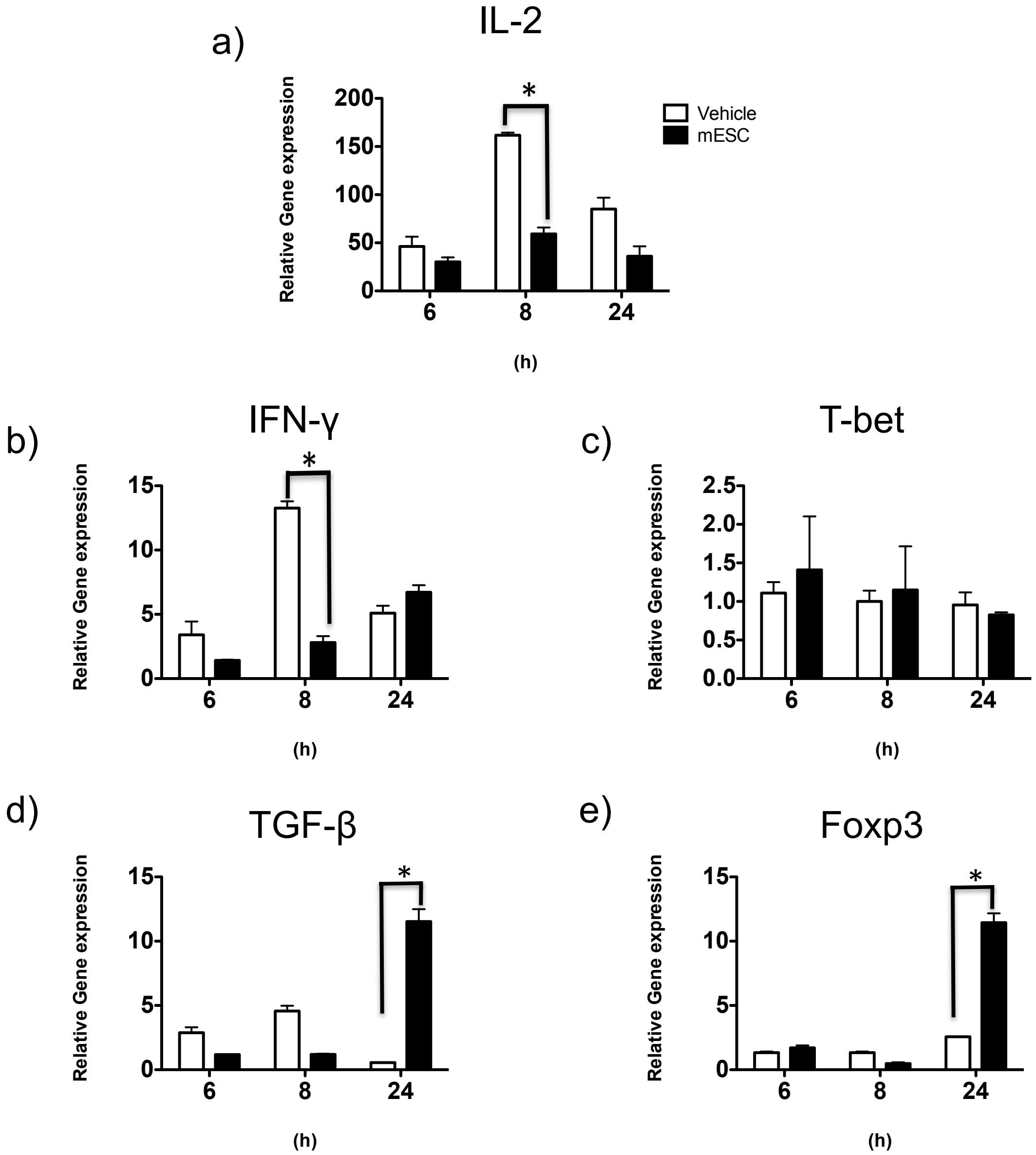

a)
IL-2
Relative Gene expression
200
150
100
50
0
6
8
24
(h)
*
Vehicle
mESC
b)
IFN-γ
Relative Gene expression
15
10
5
0
6
8
24
(h)
*
c)
T-bet
Relative Gene expression
2.5
2.0
1.5
1.0
0.5
0.0
6
8
24
(h)
d)
TGF-β
Relative Gene expression
15
10
5
0
6
8
24
(h)
*
e)
Foxp3
Relative Gene expression
15
10
5
0
6
8
24
(h)
*

Figure 12. ESC extracts skew T cell helper responses towards T regulatory cells. C57BL/6 splenocytes were treated with ESC extracts and stimulated with anti-CD3 and anti-CD28 for 3 days. The cells were harvested and stained for CD3, CD4 and CD25. Subsequently, cells were fixed, permeabilized and stained for Foxp3. Gates were set on CD3$^+$ followed by CD4$^+$ cells. Results are representative of at least 3 separate experiments.

