

# **Immunomodulation of Embryonic Stem Cell-produced MFG-E8 on T cells**

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## Abstract

Embryonic stem cells (ESCs) possess certain immunomodulatory properties; the defined components in ESCs, however, are largely unknown. Based on proteomic database, I report here that milk fat globule epidermal growth factor 8 (MFG-E8) is a key component in ESCs to suppress T cell activation and regulate T cell polarization. MFG-E8 is enriched in undifferentiated ESCs while diminishing in differentiated ESCs.

Neutralizing ESC-derived MFG-E8 substantially ameliorates the suppressive effects of ESCs on T cell activation and proliferation. Additionally, MFG-E8 in ESCs is capable of up-regulating T regulatory cells. I further prove that MFG-E8 suppresses T cell activation and regulates T cell polarization through inhibiting PKC $\theta$  phosphorylation. *In vivo* teratoma formation assay reveals an increase in ESC engraftments across allogeneic barriers with less immunologic rejection by up-regulation of MFG-E8 expression in ESCs, further validating the immunosuppressive properties of MFG-E8. Identifying an important immunoregulatory component in ESCs will greatly facilitate stem cell-based therapies.

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## LIST OF ABBREVIATIONS

APC	Antigen presenting cell
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CD	Cluster of differentiation marker
CTL	Cytotoxic T lymphocytes
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified eagle's Medium
ESC	Embryonic stem cell
EB	Embryoid Body
EDTA	Ethylenediaminetetraacetic acid
FOXP3	Factor forkhead box P3
FITC	Fluorescein isothiocyanate
GVHD	Graft versus host disease
HLA	Human leukocyte antigen
iPSC	Induced Pluripotent stem cells
IFN $\gamma$	Interferon- $\gamma$
IL-17	Interferon-17
IL-4	Interferon-4
MHC I	Major histocompatibility class I
MHC II	Major histocompatibility class II
MSC	Mesenchymal stem cell
MFG-E8	Milk fate globule Epidermal Growth factor 8
MLR	Mixed lymphocytic reaction
MEF	Mouse embryonic fibroblast cells
MS	Multiple sclerosis
NF $\kappa$ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
PMA	Phorbol myristate acetate

PBS	Phosphate buffer saline
PKC $\theta$	Protein kinase C theta
qPCR	Quantitative Polymerase chain reaction
Treg	Regulatory T cells
RA	Rheumatoid arthritis
SCNT	Somatic cell nuclear transfer
SD	Standard deviation
SDS	Sodium dodecyl sulfate
TCR	T cell receptor
Teff	Effector T cells
Th	T helper cells
TGF $\beta$	Transforming growth factor-beta
TBST	Tris-buffered saline and Tween 20

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## **Chapter 1. INTRODUCTION**

### **(1) Immune System Overview**

#### **(i) Innate and Adaptive Immunity**

To protect the body against a wide variety of foreign pathogens, the immune system has the capacity to activate two levels of immunity – innate and adaptive immunity.

Although each level functions with distinctive mechanisms, the interconnecting molecules and cells are of vital importance to maintain an optimal health condition.

Innate immunity comprises of physical barriers and a battery of fast responding cells mostly without the needs for priming and/or memory. Adaptive immunity, in contrast, facilitates specific antigen recognitions for more specialized immune responses, as well establishes a prolonged immune memory.

#### **(ii) Balance of T cell Immunity**

In adaptive immunity, T cells function to initiate, regulate and maintain diverse adaptive immune responses in an antigen specific manner. T cells are a type of lymphocyte uniquely characterized by the presence of surface T cell receptor (TCR), through which acquisition of antigen specificity is achieved by its interaction with major histocompatibility complex (MHC) on specialized antigen presenting cells (APCs) (1, 2). Unlike innate immunity with limited antigen specificity, clonal selection theory for T lymphocytes postulates major functions of adaptive immunity to generate highly targeted responses that maximize pathogen clearance and minimize healthy tissue damage (3, 4).

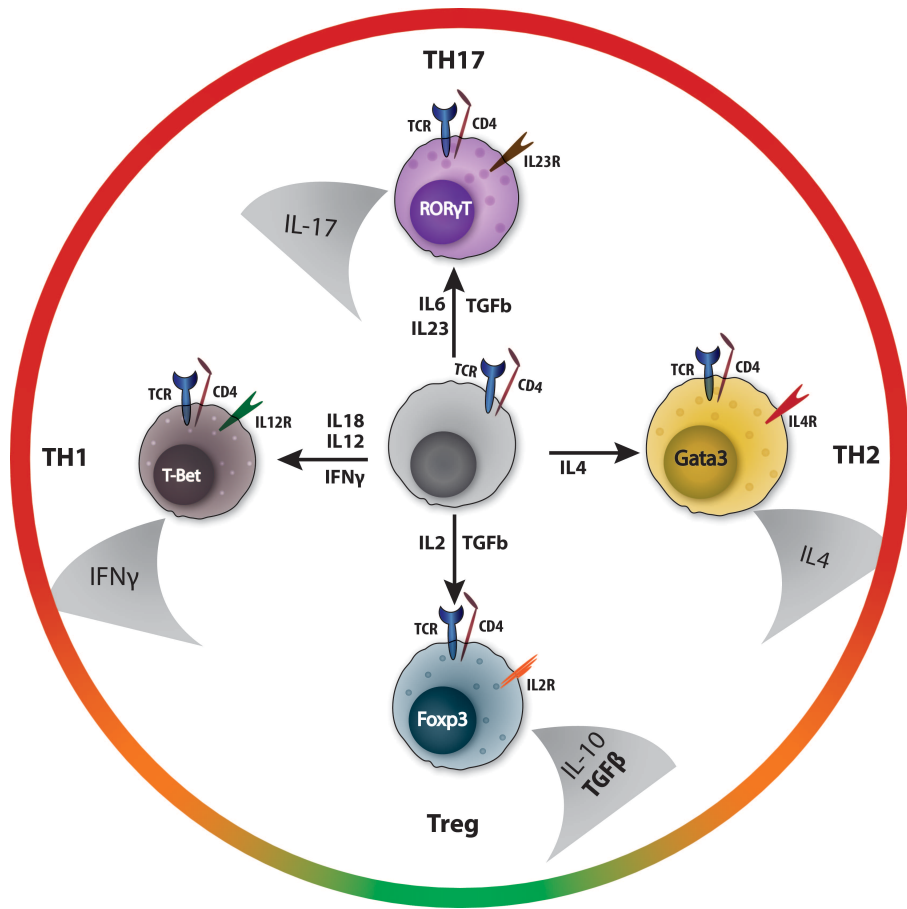
Expression of mutually exclusive co-receptors, either CD4 or CD8, subdivides T cells into CD4+ T helper cells or CD8+ cytotoxic T lymphocytes (CTLs). While CTLs can induce direct cell death to infected or dysfunctional cells through release of granzyme and perforin (5), CD4+ T helper cells mainly act indirectly to orchestrate the full panoply of immune responses by means of aiding in antibody production (6) and isotype switch

of B cells (7, 8), enhancing antimicrobial activity of macrophages (9) and recruiting activated leukocytes and lymphocytes to sites of infection (10). In 1986, the groundbreaking works of Mossman and Coffman introduced two distinct T helper cells subpopulations: those with predominant IFN $\gamma$  production and those with IL-4 as their signature cytokine (11), and hence the assumption that T helper cells function as a unity set of cells has since been disputed. Over the past 2 decades, our understanding of distinctive functional involvements of each T helper subsets and of mechanistic approaches to achieve each differentiated state has vastly expanded. According to current knowledge, after engagement with APCs through TCR-MHC and co-stimulatory molecules - B7.1/CD80 and B7.2/CD86 - interaction, naïve T cells could activate and differentiate into primarily 4 distinct functional active subsets (and possibly more). Such fate determination is established through an array of cytokine signals that T cells receive during an initial interaction with antigens (12). These well-recognized 4 subpopulations include Th1, Th2, Th17 and T regulatory (Treg) cells. Th1, Th2 and Th17 are considered as T effector cells, all of which encompass critical immunologic abilities to eradicate pathogens. Th1 cells fight intracellular microorganisms (13), Th2 cells target extracellular parasite (14) and Th17 cells clear extracellular bacteria and fungi infection (15, 16). On the contrary, Treg cells via production of anti-inflammatory cytokines – IL10 and TGF $\beta$  – and expression of surface negative regulators exert suppressive functions to maintain self-tolerance and control proper levels of immune responses (17) (Figure 1).

### **(iii) Autoimmunity and Self-Tolerance**

The immune system has uniquely evolved to possess the properties of distinguishing “self” and “non-self” and fighting disease-causing pathogens and abnormal cells. As T cell being a pivotal controller of immune responses, disruption of its balance could render breakdown of the common immunologic self-tolerance mechanisms, leading to the development of organ-specific or systemic autoimmunity. During T cell development in the thymus, multiple selection processes are employed to ensure that matured T cells entering the periphery have maintained a minimal reactivity towards self-antigens (18).

**Figure 1. Summary of CD4+ T cell polarization: their signature cytokines produced, their characteristic transcriptional factors, and condition crucial for fate determination.** Once activated, CD4+ T cells are instructed to differentiate into Th1, Th2, Th17 or Treg lineages in response to the unique environmental cues. Each T effector subset secretes its signature cytokines, involving in distinct immune responses.



However, a trade-off between TCR binding affinity and auto-reactivity results in a proportion of T cells that have low affinity to self-antigen survived the selection process, and thus it is heavily relied on Treg cells, a key component of peripheral tolerance, poised to prevent autoimmune reactions (19).

Errors in the developmental abnormalities or molecular mimicry from exogenous agents could trigger activation of autoreactive T cells and thereby initiate tissue destruction by targeting self-antigens. Numerous autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, and systematic lupus erythematosus (SLE) etc., are attributable to the actions of T cells (20). Autoreactive T effector cells, once activated, provoke a series of harmful inflammatory cascade indirectly via cytokine secretion or directly via cell-cell interaction with macrophages and B cells (21–23). To reestablish the disrupted T cell balance in autoimmune diseases, evoking Treg activation has been shown beneficial in restoring tolerance and suppressing the progression of autoimmune diseases (24).

Similarly, the ultimate goal of organ transplantation is the induction of immune tolerance to ensure the long-term survival of engrafted organ or tissue. It is evident that allograft rejection is mediated by a primary response from T cells, followed by infiltration of the graft with a mixture of pro-inflammatory immune cells (25–27). In a cohort of liver transplant studies, occurrence of acute cellular rejection has been recorded in 80% of patients and associated with an increases in morbidity and hospitalization rates (28). Even with advancements in immunosuppressive regimens, chronic rejections to therapy lead to the loss of up to 20% of grafts (29), and remain as one of the biggest challenges in clinical transplantation. Hence, the search continues for a novel, effective and specific immunomodulator that could restore the balance by retarding or promoting T cell responses. One of the interesting candidates is embryonic stem cells (ESCs).

## **(2) Embryonic Stem Cells**

ESCs are well recognized by their capacity to unlimited self-renew and ability to differentiate into virtually all cell types. The discovery of these cells has been one of the

most defining moments in the development of modern regenerative medicine and cell-replacement therapy, but understanding and potential clinical application of these cells have been complicated.

#### **(iv) Discovery**

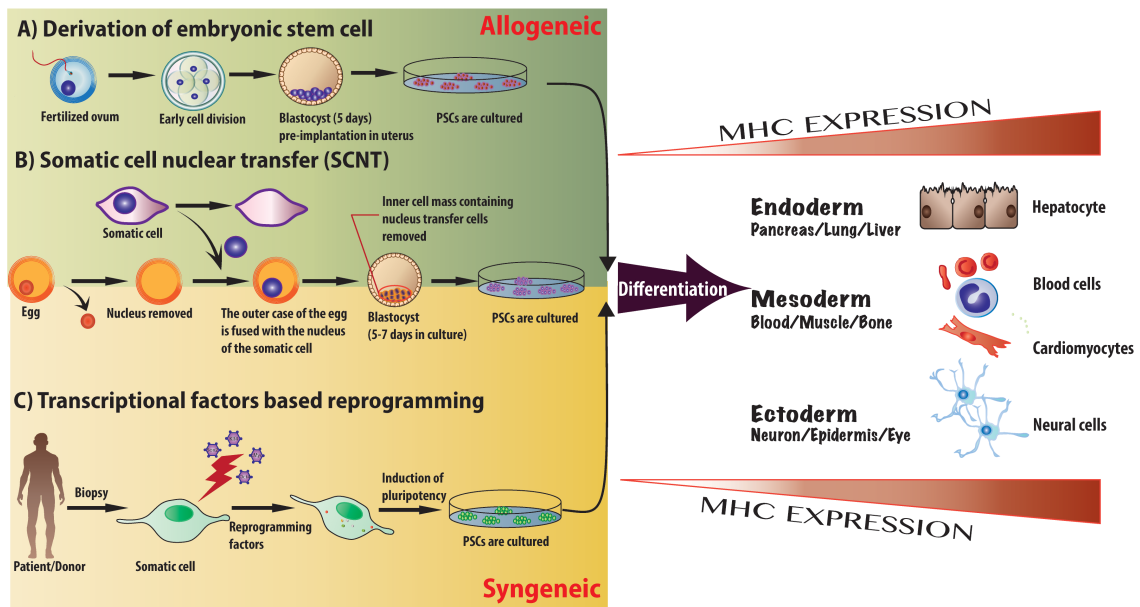
In 1981, two groups of scientists independently isolated cells from the inner cell mass of 3.5 day blastocyst, and these cells were successful subcultured *in vitro* showing undisrupted pluripotent potentials, thereby termed “embryonic stem cells”. Over a decade later, James Thomson developed techniques to isolate and culture human ESCs (30). Nevertheless, hurdles over the ethics concerns of destruction of viable embryos and directed differentiation impede the usage of human ESCs in clinical settings and render the progress of cell-based therapy limited to laboratory environment (31). It is not until several other techniques developed, which could be the possible answers to issues pertaining ESCs, that stem cell researches are truly jumpstarted.

To seek an alternative approach to obtain stem cells, Shinya Yamanaka developed the technology of converting adult cells to induced pluripotent stem cells (known as iPSCs) by the introduction of four transcription factors (Oct3/4, Sox2, c-Myc, Klf4) (32). The aim of carrying out this procedure was to bypass the need for embryos and to generate pluripotent cells that are genetically perfect-matched to the donor, thereby avoiding immune rejection during engraftments. (Figure 2.)

#### **(v) Feto-maternal Tolerance**

Acceptance of the fetus, which carries half of paternally inherited alloantigens, exemplifies a natural circumstance where the immune system restructures an alloimmune response to a state of tolerance. The fetus is considered as a foreign entity to the maternal immune system, but this allograft is not rejected (33). Even though mechanisms remain elusive, many contributing factors have been demonstrated to offer protection to the fetus from the maternal immune system, including expression of nonclassical MHC molecules – HLA-G (34, 35), tryptophan catabolism by IDO (36), induction of T cell apoptosis (37), involvement of Tregs (38–41), and surface inhibitory

**Figure 2. Different approaches for derivation of pluripotent stem cells.** A) ESCs can be derived by isolating the inner cell mass of a blastocyst 5-6 days post-fertilization and pre-implantation. B) The SCNT method facilitates the transfer of the nucleus from patient somatic cells into enucleated donor oocytes, the inner cell mass containing nucleus transfer cells is removed from the blastocyst after 5 to 6 days, and subsequently cultured to generate PSCs. C) Four transcriptional factors, Sox2, Oct3/4, c-Myc and Klf4, are transduced into somatic cells via viral vectors. Induced pluripotent stem cells (iPSCs, syngeneic patient specific PSCs) can be generated by repetitive passaging and subculture. Once the PSC cell line has been established from different sources, the cells have the capacity of self-renewal and unlimited division. PSCs can be differentiated into three germ layers – endoderm, mesoderm and ectoderm with clinical application potential. Reproduced with permission from (Tan *et al.*, Curr Stem cell Res Ther, 2014) License Number: 3580790696225 (24).



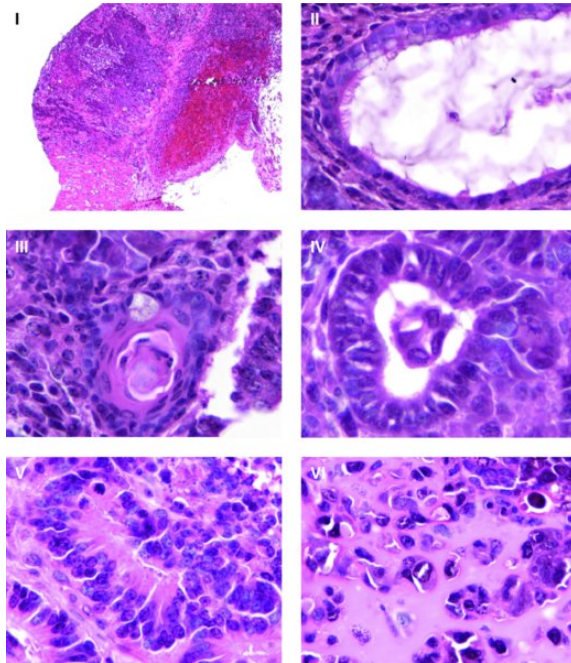
costimulatory molecule programmed death ligand (PDL) 1 (42). Of particular noteworthy, it is evident that fetal proteins can be detected in the maternal circulation, and yet these embryonic tissues could evade immune recognition. Given the origin and shared characteristics with an embryo, ESCs offer new strategies for examining and developing novel approaches for induction of tolerance.