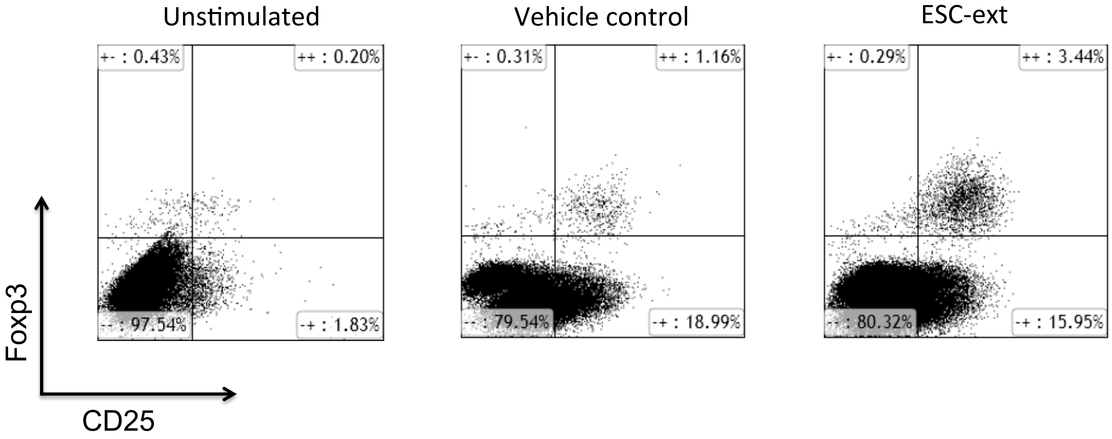
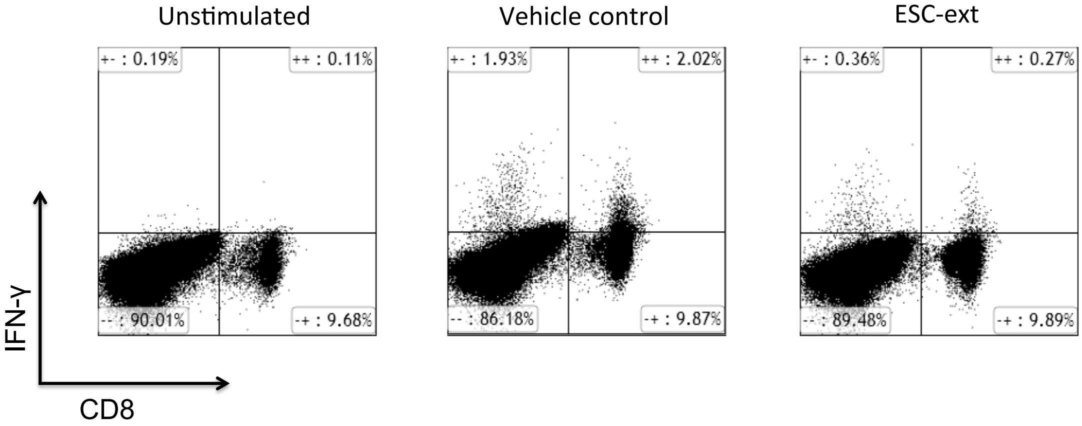

Unstimulated
+- : 0.43%
++ : 0.20%
-- : 97.54%
-+ : 1.83%
Vehicle control
+- : 0.31%
++ : 1.16%
-- : 79.54%
-+ : 18.99%
ESC-ext
+- : 0.29%
++ : 3.44%
-- : 80.32%
-+ : 15.95%
Foxp3
CD25

Figure 13. ESC extracts decrease IFN-γ positive CD8$^+$ T cells. C57BL/6 splenocytes were pre-treated over night with ESC extracts and stimulated with PMA and Ionomycin or anti-CD3/anti-CD28 for 6 hours. Protein transport inhibitor cocktail was added to the cells 1 hour following stimulation. Cells were harvested and stained for surface CD8 marker. After washing, the cells were fixed, permeabilized and stained for intracellular IFN-γ. Results are representative of at least 3 separate experiments.


Unstimulated
Vehicle control
ESC-ext
+- : 0.19%
++ : 0.11%
-- : 90.01%
-+ : 9.68%
+- : 1.93%
++ : 2.02%
-- : 86.18%
-+ : 9.87%
+- : 0.36%
++ : 0.27%
-- : 89.48%
-+ : 9.89%
IFN-γ
CD8

3.3 MFG-E8 expressed by ESCs contributes to the inhibition of T cell activation.

Treating ES cell lysates with proteinase K abrogates extract-mediated suppression of PBMC proliferation whereas RNase treatment had no influence on the extracts' suppressive function (70). Therefore, factors involved in ESC-mediated immune modulation are very likely to be proteinaceous in nature. According to proteomics databases, few proteins, including MFG-E8, with immune modulatory function appear to be highly expressed in undifferentiated ES cells while decreasing in differentiated ES cells (87-90). Based on current literature, milk fat globule-EGF factor 8 protein (MFG-E8) may contribute to T cell polarization and immune suppression (94, 96, 112). I reasoned that high MFG-E8 expression by ESCs but not adult cells may contribute to the inhibition of T cell activation.

3.3.1 MFG-E8 expression in ESCs.

To determine the expression of MFG-E8 in ESCs, I first verified its expression at the mRNA level in B6 and D3 ESCs by Q-PCR and compared it to MFG-E8 mRNA levels in bone-marrow cells (low expression) and RAW264.7 cell line (high expression) as controls (**Figure 14**). I found that MFG-E8 mRNA expression in ESCs is significantly higher compared to its expression in bone marrow cells. Next, I determined MFG-E8 expression at the protein level by western immunoblotting. I found high levels of MFG-E8 protein expression in ESCs comparable to the expression level in the RAW264.7 cell line and cells over-expressing MFG-E8 (**Figure 15**).

3.3.2 MFG-E8 neutraliztion partially abrogates ESC extract-mediated inhibition of T cell activation.

To test whether MFG-E8 indeed contributes to the immune modulatory effect of ESC extracts on T cell activation, I attempted to neutralize MFG-E8 in ESC extracts. At the beginning, I used whole splenocytes in the experiments and did not find significant differences. I reasoned that monocytes known to produce MFG-E8 may interfere with neutralization experiments. Accordingly, ESC extracts were incubated with MFG-E8 blocking antibodies and a purified T cell activation assay was carried out as described in section **3.1.3**. I found that neutralization of MFG-E8 partially abrogate ESC extract-mediated reduction in CD25 surface expression on both CD4$^+$ and CD8$^+$ T cells (**Figure 16,17**). In addition, MFG-E8 neutralization abolished ESC extract-induced decrease of CD44 and CD69 expression on T cells (**Figure 16,17**). Taken together, the results show that MFG-E8 blockade dampens ESC extract-mediated inhibition of T cell activation. Since these results were reproducible when using different strains of mouse T cells and ESC extracts (data not shown), it is likely that this phenomenon is not mouse strain specific.

Partial restoration of T cell activation upon MFG-E8 blockade indicates that it, together with other ESC-derived factors, inhibit T cell functions. One ongoing project in the way is to define these factors using proteomic analysis followed by functional assay, which may reveal known and novel bioactive candidate proteins.

Figure 14. MFG-E8 mRNA expression. RAW, B6, D3 and fresh B6 bone marrow cells were harvested and total cellular RNA was isolated. Subsequently, cDNA was synthesized and the expression of MFG-E8 mRNA was measured by QPCR and normalized to GAPDH mRNA levels. mRNA level in RAW cells was set at 1.0 (baseline) and all other normalized mRNA levels were plotted relative to that value. Results are representative of 3 separate experiments. Data points represent mean +/- SD. * indicates *p* value<0.05. Results are representative of 3 separate experiments.

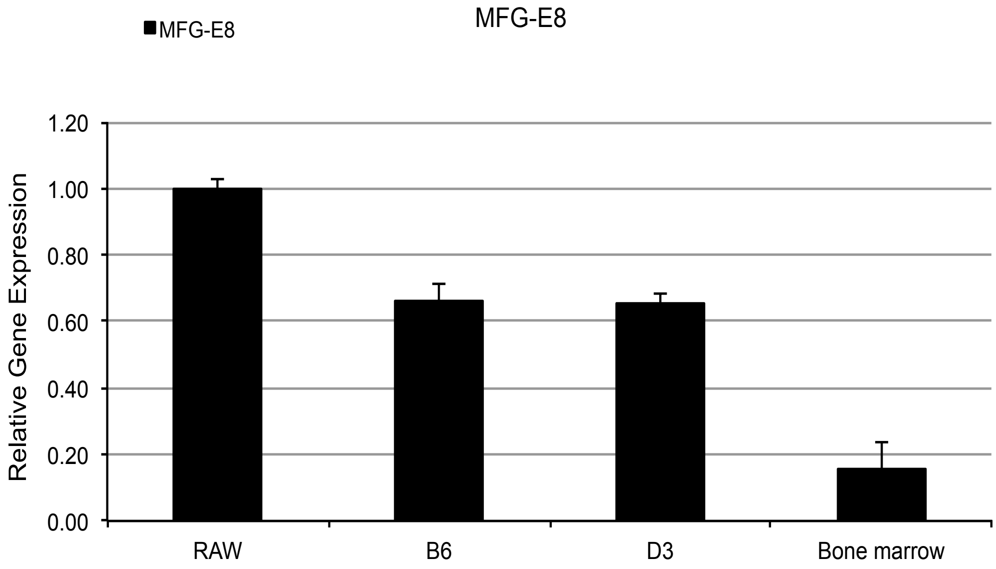

■MFG-E8
MFG-E8
Relative Gene Expression
1.20
1.00
0.80
0.60
0.40
0.20
0.00
RAW
B6
D3
Bone marrow

Figure 15 MFG-E8 expression. Cells with over-expressed MFG-E8, RAW cells and ESCs were harvested and lysed. Lysates were centrifuged at 15000 g for 15 minutes. Subsequently, supernatants were examined by western immunoblotting for MFG-E8. Results are representative of 3 separate experiments.

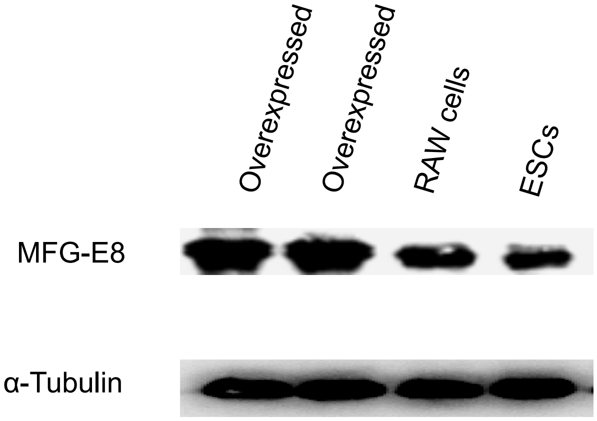

Overexpressed
Overexpressed
RAW cells
ESCs
MFG-E8
α-Tubulin

Figure 16. MFG-E8 blockade abrogates ESC extract mediated inhibition of activation markers CD25, CD44 and CD69 on CD4$^+$ T cells. Negatively isolated C57BL/6 CD3$^+$ T cells were stimulated with plate bound anti-CD3/anti-CD28 antibodies in the presence of ESC extracts, ESC extracts incubated with anti-MFG-E8 antibody or vehicle control. CD4 positive T cells were examined for CD25 and CD44 expression after 24 hours and CD69 expression after 6 hours of stimulation. Results are representative of 3 separate experiments.