#### **(vi) Immune privileged**

To achieve the aims of regenerative therapy for restoring dysfunctional or damaged tissues and organ, approaches involve transplantation of cells or combinations of cells and materials. For cells of syngeneic or autologous origin, immunological rejections are low. Transplantation of allogeneic cells, tissues or organs, however, would confront immunological barriers by allorecognition, which consequently induces rejection of engraftment. Studies of ESC-derived tissue replacement, however, seem to lack the concerns for potential immunologic rejection (43). More recently, ESCs have been described to exert certain levels of immune privileges in specific circumstances. To begin with, ESCs would not be subjected to allorecognition during MHC mismatch-induced immune rejections, as documented in studies that human (44), rat (45) and mouse (46) ESCs express extremely low levels of MHC class I molecules, and no MHC class II or costimulatory molecules. Moreover, observations that ESCs fail to induce allogeneic T cell proliferation in a mixed leukocytes reaction (MLR) support the concept of direct ESC-mediated immunomodulation (47). One of the proposed mechanisms for how ESCs modulate immune responses is through depletion of L-arginine at local sites to arrest T cell responses by the expression of surface Arginase I. Furthermore; our previous paper revealed the involvement of immunosuppressive soluble factors in ESCs. Soluble extracts from ESCs have found to retain the immunomodulatory properties of intact cells, showing by their capacities of suppressing T cell activation and alternating T cell polarization via inhibitive effects on the PKC $\theta$  pathway. While this provides a new approach to circumvent hurdles of teratoma formation when using live ESCs in therapeutics (Figure 3), the defined immunomodulatory molecules remains unknown.

**Figure 3. Histological characterization of teratoma formed from embryonic stem cells (ESCs).** Section of tumor tissues with remnant of three germ layers after 4-week inoculation into SCID mice. Reproduced with permission from (Cao *et al.*, *Circulation*. 2005), License Number: 3576040269245 (48).

- i. Myocardium, mesoderm
- ii. Respiratory Epithelium with ciliated columnar, endoderm
- iii. Squamous cells, ectoderm
- iv. Columnar gland, endoderm
- v. Neuronal rosette, ectoderm
- vi. Osteoid, mesoderm



### (3) MFG-E8

To pinpoint a specific contributor in ESC-mediated immunosuppression, we extensively searched through proteomic databases, and milk fat globule-epidermal growth factor 8 (MFG-E8), known as lactadherin in human, was considered as a potential candidate.

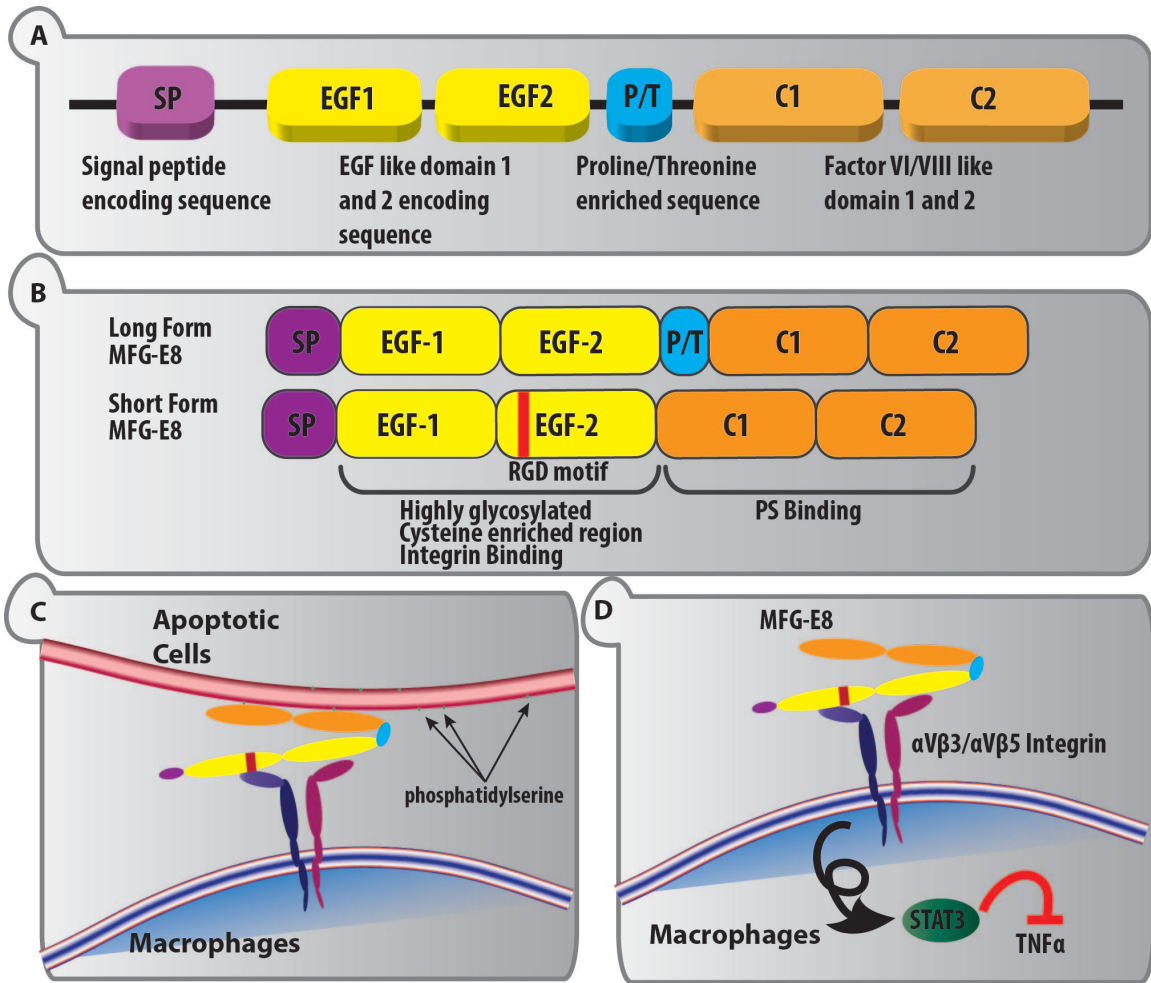
MFG-E8, a secreted glycoprotein, is first identified in mammary gland epithelial cells, but later becomes more well-known for its anti-inflammatory functions in macrophages where it assists phagocytosis signaling through  $\alpha V\beta 3/\alpha V\beta 5$  integrins (49–51). MFG-E8 is localized closely to the plasma membrane, encased within small-exosomes and secreted upon environmental stimuli (52).

MFG-E8 exhibits bi-motif functions: the N-terminal site of MFG-E8 peptide contains a peptide sequence that directs its secretion, and two epidermal growth factor (EGF) repeats. Within the second EGF repeat, there is a highly conserved arginine-glycine-aspartate (RGD) motif that facilitates binding to  $\alpha V\beta 3/\alpha V\beta 5$  integrin on the surface of phagocytes. The C-terminal factor V/VIII like domains recognize phosphatidylserine (PS) on apoptotic cells, and thereby allow MFG-E8 to scavenge for dying cells (53–55) (Figure 4.). With such unique molecular structure, MFG-E8 has proven to be a vital factor to ensure the proper clearance of dying cells and to control the autoimmune responses.

MFG-E8 expression is activated to attenuate pro-inflammatory cytokine production and to promote synthesis of anti-inflammatory cytokine IL10, which facilitates the establishment of a tolerant state during LPS-stimulated condition (56). Miksa *et al.* (57) have proposed that MFG-E8 interferes with LPS-TLR4 signaling by reducing activation and phosphorylation of MAP kinases and NF $\kappa$ B, thereby impeding pro-inflammatory cytokines production. Treatment with recombinant MFG-E8 (rMFG-E8) has also imposed considerable improvements to those diseases where the phagocytosis of apoptotic cells is not impaired. In a model of colitic mice, researchers have found that MFG-E8 ameliorates inflammation via competitive binding with the potent pro-inflammatory inducer to the  $\alpha V\beta 3$ -integrin receptor (58, 59).

**Figure 4. Function and structure of MFG-E8.**

- a) MFG-E8 transcript
- b) Two alternative spliced forms of MFG-E8. Long form of MFG-E8 begins with N-terminal signal peptide (SP), followed by two epidermal growth factor domains (EGF-1 and EGF-2). The highly conserved arginine-glycine-aspartate (RGD) motif is contained with the second EGF domain. C-terminal factor VIII domains C1 and C2 are followed by proline/threonine (P/T) repeats. The shorter spliced form of MFG-E8 lacks the P/T repeats.
- c) Bi-motif structural feature of MFG-E8 enabling it to bridge between phagocytes and apoptotic cells.
- d) RGD motif binds to integrin  $\alpha V\beta 3/\alpha V\beta 5$  on cell surface.