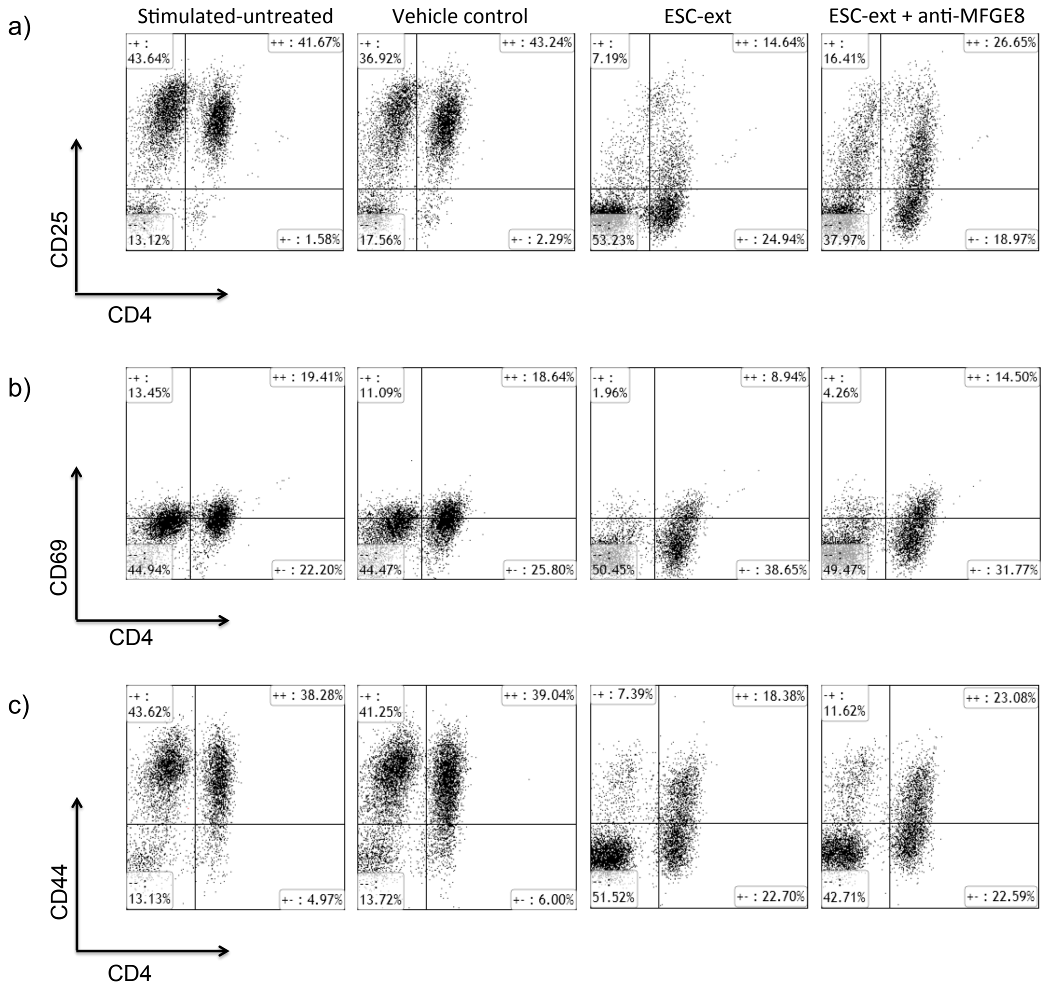
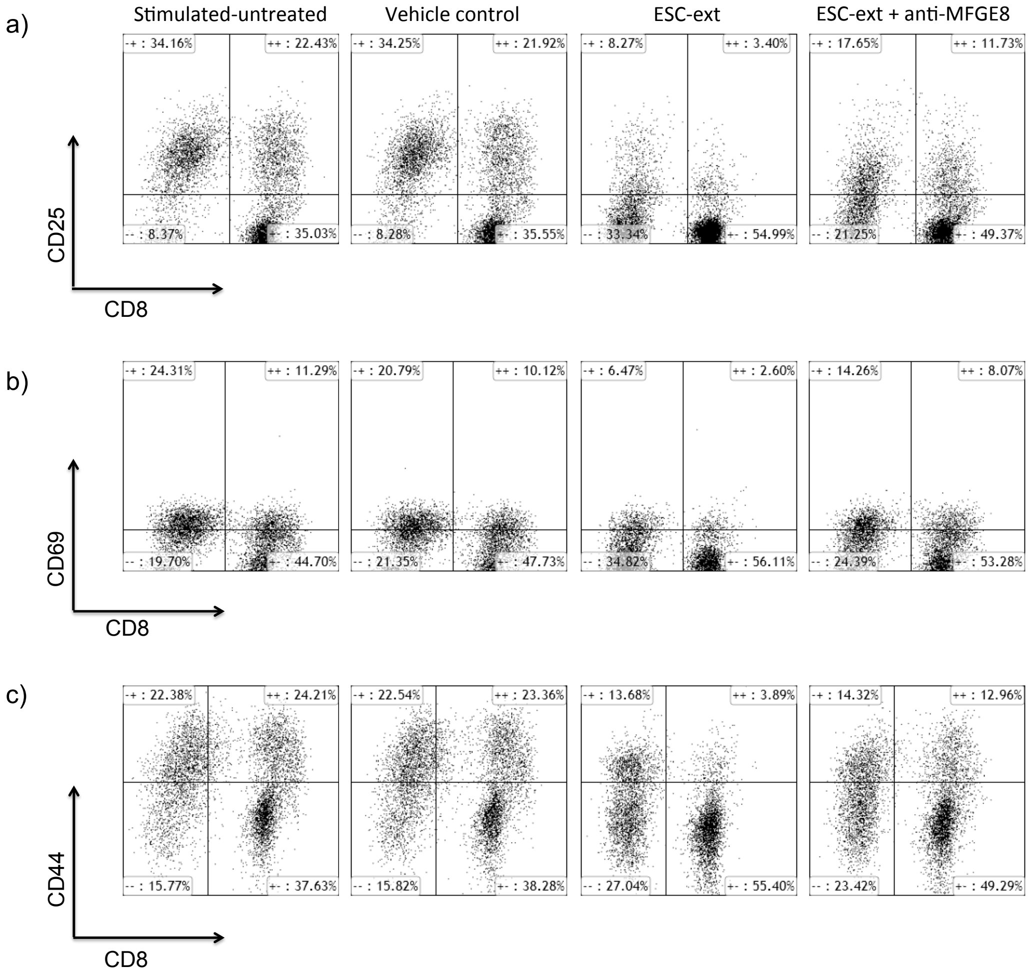