MFG-E8 is naturally expressed abundantly in intracellular exosomal compartment of immature dendritic cells (DCs) and undifferentiated macrophages (49, 50). To maintain homeostatic balance, MFG-E8 expression is under tight regulation. Differential MFG-E8 expression from its basal level is associated with pathological conditions. For instance, augmented tumorigenicity and metastatic capacity in tumors is correlated with elevated MFG-E8 expression (60, 61); development of autoimmunity is linked to down-regulated MFG-E8 level (62, 63).

#### **(4) Rationale**

Given the facts that MFG-E8 exhibits beneficial effects in inflammatory diseases (64–66) and that Treg infiltration increases in tumors with up-regulated MFG-E8 levels (67), MFG-E8, therefore, may be a key contributor in ESC-mediated T cell immunomodulation. Though none has yet linked MFG-E8 to T cell responses, MFG-E8 knockout mice exhibit SLE-like autoimmunity and AIDS symptoms, both of which have well been considered to be T-cell driven. Additionally, several studies have recorded lowered serum level of MFG-E8 in SLE patients (62) and elevated expression in different type of tumors (51, 68) that contributes to evasion of immune surveillance. All in all, it thereby suggests a potential role of MFG-E8 in T cell immunity.

#### **(5) Hypothesis**

Based on the aforementioned findings, I hypothesized that MFG-E8 is the key contributor in ESCs that suppresses T cell activation and regulates T cell polarization via inhibition of PKC $\theta$ .

#### **(6) Objectives**

To explore the role of MFG-E8 in ESC-mediated immunomodulation, I sought to: 1) confirm the expression level of MFG-8 in differentiated and undifferentiated ESCs; 2) examine whether MFG-E8 in ESCs is capable of suppressing T cell activation; 3) determine whether MFG-E8 induces T cell suppression through PKC $\theta$  pathway; 4) study whether ESC-derived MFG-E8 are able to modulate T effector/regulatory cell function and polarization; 5) further validate MFG-E8 mediated T cell immunosuppression via *in*

*vivo* teratoma formation assay. Above questions outline my MSc. research project, all of which will be addressed in this thesis.

## **Chapter 2. MATERIALS AND METHODS**

### **(1) Cell lines and animals**

Mouse ESC C57BL/6 (B6) cell line (69) was obtained from American Type Culture Collection (ATCC SCRC-1002). Mouse ESC D3 cells (70), derived from 129/SvJ mouse strain, were kind gifts from Dr. Qiao Li. Mouse ESCs were maintained on mitomycin-treated MEF feeder cells in Dulbecco's modified eagle Medium (DMEM) supplemented with 15% FBS (HyClone, Logan, UT, USA), 4.0mM L-glutamine, 1.0% non-essential amino acids, 0.1  $\mu$ M 2-ME,  $1.0 \times 10^2$  units of Penicillin and Streptomycin and  $1.0 \times 10^3$  units/mL of LIF (Millipore, USA). Cells were subsequently passed on 0.10% gelatin coated plates to eliminate MEF cells. Jurkat T cells were maintained in RPMI supplemented with 10% FBS (HyClone, Logan, UT, USA). RAW 264.7 cell line was obtained from Dr. Makrigiannis, which were grown in Dulbecco's modified eagle Medium (DMEM) supplemented with 10% FBS (HyClone, Logan, UT, USA). C57BL/6 (H-2<sup>b</sup>) and Balb/c (H-2<sup>d</sup>) mice (6-8 weeks of age) were obtained from Charles River Laboratories, and maintained at the University of Ottawa in accordance with the Canadian Council on Animal Care guidelines under protocols approved by the Animal Use Subcommittee at The University of Ottawa (Permit No. BMI-137/2025).

### **(2) Mouse ESC differentiation**

For differentiation of ESCs to embryoid bodies (EBs), mouse D3 and B6 ESCs were trypsinized and re-suspended in ESC medium without LIF supplementation to generate single-cell suspension, which was pipetted onto the lid of a cell culture dish for 2 days. Cells aggregated at the bottom of the droplets by gravitational force, thereby forming EBs. Afterwards, EBs were passed to ultra-low culture plate for another 2 days of culture. Spontaneous differentiation was finalized after transferring EBs to normal culture plate with differentiated medium (DMEM with 10% FBS, 4.0mM L-glutamine,

1.0% non-essential amino acids, 0.1  $\mu$ M 2-ME,  $1.0 \times 10^2$  units of Penicillin and Streptomycin each) for 15-17 days.

### **(3) Soluble factor extraction**

After plating of mouse D3 and B6 ESCs on 0.1% gelatin for at least two passages, cells were harvested by trypsin treatment and dissociated into single cells suspension. ESCs were then washed twice with ice cold PBS, and subsequently resuspended in lysis buffer (50 mM HEPES, 50 mM NaCl, 1.0 mM EDTA, 1.0 mM DTT, 50 mM L-arginine, pH 8.2) with protease inhibitor at 1:1000 (4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin, and aprotinin dissolved in DMSO, Sigma Aldrich). After 30 minutes incubation at 4°C, cells were sonicated until complete lysis of cell was achieved. The sonicated cell lysates were then centrifuged at 15000g for 15min in cold, and ESC soluble extracts were separated from the insoluble debris. Protein concentration was determined using NanoDrop 1000 (Thermo Fisher Inc., USA). Soluble factor extraction for other cell types and differentiated ESCs was done following same procedures.

### **(4) Mouse splenocyte and CD3+ T cell isolation**

Mice were sacrificed by cervical dislocation. Mouse spleens were removed aseptically and gently homogenized with the frosted ends of two sterile microscope slides. After being filtered through a 45 $\mu$ m mesh filter, cells were washed twice with PBS, and red blood cells were lysed using ACK red blood cell lysis buffer (Cederlane Laboratories Ltd.). After another two washes with PBS, splenocytes were re-suspended in media (RPMI medium with 10% heat-inactivated FBS, 2 mM L-glutamine,  $1.0 \times 10^2$  units of Penicillin and Streptomycin, 1.0 mM Non-essential amino acids, 50 mM 2-ME). Purified CD3+ T cells were obtained by negative selection using a magnetic labeling kit (StemCell Technologies Inc.) according to manufacturer instructions (Purity was 92% for CD3 marker).

### **(5) CFSE proliferation Assay**

Isolated T cells were suspended in serum free RPMI media at  $1.0 \times 10^6$ /mL and stained with  $0.01 \mu\text{M}$  of carboxyfluoresceindiacetatesuccinimidyl ester (CFSE) (Sigma Aldrich Inc) for 40 minutes at  $37^\circ\text{C}$ . After two washes with PBS, cells were plated into 96 well plate with growth media and stimulated with plate-bound anti-CD3/CD28 in the presence or absence of mESC-derived factors or rmMFG-E8 (R&D system). Cell proliferation proceeded for 2-3 days, and dilution of intracellular CFSE was analyzed by Beckman Coulter Cyan flow cytometer.

### **(6) Flow cytometry – staining of surface markers**

T cell activation was measured using fluorochrome-conjugated antibodies to CD3, CD4, CD8, CD25, CD44 and CD69 (eBioscience Inc.). Negatively selected CD3<sup>+</sup> T cells were first treated under certain reagent combination treatments as stated in the result, then followed by 48hr stimulation with plate coated anti-CD3 and anti-CD28 ( $1.5 \mu\text{g}/\text{mL}$  for each) antibodies (eBioscience Inc.). Cells were harvested and washed with FACS washing buffer. Polyclonal IgG was used to block unspecific binding by incubating in  $4^\circ\text{C}$  for 15 minutes. Cells were stained with CD4, CD8 and surface activation markers according to manufacturer's recommendations. At the end of 30-minute incubation period, analysis was carried out in Beckman Coulter Cyan cytometer, and data was analyzed using Kaluza and/or Flowjo.

### **(7) Qualitative PCR (qPCR)**

Total RNA was isolated using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions and reversed transcribed into cDNA using iScript cDNA Synthesis Kit (Qiagen). Following cDNA synthesis,  $0.5 \mu\text{L}$  of cDNA from each sample,  $10 \mu\text{L}$  of iQ SYBR Green supermix (Bio-Rad Ltd.) and  $9.5 \mu\text{L}$  primer-mix was used to performed qPCR on iQ-iCycler (Bio-Rad) with hot start for 90 seconds at  $94^\circ\text{C}$  followed by 40 cycles set for 10 seconds at  $94^\circ\text{C}$ , 30 seconds at  $60^\circ\text{C}$ , and 30 seconds at  $72^\circ\text{C}$ . Primers used are listed in Table 1. Fold changes in transcript levels were calculated using threshold cycle (Cq) normalized to  $\beta$ -actin.