a)
Stimulated-untreated
Vehicle control
ESC-ext
ESC-ext + anti-MFGE8
-+ : 43.64%
++ : 41.67%
-- : 13.12%
+- : 1.58%
-+ : 36.92%
++ : 43.24%
-- : 17.56%
+- : 2.29%
-+ : 7.19%
++ : 14.64%
-- : 53.23%
+- : 24.94%
-+ : 16.41%
++ : 26.65%
-- : 37.97%
+- : 18.97%
CD25
CD4
b)
-+ : 13.45%
++ : 19.41%
-- : 44.94%
+- : 22.20%
-+ : 11.09%
++ : 18.64%
-- : 44.47%
+- : 25.80%
-+ : 1.96%
++ : 8.94%
-- : 50.45%
+- : 38.65%
-+ : 4.26%
++ : 14.50%
-- : 49.47%
+- : 31.77%
CD69
CD4
c)
-+ : 43.62%
++ : 38.28%
-- : 13.13%
+- : 4.97%
-+ : 41.25%
++ : 39.04%
-- : 13.72%
+- : 6.00%
-+ : 7.39%
++ : 18.38%
-- : 51.52%
+- : 22.70%
-+ : 11.62%
++ : 23.08%
-- : 42.71%
+- : 22.59%
CD44
CD4

Figure 17. MFG-E8 blockade abrogates ESC extract mediated inhibition of activation markers CD25, CD44 and CD69 on CD8$^+$ T cells. Negatively isolated C57BL/6 CD3$^+$ T cells were stimulated with plate bound anti-CD3/anti-CD28 antibodies in the presence of ESC extracts, ESC extracts incubated with anti-MFG-E8 antibody or vehicle control. CD8 positive T cells were examined for CD25 and CD44 expression after 24 hours and CD69 expression after 6 hours of stimulation. Results are representative of 3 separate experiments.


a)
Stimulated-untreated
Vehicle control
ESC-ext
ESC-ext + anti-MFGE8
-+ : 34.16%
++ : 22.43%
-- : 8.37%
+- : 35.03%
-+ : 34.25%
++ : 21.92%
-- : 8.28%
+- : 35.55%
-+ : 8.27%
++ : 3.40%
-- : 33.34%
+- : 54.99%
-+ : 17.65%
++ : 11.73%
-- : 21.25%
+- : 49.37%
CD25
CD8
b)
-+ : 24.31%
++ : 11.29%
-- : 19.70%
+- : 44.70%
-+ : 20.79%
++ : 10.12%
-- : 21.35%
+- : 47.73%
-+ : 6.47%
++ : 2.60%
-- : 34.82%
+- : 56.11%
-+ : 14.26%
++ : 8.07%
-- : 24.39%
+- : 53.28%
CD69
CD8
c)
-+ : 22.38%
++ : 24.21%
-- : 15.77%
+- : 37.63%
-+ : 22.54%
++ : 23.36%
-- : 15.82%
+- : 38.28%
-+ : 13.68%
++ : 3.89%
-- : 27.04%
+- : 55.40%
-+ : 14.32%
++ : 12.96%
-- : 23.42%
+- : 49.29%
CD44
CD8

Chapter 4: DISCUSSION

ESC studies have generated fundamental knowledge in the field of developmental and cellular biology, which represents one of the most promising areas of biomedical research in history. Importantly, on the world stage a wide variety of patients with serious genetic and degenerative conditions are seeking cures, or at least disease remission, in light of the promising potential of ESCs in cell replacement therapy (54, 56, 57, 59, 125-127). A recent FDA approved clinical trial and preliminary positive results have started to increase the hope for fulfilling some of those promises in patients with macular degenerative disease treated with hESC-derived retinal pigment epithelium transplant (128).