**Table 1. List of qPCR primer sequences**

Primers	Sequences	Product Size
Mouse MFG-E8	Forward: 5'-ATATGGGTTTCATGGGCTTG-3' Reverse: 5'-GAGGCTGTAAGCCACCTTGA-3'	218
IFN $\gamma$	Forward: 5'-GAAATCCTGCAGAGCCAGA-3' Reverse: 5'-TGAGCTCATTGAATGCTTGG-3'	214
T-bet	Forward: 5'-GCCAGGGAACCGCTTATATG-3' Reverse: 5'-GACGATCATCTGGGTCACATTGT-3'	136
ROR $\gamma$ T	Forward: 5'-GGAGCTCTGCCAGAATGAGC-3' Reverse: 5'-CAAGGCTCGAAACAGCTCCAC-3'	148
TGF $\beta$	Forward: 5'-GCAACAATTCCTGGCGATACCTC-3' Reverse: 5'-AGTTCTTCTCCGTGGAGCTGAAG-3'	354
GATA3	Forward: 5'-GGCGAGATGGTACCGGGCACTA-3' Reverse: 5'-CCCCATTAGCGTTCCTCCTCCAGA-3'	180
Foxp3	Forward: 5'-CGAAAGTGGCAGAGAGGTATTGA-3' Reverse: 5'-ACTGTCTTCCAAGTCTCGTCTGAA-3'	193

## **(8) Western Blot**

Cells were harvested and lysed in RIPA buffer (25 mM Tris-HCl, 0.15 M NaCl, 5.0 mM MgCl<sub>2</sub>, 1.0% NP-40, 1.0 mM DTT, 5.0% glycerol, [pH 7.5]) supplemented with 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). After 30 minute incubation in cold, cell lysates were centrifuged at 13500rpm for 15 minutes at 4°C. An equivalent volume of Laemmli Sample buffer (Bio-Rad Laboratories Ltd.) with 10% β-ME (Bio-Rad Ltd.) was then added to the sample supernatants. Samples were boiled for 10 minutes and ran on an 8% SDS-PAGE gel, which subsequently transferred to Polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% powder nonfat milk (Santa Cruz Biotechnology Inc.) or 5% Albumin Bovine Serum (Fisher BioReagents, Thermo Fisher Scientific Inc.) in TBS-T for 1 hour at room temperature. Membranes were probed with rabbit anti-mouse phospho-PKCθ (Thre538), total PKCθ antibodies at 1:1500 (Santa Cruz Biotechnology Inc.) overnight at 4°C. At the next day, after washed 3 times with TBS-T for 10 minutes of each, membranes were probed with appropriate secondary horseradish peroxidase (HRP)-conjugated antibody at 1:10,000 dilution at room temperature for 1 hour, followed by another 3 times TBS-T washes. The bands were visualized with enhanced chemiluminescence solution (Pierce, Thermo Scientific, USA). A similar procedure was carried out for anti-mouse MFG-E8 antibodies (Dr. Jinushi, Japan), and α-Tubulin as the loading control (T9026, Sigma-Aldrich, St. Louis, MO, USA).

## **(9) Intracellular staining**

Isolated T cells were stimulated with plate-bound anti-CD3/CD28 for 2 days, and then treated with either ESC extracts or rmMFG-E8 (R&D system). Those pre-treated cells were then secondarily stimulated with PMA for 6 hours. During the last 2hr of stimulation, T cells were treated with Brefeldin in 1:1000 (eBioscience Inc.). Following by two washes with FACS buffer, cells were first stained for surface markers CD4 and CD25/CD69 (eBioscience Inc.) for 30 minutes in cold. At this point, cells were fixed and

permeabilized with Foxp3 Fixation/Permeabilization Concentrate kit (eBioscience Inc.). Foxp3, IFN $\gamma$  and IL-17 antibodies (eBioscience Inc.) were added according to manufacturer's instructions, and then analyzed by CyAn flow cytometer (Beckman Coulter).

#### **(10) Lentiviral production and MFG-E8 transduction**

293 T cells were plated at 80% confluence in 100-mm dishes and transfected with the MFG-E8 lentivirus vector (plasmid map shown in appendix), encoding full length MFG-E8 and a GFP marker (Addgene plasmid #46847; Addgene, Cambridge, MA, USA), and two packing plasmids psPAX2 and pMD2.G (Addgene plasmid 12259 and 12260) using Lipofectamine 2000 (Invitrogen) for 15 minutes. The virus containing supernatant was harvested after 48 hours of transfection. Harvested lentiviruses were then concentrated with Lenti-X concentrator (Cloneteck) as manufacturer's instruction and resuspended in growth medium later used for lentivirus infection. B6-WT ESCs were seeded onto feeder cells in 6-well plate. After overnight incubation, lentivirus medium with 10 $\mu$ g/mL polybrene (Sigma, St. Louis, MO, USA) was replaced the culture medium. Fresh ESC growth medium was changed after 24 hours of lentiviral transduction supplemented with 2 $\mu$ g/mL puromycin. Drug selection process was carried out for 2 weeks with daily medium changes. After establishing stable cell line with up-regulated MFG-E8 (B6-M ESCs), cells were cultured in ESC growth medium with 1  $\mu$ g/mL puromycin.

#### **(11) Characterization of B6-M ESC with fluorescence microscopy**

B6-M ESCs were cultured on cover glasses (Fisher, USA) after two passages on 0.1% gelatin coated plate to eliminate MEF feeders. After being culture in ESC growth medium with 15% FBS for 24 hours, cells were washed three times with PBS for 5 minutes each, and then fixed with 10% formalin at 4°C overnight. After another 3 washes with PBS, samples were incubated for 15 minutes with 4', 6-diamidino-2-phenylindole-dihydrochloride (DAPI) (Sigma, USA) (300ng/mL in PBS), following another 3 washes with PBS. Cover slide was mounted with 50% glycerol/50%PBS and examined under a fluorescence microscope (Zeiss AxioObserver. Z1).

### **(12) Teratoma Formation and histological analysis**

ESCs were harvested. One or three million cells were counted and later injected subcutaneously into the flank region of C57BL/6 or Balb/c mice. Tumors were measured and surgically removed from euthanized mice after four to six weeks. Teratomas were freshly frozen in optimal cutting temperature (OCT) compound, and sections were cut at 10 micron. After fixation with cold acetone for 10 minutes, samples were stained with haematoxylin and eosin and observed under the microscope (Zeiss).

### **(13) Statistical analysis**

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

## **Chapter 3. Result**

### **(1) High MFG-E8 expression in mouse embryonic stem cells**

Proteomic databases indicate high expression level of MFG-E8 in ESCs, and preliminary qualitative PCR (qPCR) analysis also revealed high MFG-E8 mRNA expression in two mouse ESC lines, B6 and D3. To further validate MFG-E8 expression profile, I cultured mESCs of B6 and D3 lines, RAW macrophages, C2C12 myoblasts, and harvested bone marrow from mice. Additionally, I induced spontaneous differentiation of D3 ESCs through formation of embryoid body (EB) using the hanging drop method. At day 14 after EBs re-plated, complete dissociation of typical EB structure was observed (Figure 5.) together with a drastic reduction in pluripotency gene (Sox2, Oct4 and Nanog) expressions (Figure 6.). Western blot results confirmed an abundance of MFG-E8 protein in both mouse ESC lines when compared to cells (RAW, bone marrow, C2C12) with high, moderate and no MFG-E8 expression respectively (Figure7.). Most interestingly, a marked decrease in MFG-E8 protein expression in differentiated mESCs was observed (Figure 7.). Herein, data from these experiments presented a differential MFG-E8 expression pattern between undifferentiated and differentiated ESCs, indicating a specialized function of MFG-E8 in undifferentiated ESCs.

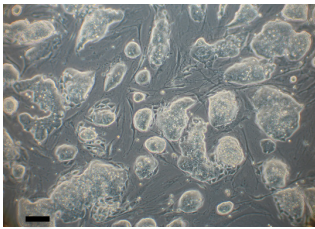
### **(2) Suppressive effects of MFG-E8 on T cell activation and proliferation**

Although we previously reported the direct immunosuppressive functions of ESC-derived factors on T cell activation and proliferation (71), the pivotal molecule contributing for such actions was undefined. Preliminary data showed that while ESC-derived factors alone significantly suppressed the up-regulation of T cell activation markers (CD25, CD69 and CD44) significantly on both CD4+ and CD8+ effector T cells, neutralization of MFG-E8 in ESC-derived soluble factors was able to notably mitigate this suppressive effects on T cells.

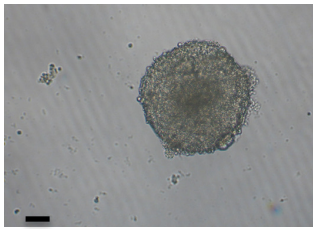
**Figure 5. Induction of Spontaneous differentiation of mESCs.**

D3 mESCs were first dissociated to single cell suspension. Hanging drop method was performed to induce mESCs to form embryoid body (EB), and EBs were then transferred to plate and cultured under mESC differentiation medium for spontaneous differentiation. Fourteen days later, ESCs were almost fully differentiated in the absence of any typical ESC colonies or EB structures.