Understanding of ESC immune modulation will open a new research avenue and may hold strong application potential. Several studies have shown that ESCs not only are hypoimmunogenic but also have immune regulatory properties. ES cells from human, mouse and rat have been shown to be non-immunogenic, as they do not elicit immune responses in the presence of allogeneic immune cells (48, 56, 62, 65, 116, 129, 130). In addition to non-immunogenic properties, *in vitro* studies have demonstrated that ES cells are able to inhibit T cell proliferation (42, 48, 54, 116, 129). Moreover, ES cells have been shown to prevent immune activation in response to third party antigen presenting cells (APCs) *in vitro* and have the capacity to promote allograft survival *in vivo*. Several groups have demonstrated that ESCs have immune modulatory properties that not only allow them to survive across the allogeneic immune barrier but also provide protection to solid organ transplants (58, 60, 62, 63, 65-68, 70, 72, 78, 131). Understanding and deep analysis of ESCs' immune-privileged nature is of great importance and may lead to the discovery of specific components for broad-scale clinical applications in autoimmune diseases, allergies and transplantation.

As an attempt to identify specific factors in ESCs that modulate immune responses, the way lab has demonstrated that ESC extracts also possess immune inhibitory property. Previous results have clearly demonstrated that cellular extracts derived from both human and mouse undifferentiated ES cells retain the immune modulatory effects of intact cells. Previously it was shown that hESC extract treatment containing 12-24µg of total protein could effectively inhibit T cell proliferation in allogeneic mixed lymphocyte reactions (MLR), whereas control extracts from fibroblast cells have been found to enhance proliferation (70). Based on these results I decided to examine the effects of ESC extracts on T cell activation and polarization, investigate the molecular mechanisms behind these affects and finally define the immune modulatory components of ESC extracts.

Previous work provided the first evidence regarding the cellular mechanism underlying the effects of ESC extracts on monocyte derived dendritic cells (mDC) maturation (70). Treatment of mDCs with ESC extracts resulted in reduced surface expression of co-stimulatory and maturation markers CD80, HLA-DR and CD83 and lower levels of secreted IL12p40. Accordingly, ESC extract-treated DCs were also poor stimulators of purified allogeneic T cells in contrast to DCs treated with vehicle or fibroblast control extracts (70).

Here I have further demonstrated that ESCs have the capacity to directly inhibit T cell proliferation and activation. In addition, I found that ESC derived factors skew T helper responses from Th1 towards a regulatory phenotype by decreasing the expression of IL-2 and IFN-γ and increasing the expression of TGF-β and Treg transcription factor Foxp3. I also found that MFG-E8 in ESC extracts is one of the active immune-modulators. Recently, a potential mechanism wherein ESC-derived factors directly act on mitogen stimulated $CD3^+$ purified T cells and suppress PKC-θ phosphorylation without affecting upstream signaling events originating from CD3 and CD28 receptors on T cells was proposed (85).

4.1 ESC extracts directly inhibit T cell activation and polarization

Here I have found that ESC-derived factors directly inhibit T cell proliferation in a dose dependent manner wherein levels as low as 0.15 mg/mL of protein concentration can partially suppress T cell proliferation (**Figure 5**). In contrast, the control cell line C2C12-extracts stimulated T cell proliferation. In addition, CD44, CD69 and CD25 expression was markedly down-regulated in purified T cell activation experiments after treatment of ESC extracts (**Figure 8 and 9**). As discussed earlier, it has become apparent that ESCs can modulate T cell activation, however, the influence of ESC extracts on T cell proliferation, activation and polarization can be attributed to either a direct impact on T cells or an indirect influence through APCs. Attenuation of splenocyte proliferation as a result of anti-CD3 and anti-CD28 stimulation, not involving the help from APCs, indicates a direct effect on T cells.

Evidently, ESC-derived factors not only resulted in the attenuation of purified CD3$^+$ T cell activation, but also skewed the T cell response from a T helper to a T regulatory phenotype by down regulating IFN-γ, IL-2 mRNA and up regulating TGF-β and Foxp3 transcripts. Conversely, MLR treated with vehicle control induced an opposite response that seems to promote Th1 cells with increased IL-2 and IFN-γ production. Interestingly, there was little difference in the level of Th1 transcription factor T-bet expression. T-bet is known to be a Th1-specifying transcription factor but also required for T regulatory cells. For example, Koch *et al.* has found that Tregs require T-bet for proper function and homeostasis in response to Th1-mediated inflammation (132). Examination of intracellular T-bet protein level in Th1 cells by using FACS analysis may provide a clear insight.

The *il-2* gene is essential for T cell activation and subsequent proliferation as it is a downstream target of TCR signaling. However, IL-2 is instrumental in Treg differentiation as well. ESC extract treatment did not completely abolish IL-2 expression. Instead, IL-2 showed lower expression levels without significant change from the initial expression at the 6 hour time-point. Indeed, my results have shown that ESC-derived factors promote Foxp3$^+$ CD4$^+$ CD25$^+$ T cell differentiation. A shift towards Treg cells is considered a protective response against allograft rejection and autoimmunity. In the absence of DCs, CD44, CD69 and CD25 were markedly down-regulated in purified T cell activation experiments after treatment with ESC extracts. Collectively, my results demonstrated a direct effect of ESC extracts on T cell activation and polarization.

4.2 Identification of immune modulatory factors from ESC extracts

Since ESCs strongly inhibit both DCs and T cells, I sought to investigate a few candidate proteins with known immune modulatory functions that appear to have high expression in undifferentiated ES cells and little or no expression in differentiated ES cells. MFG-E8, among other proteins was on the list. One such protein is MFG-E8 was found to be highly expressed in undifferentiated ESCs with immune modulatory potential, as opposed to differentiated ESCs (**Figure 14 and 15**) (87-90). Based on the current literature, MFG-E8 may partially contribute to T cell polarization and immune suppression (94, 96, 112). Autoimmunity associated with MFG-E8 deficiency in mouse models suggests a strong correlation between this protein and T cell function.

MFG-E8 blockade with anti-MFG-E8 antibodies (Abs) in my T cell experiments had an antagonistic effect on T cell activation. My data show that neutralizing MFG-E8 in ESC extracts rescues down-regulation of CD44 in purified T cells upon stimulation. These observations are supported by studies showing strong association between MFG-E8 deficiency in knockout (KO) mouse model and decreased CD44 expression on T cells in conjunction with symptoms of autoimmunity. Consistent with these results, MFG-E8 has also shown to be involved in the inhibition of T cell activation and dendritic cell maturation (101, 112). It has also been shown to promote Treg differentiation in a tumour micro-environment (94, 96).

The role of MFG-E8 in immune modulation and Treg polarization has been recently explored by several studies. One proposed mechanism behind MFG-E8 immune modulation relies on accelerating clearance of apoptotic cells by facilitating Ag presentation and phagocytic signal conduction (133-136). MFG-E8 is characterized as an opsonin that bridges phosphatidyl-serine (PS) on apoptotic cell and αvβ3/αvβ5 integrins on APCs in order to enhance apoptotic cell phagocytosis by APCs (95, 134-137). Proper phagocytosis results in prevention of auto-Ags, nuclear and cellular debris from stimulating T cells (138-140). Another mechanism by which MFG-E8 could ameliorate inflammation is through competitive binding with αvβ3/αvβ5 integrins on phagocytes to enhance efferocytosis compared to other proteins that play an inhibitory role. In this particular case, Friggeri *et al.* have recently shown that an inflammatory cytokine, high-mobility group protein B 1 (HMGB1), binds αvβ3-integrin, leading to inflammation by impairing apoptotic cell clearance (141). In contrast, MFG-E8 has a high affinity to αvβ3-integrin and is capable of restoring macrophage phagocytic potential in the presence of HMGB1, resulting in amelioration of inflammation and autoimmunity (141).

Of interest is the observation that the inhibitory effect on T cell proliferation and activation by ESC extracts is not a result of cell death or apoptosis. The percentage of viable cells after ESC extract treatment is in fact slightly higher than vehicle and stimulated-untreated controls. Similarly, the percentage of annexin V positive events in ESC extract treated groups are slightly lower than control groups (**Figure 7**). This observation together with the report showing that MFG-E8 promotes resistance to apoptosis (96) strongly suggest that immune modulation of ESC extracts is unrelated to cell death. The contribution of MFG-E8 in tumour invasion and metastasis has been associated with inducing Foxp3^{+} Tregs while suppressing Th1 response and inhibiting CD8^{+} T cells and NK cells (108). In agreement with these results, MFG-E8 blockade, in other independent studies, has been shown to enhance drug-induced apoptosis and tumour invasion (94, 96, 108).

αvβ3 integrin signaling affects cell migration and cell adhesion. Cells in MLRs treated with ESC extracts show less aggregation compared to untreated and vehicle-treated controls that show more cellular clumps (**Figure 6**). αvβ3 integrins regulate DC and T cell cytokine profile and polarization (138, 142, 143). MFG-E8 has been shown to act as a bridging molecule, however, evidence of a direct anti-inflammatory effect, not related to apoptotic conditions, involving inhibition of TNF-α has also been elucidated recently (144). Several studies suggested a role of MFG-E8 in inhibiting TNF-α through direct or indirect mechanisms. A report by Aziz *et al.* demonstrated that recombinant mouse MFG-E8 attenuates TNF-α production via a STAT-mediated pathway (145). While TNF-α has been reported to negatively regulate both naturally occurring and induced Treg cells (146, 147) and the inhibition of PKC-θ plays an essential role in augmenting Treg suppressive function (148), both PKC-θ and TNF-α are demonstrated to act collaboratively in suppressing Tregs (148). PKC-θ is found to be sequestered at the distal pole of Treg cells which prevents its recruitment to the immunological synapse. Interestingly, in the Treg immunological synapse, TNF-α is instrumental in unleashing PKC-θ, which leads to suppression of Treg function and proliferation and an increase in effector T cell IFN-γ secretion (148). A recent finding by the way lab demonstrated a down-regulation in T cell PKC-θ phosphorylation and its subsequent activity upon receiving ESC extracts compared to control vehicle treatment (85). Furthermore, ESC extracts inhibited PKC-θ-mediated translocation of NFκB to the nucleus and prevented the degradation of NFκB inhibitor IκB-α (85). One possible mechanism underlying the inhibitory effect of MFG-E8 in ESC extracts on T cell activation may be through interfering with NFκB translocation to the nucleus as reported recently by Aziz and coworkers using a different model system (145). However, the precise role of MFG-E8 in ESC extracts needs to be further investigated.