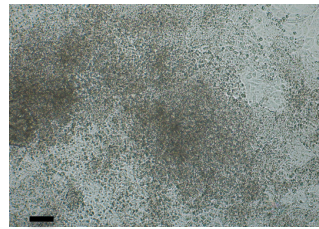
D3p35 ESCs



D3p35 ESCs  
Embryoid Body

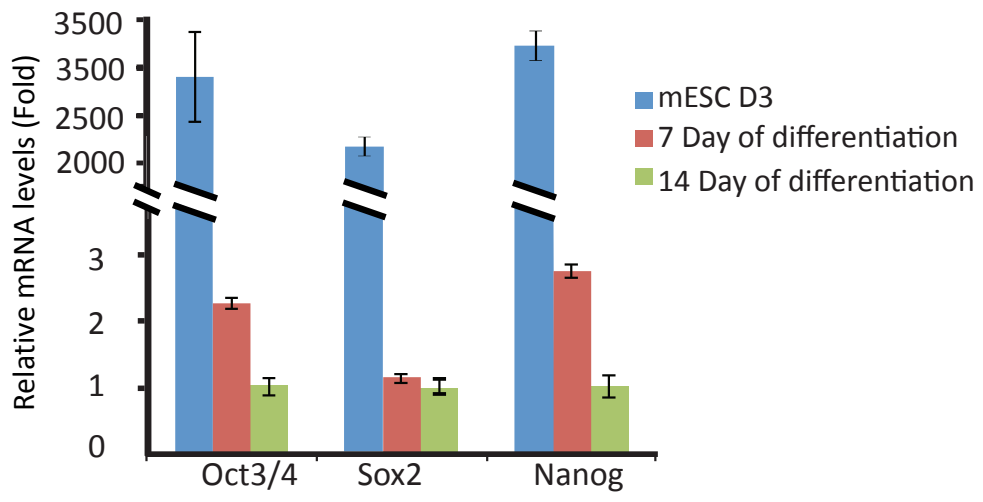


Differentiated  
D3p35 ESCs



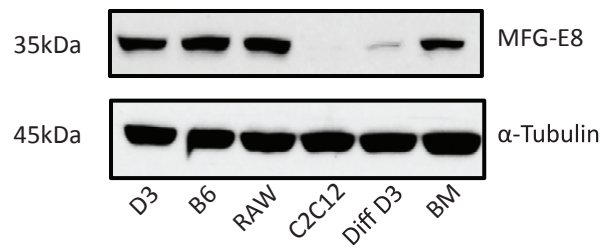
**Figure 6. Assessment of pluripotent gene expression in differentiated ESCs.**

A marked reduction in the expression of embryonic genes (Oct4, Sox3 and Nanog) is observed (qPCR analysis), indicating the differentiated of D3 mESCs. mRNA level in RAW264.7 cells was set at 1.0 (baseline) and mRNA levels in all other groups were normalized as fold changes relative to RAW. Data represent mean  $\pm$  s.d of at least three independent experiments; basal expression of  $\beta$ -actin was used for normalization.



**Figure 7. Expression of MFG-E8 in undifferentiated ESCs and other cell types.**

Western blot analysis of MFG-E8 protein expression in D3 mESCs, B6 mESCs, RAW264.7, C2C12, differentiated D3 mESCs, bone marrow cells. Membrane was probed with antibodies specific for MFG-E8 and  $\alpha$ -tubulin (as a loading control). The results are representative of three independent experiments. Abbreviation: Diff D3, differentiated D3 mESC-derived factors; BM, bone marrow.

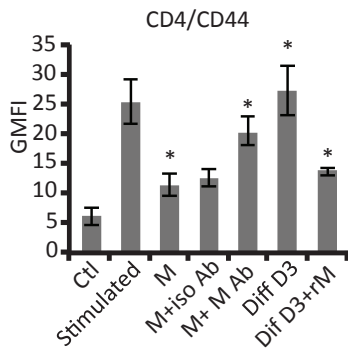
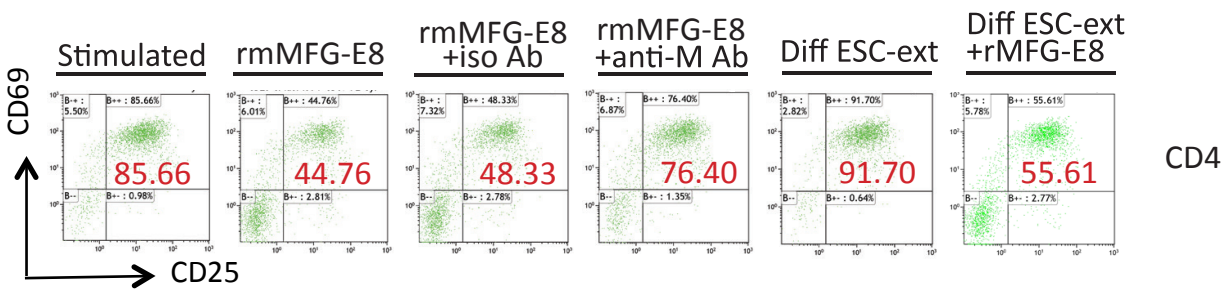
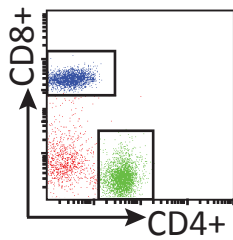


To further confirm the aforementioned immunomodulatory function of MFG-E8, purified T cells isolated from spleens of C57BL/6 mice were either subjected to treatments with recombinant mouse MFG-E8 (rmMFG-E8) alone or together with MFG-E8 blocking antibodies for 16 hours, followed by anti-CD3/CD28 stimulation for 24 hours. The level of T cell activation was determined by surface expression of activation markers; CD25, CD44 and CD69. Non-stimulated T cells were used as the negative control and stimulated T cells without treatments constituted the positive control. As expected, non-stimulated CD4<sup>+</sup> and CD8<sup>+</sup> T cells expressed low basal levels of three activation markers, all of which were drastically up-regulated on untreated stimulated T cells. Suppression of both CD4<sup>+</sup> (Figure 8.) and CD8<sup>+</sup> (Figure 9.) effector T cell activation was achieved when cells were treated with rmMFG-E8 alone. A 2-fold reduction in surface expressions of CD25, CD44 and CD69 was observed. Introduction of MFG-E8 blocking Ab but not an isotype control Ab significantly abrogated this inhibition with the marked increases of CD25<sup>+</sup>CD69<sup>+</sup> T cell population and of CD44 expression on both CD4<sup>+</sup> (Figure 8) and CD8<sup>+</sup> T cells (Figure 9).

Extending an earlier notion of reduction in MFG-E8 expression level along the course of ESC differentiation, I next investigated the possibility that withdrawal of immune privilege in differentiated ESCs might be due to a decrease in MFG-E8 level. Both CD4<sup>+</sup> (Figure 8) and CD8<sup>+</sup> (Figure 9) T cell activations were unaffected by the act of differentiated ESC-derived soluble factors, with the majority of T cells showing up-regulated activation markers post anti-CD3/CD28 stimulation. However, reconstitution of rmMG-E8 together with differentiated ESC-derived factors restored suppression of T cells, as indicated by a markedly reduction in all three activation markers (CD25, CD44 and CD69 - surface expression, Figure 8. and Figure 9. for CD4 and CD8 respectively). I further measured the immunosuppressive action of MFG-E8 on T cell proliferation based on the level of CFSE dilution in labeled CD3<sup>+</sup> T cells. Of increasing rmMFG-E8 concentration to 0.05 µg/mL, I observed a reduced number of cell divisions (Figure 10) in activated T cells as detected by decreased CFSE fluorescent dilution. Collectively, these experiments

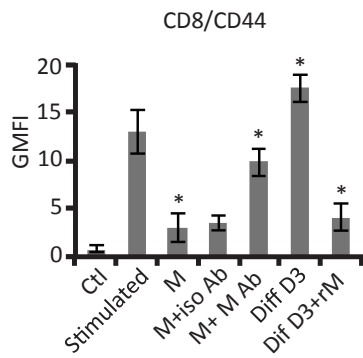
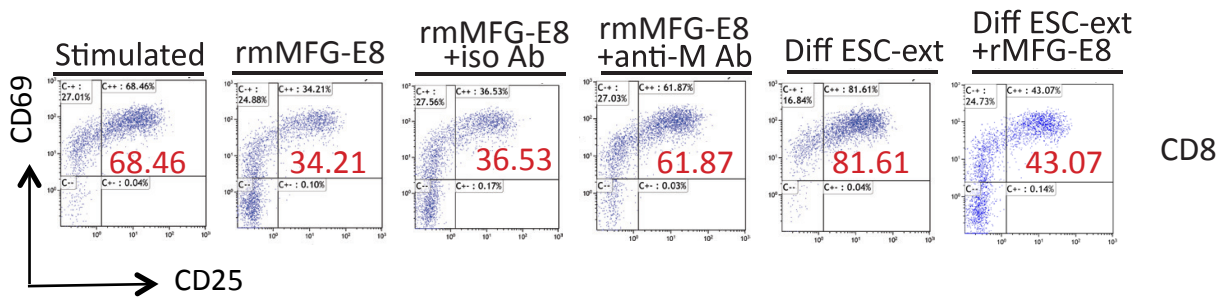
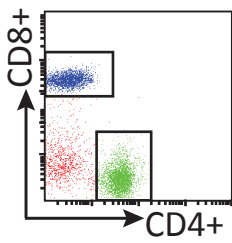
**Figure 8. ESC-produced MFG-E8 inhibits upregulation of activation markers CD25, CD44 and CD69 on CD4+ T cells.**