The Identification of molecules involved in ESC-immune modulation is currently under investigation. Robertson *et al.* have ruled out molecules such as IL-10, TGF-β1, arginase-1, arginase-2, and endoleamine-2,3-dioxygenase as possible immune modulators in mouse ESCs (67). One of the candidate mechanisms, however, may involve TGF-β in mouse ESCs (62). Meanwhile, FasL has been implicated in rat ESC immune modulation (62, 78). In human ESCs, studies suggest a role of arginase-1 and HLAG in immune modulation (72, 131). In contrast, the use of 50 mM L-Arginine in the lysis buffer (Vehicle), as a supplement in my experiments to enhance protein stability and prevent dimer formation (149, 150) does not significantly suppress T cell activation and polarization. This suggests a multi-factorial effect rather than a single protein in ESC immune modulation. Recently, the way lab (85) suggested a role of ESC extracts in direct inhibition of murine T cell activation and polarization while another study demonstrated indirect mechanisms involving DC maturation (70). It appears that numerous mechanisms and multiple factors are involved in ESC immune modulation. Ultimately, it is of great importance to step forward and define other potential molecules involved in immune modulation in addition to MFG-E8 and investigate possible mechanisms *in vitro*, followed by *in vivo* verification.

4.3 Future directions

While these findings provide a basis and open doors for further studies, they also raise several important questions that need to be addressed. First, It would be interesting to elucidate how MFG-E8:αvβ3-integrin signaling affects T cell activation and whether MFG-E8 in ESC extracts targets PKC-θ activation or whether it is associated with NF-κB translocation and TNF-α down-regulation. The best direction to take would be to investigate the mechanism by which MFG-E8 modulates T cell activation by the use of MFG-E8-null ESC extracts. Second, it remains unknown what other factors involved in ESC extracts modulate immune responses and what specific roles they are playing. Thus, proteomic studies are needed to reveal alternative molecules and their functions in immune suppression. Without a doubt, *in vivo* verification of ESC extracts efficacy will amplify the value of *in vitro* results. Finally, these findings can be translated into future therapies after further understanding of the sequential events and suppressive mechanisms of ESC-derived factors.

4.3.1 Immune modulatory effect of ESC extracts on NK cells

There has been considerable controversy regarding ESC susceptibility to NK cells since the discovery of ESC immune privilege. Some researchers find low expression of MHC I on ESCs could theoretically render them targets for NK cell mediated killing while others present an opposing argument. NK cell function is regulated through the sum of activating and inhibitory receptors, and equilibrium between opposing signals determines NK cell activity (151). Down regulation of class I MHC molecules (ligand for inhibitory NK cell receptors), as seen in virus infected and tumor cells, render them targets for NK cell mediated lysis, whereas sufficient expression of self MHC I inhibits NK cell responses, a theory termed “missing-self” (152, 153). Cells also express ligands for NK cell activating receptors that are sufficient to trigger NK cell lysis (154); Dressel *et al.* reported that pluripotent stem cells remain susceptible targets for NK cells, despite reduced MHC expression (155). On the other hand, in spite of low expression of MHC I, Giuliani *et al.* have demonstrated that human embryonic-MSCs and iPS-MSCs disrupt NK cell immunological synapses and their lysis machinery (156). During pregnancy, embryonic tissue down-regulates MHC I antigens and retain HLA-C, HLA-E and HLA-G molecules to interact with uterine NK cells as a mechanism of escaping maternal immune surveillance (157, 158). A similar situation is seen in human

ESC-derived mesenchymal progenitors possess strong immune suppressive effects towards NK cells as well as T cells whereby HLA-G interacts with NK cells (159). Other studies have found reduced expression of activating ligands on both human and mouse ESCs to protect them against NK cells (61, 73). Erythrocytes and neuronal tissue are other examples of cells with no or low expression of MHC I, and yet are able to escape NK cell surveillance (160, 161). The presence of as yet unidentified inhibitory ligands or the absence of as yet unidentified activating ligands on these cells could explain this phenomenon. Hence, whether ESC-derived factors also modulate NK cell responses remain to be determined.

Concluding remarks

In summary, the studies described in this thesis suggest that ESC extracts have the capacity to directly modulate T cell responses. ESC extract treatment hinders T cell proliferation and suppresses surface expression of essential T cell activation markers. ESC extract treatment seems to skew T cell polarization by favouring a $CD4^+$, $CD25^+$, $Foxp3^+$ T regulatory phenotype over a Th1 response, leading to a decrease in IFN-γ secretion in $CD8^+$ T cells. Moreover, MFG-E8 is a protein with immune modulatory properties and is expressed in ESCs. Furthermore, interfering with MFG-E8 in ESC extracts can partially increase the number of activated $CD3^+$ T cells.

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