Negatively selected C57BL/6 CD3+ T cell were first incubated under each of treatment conditions and followed by stimulation with plate-bound anti-CD3/anti-CD28 antibodies. Flow cytometric analysis of surface expression of CD25, CD44 and CD69 was performed on CD4+ T cells treated with rmMFG-E8 (0.05µg/mL for 12hr), rmMFG-E8 with isotype antibody (0.05µg/mL rmMFG-E8 and 0.5µg/mL antibody for 12hr), rmMFG-E8 with anti-MFG-E8 antibody (0.05µg/mL rmMFG-E8 and 0.5µg/mL antibody for 12hr), differentiated D3 mESC derived factors (0.23mg/mL for 12hr), or differentiated D3 mESC derived factors with rmMFG-E8 (0.23mg/mL of differentiated D3derived factors and 0.05µg/mL of rmMFG-E8 for 12hr). After 24hr of anti-CD3/anti-CD28 stimulation, cells were stained with CD25, CD44 and CD69. Dot plots are representative of cells stained with CD25 and CD69 markers, and histograms represent the geometric mean fluorescent intensity (GMFI) of CD4+ T cells with CD44 expression for each treatment group. Similar results were obtained from three independent experiments. Error bars indicate mean ± s.d. (\* $p < 0.05$ .) Abbreviation: M, rmMFG-E8; M+iso, rmMFG-E8 with isotype antibodies; M+Mab, rmMFG-E8+anti-MFG-E8 antibodies; Diff D3, differentiated D3 mESC-derived factors; Diff+M, differentiated D3 mESC extract with rmMFG-E8.



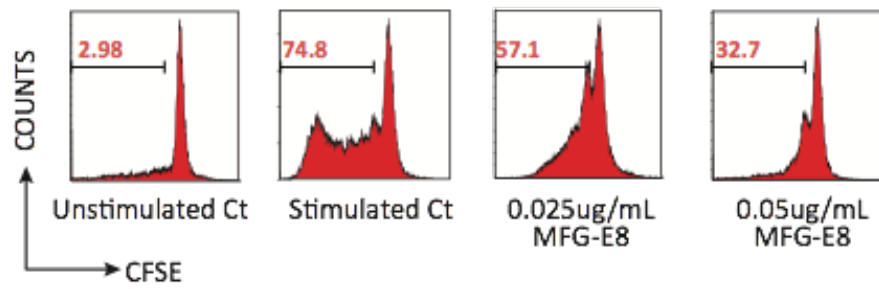
**Figure 9. ESC-produced MFG-E8 inhibits upregulation of activation markers CD25, CD44 and CD69 on CD8+ T cells.**

Negatively selected C57BL/6 CD3+ T cell were first incubated under different treatment conditions, followed by stimulation with plate bound anti-CD3/anti-CD28 antibodies. Flow cytometric analysis of surface expression of CD25, CD44 and CD69 was performed on CD8+ T cells treated with rmMFG-E8 (0.05µg/mL for 12hr), rmMFG-E8 with isotype antibody (0.05µg/mL rmMFG-E8 and 0.5µg/mL antibody for 12hr), rmMFG-E8 with anti-MFG-E8 antibody(0.05µg/mL rmMFG-E8 and 0.5µg/mL antibody for 12hr), differentiated D3 mESC derived factors (0.23mg/mL for 12hr), or differentiated D3 mESC derived factors with rmMFG-E8 (0.23mg/mL of differentiated D3derived factors and 0.05µg/mL of rmMFG-E8 for 12hr). After 24hr of anti-CD3/anti-CD28 stimulation, cells were stained with CD25, CD44 and CD69. Dot plots are representative of cells stained with CD25 and CD69 markers, and histograms represent the geometric mean fluorescent intensity (GMFI) of CD8+ T cells with CD44 expression for each treatment group. Similar results were obtained from three independent experiments. Error bars indicate mean ± s.d. (\* $p < 0.05$ . \*\* $p < 0.01$ ) Abbreviation: M, rmMFG-E8; M+iso, rmMFG-E8 with isotype antibodies; M+Mab, rmMFG-E8+anti-MFG-E8 antibodies; Diff D3, differentiated D3 mESC-derived factors; Diff+M, differentiated D3 mESC extract with rmMFG-E8.



**Figure 10. MFG-E8 inhibits T cell proliferation.**

Proliferation assay was performed on CFSE-labeled CD3<sup>+</sup> selected lymphocytes fractionated from C57BL/6 mouse spleen. After treatment with different concentrations of rmMFG-E8 (0.025 $\mu$ g/mL, 0.05 $\mu$ g/mL), CFSE-labeled cells were stimulated with plate-bound anti-CD3/CD28 antibodies for 48hr. The dilution of CFSE fluorescent intensity (indicating proliferation) was measured by flow cytometry. Similar results were obtained from three independent experiments.



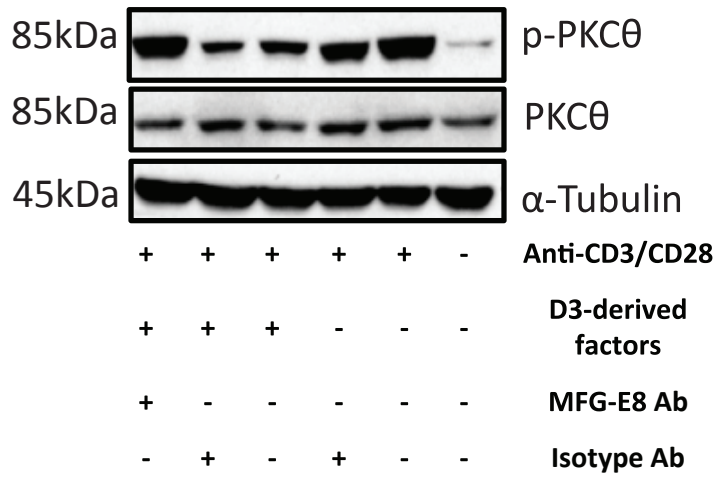
highlight the ability of MFG-E8 in ESCs to potentiate suppression on T cell activation and proliferation.

### **(3) ESC-produced MFG-E8 suppresses T cell activation via inhibition of PKC $\theta$ phosphorylation**

Much progress has been made revealing the indispensable roles of PKC $\theta$  in T cell responses, and analysis of PKC $\theta$  deficient mice confirmed that PKC $\theta$  is required for activation and proliferation of mature T cell when encountered infections (72). In our early efforts, we delineated that ESC-derived soluble factors inhibited PKC $\theta$  phosphorylation, resulting in the suppression of T cell activation and functions (71). In this context, I speculated that MFG-E8 might facilitate immunomodulation of T cells through PKC $\theta$ . Treatment and stimulation procedures were identical to those described in the previous section. Consistent with inhibitory functions of ESC-derived soluble factors on PKC $\theta$  activation (Figure 11), treatment with rmMFG-E8 significantly suppressed PKC $\theta$  phosphorylation in activated T cells (Figure 12). The capacity of MFG-E8 in inhibiting PKC $\theta$  signaling was further proven by the introduction of a specific anti-MFG-E8 blockade. As shown in Figure 11. and Figure 12., ESC-derived soluble factors or rmMFG-E8 in combination with anti-MFG-E8 antibody rendered markedly less efficacious in suppressing PKC $\theta$  phosphorylation. Additionally, correlating with down-regulated MFG-E8 expression, activated T cells displayed unaffected PKC $\theta$  phosphorylation when treated with differentiated ESC-derived soluble factors or terminally differentiated C2C12 cell extracts. However, inhibition was restored when differentiated ESC-derived factors was supplemented with rmMFG-E8 (Figure 13). To complete the MFG-E8 signaling pathway, a specific antibody capable of blocking MFG-E8 integrin receptors,  $\alpha_v\beta_3/\alpha_v\beta$ , was introduced. Western blot analysis revealed that, by blocking the binding of MFG-E8 to its receptor with anti-CD51 ( $\alpha_v$  component) antibody, ESC-derived soluble factors or rmMFG-E8 showed a significant reduction in their capacity of inhibiting PKC $\theta$  phosphorylation (Figure 14). The analysis of the PKC $\theta$  pathway supports and extends the aforementioned demonstration that MFG-E8 is an important determinant of ESC-induced T cell suppression in a PKC $\theta$  dependent fashion.

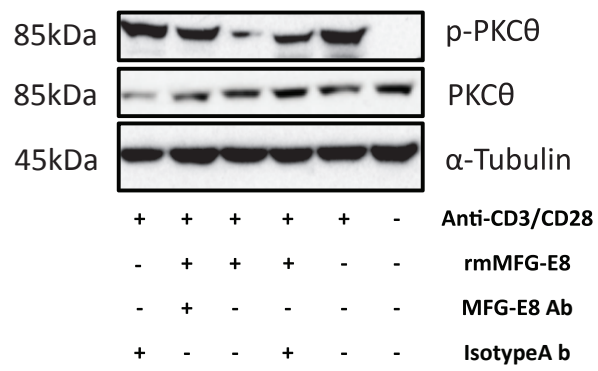
**Figure 11. ESC-produced MFG-E8 suppresses PKC $\theta$  activation.**

T cells were treated with mESC-derived factors (0.23mg/mL for 12 hr). Cells were then stimulated with anti-CD3/anti-CD28 antibodies for an additional 24hr. After stimulation, cells were lysed and subsequently probed with phosphorylated-PKC $\theta$  (Ther538), total PKC $\theta$  and  $\alpha$ -tubulin (as a loading control) for western blot analysis. Results are representative of at least three independent experiments.



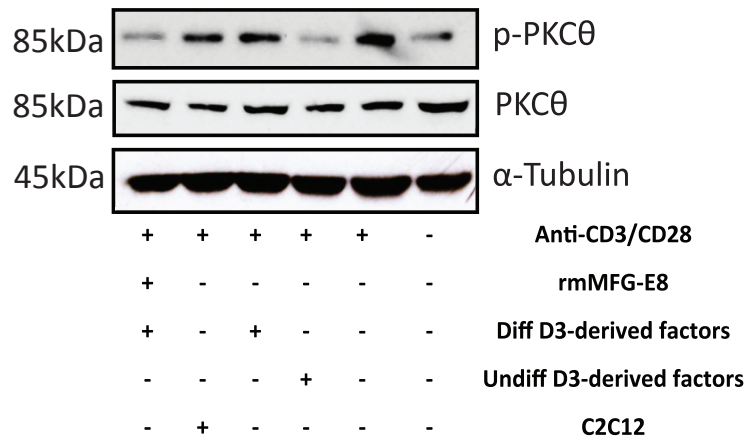
**Figure 12. MFG-E8 suppresses PKC $\theta$  activation.**

T cells were treated with rmMFG-E8 (0.1  $\mu$ g/mL, 1.0  $\mu$ g/mL for 12 hr). Cells were then stimulated with anti-CD3/anti-CD28 antibodies for an additional 24hr. After stimulation, cells were lysed and subsequently probed with phosphorylated-PKC $\theta$  (Ther538), total PKC $\theta$  and  $\alpha$ -tubulin (as loading control) for western blot analysis. Results are representative of at least three independent experiments.



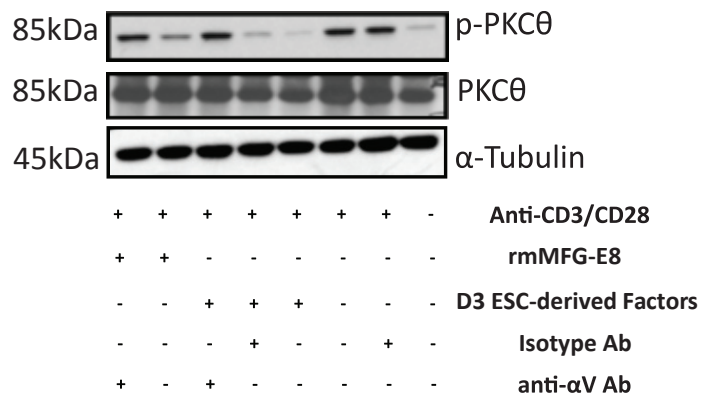
**Figure 13. Differentiated ESCs are incapable of suppressing PKC $\theta$  activity by the down-regulation of MFG-E8 expression.**

Undifferentiated D3 mESC-derived soluble factors (0.23mg/mL) and Differentiated D3 mESC-derived soluble factors (0.23mg/mL) alone or in combination with rmMFG-E8 (0.1 $\mu$ g/mL) were used to treat T cells when compared to undifferentiated D3 mESC group (0.23mg/mL) following same procedures described as above. Western blot results represented cell lysates probing with phosphorylated-PKC $\theta$  (Ther538), total PKC $\theta$  and  $\alpha$ -tubulin (as loading control). Results are representative of at least three independent experiments.



**Figure 14. Blockade of  $\alpha$ V integrin arrests ESC-derived MFG-E8-mediated inhibition on PKC $\theta$  phosphorylation.**

Following the same treatments as described in Figure 11-13,  $\alpha$ V integrin blocking antibodies (anti-CD51) was introduced to restrain the binding of MFG-E8 to its receptor. Cell lysates were then analyzed for levels of PKC $\theta$  phosphorylation, while  $\alpha$ -tubulin was used as a loading control. Results are representative of at least three independent experiments.

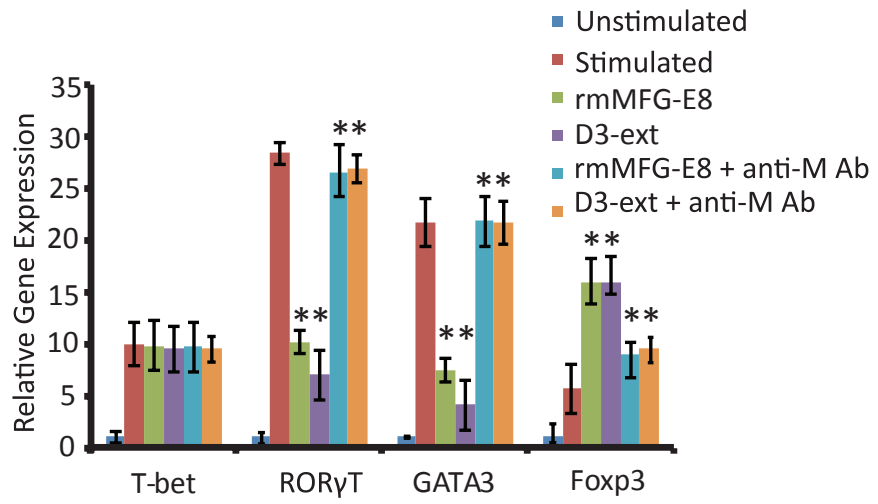


#### **(4) ESC-produced MFG-E8 is capable of skewing T cells polarization**

Because PKC $\theta$  is of vital importance in T cell polarization with particular impacts on Th1, Th2 differentiation(73), Th17 and T regulatory cell (Treg) homeostasis(74, 75), ESC-derived MFG-E8 was tested on its ability to engender modulations on T cell polarization. In order to acquire more robust responses from each CD4+ T effector subset, negatively selected T cells were first primed with plate-bound anti-CD3/CD28 antibodies for 2 days, and subsequently treated with either rmMFG-E8 or D3 ESC-derived factor in the presence or absence of MFG-E8 blocking antibody. After treated cells were secondarily stimulated by PMA, gene analyses were performed to examine the expression level of transcriptional factors (TFs) and cytokines specific to each T cell subset using qPCR. It has been well recognized that TF of T-bet and IFN $\gamma$  production designates for Th1 differentiation (76, 77), and GATA3 and IL4 production are characteristics of Th2 development (78, 79). Similarly, ROR $\gamma$ T is the master regulator gene for Th17 cells that have a signature cytokine profile of IL17 and IL21 (14). Based on the qPCR results, there were significant reductions in GATA3 and ROR $\gamma$ T expression in T cells after treatments with rmMFG-E8 or mESC-derived soluble factors. Conversely, MFG-E8 neutralization restored GATA3 and ROR $\gamma$ T expression as to those of untreated stimulated control cells (Figure. 15). Interestingly, T-bet gene expression was unaffected (Figure. 15) in all treatment conditions, yet assessment of the cytokine gene expression profile revealed a strong suppression of gene expression of Th1 specific cytokine, IFN $\gamma$  (Figure 16). In an attempt to further exploit the changes of cytokine production specialized to each subset of T effector cells, I intracellularly stained T cells with IFN $\gamma$ , IL4 and IL17 antibodies. Consistent with changes in IFN $\gamma$  gene expression, T cells treated with rmMFG-E8 encompassed a notably decreased proportion of IFN $\gamma$  producing effector cells (Figure 17A). Likewise, in accordance with down-regulated GATA3 and ROR $\gamma$ T gene expression, IL4 (Figure 17B) and IL17 (Figure 17C) producing T cell population significantly decreased. Neutralization of MFG-E8 in cells treated with rmMFG-E8 or mESCd-derived factors effectively ameliorated this suppression on T cells, showing by the

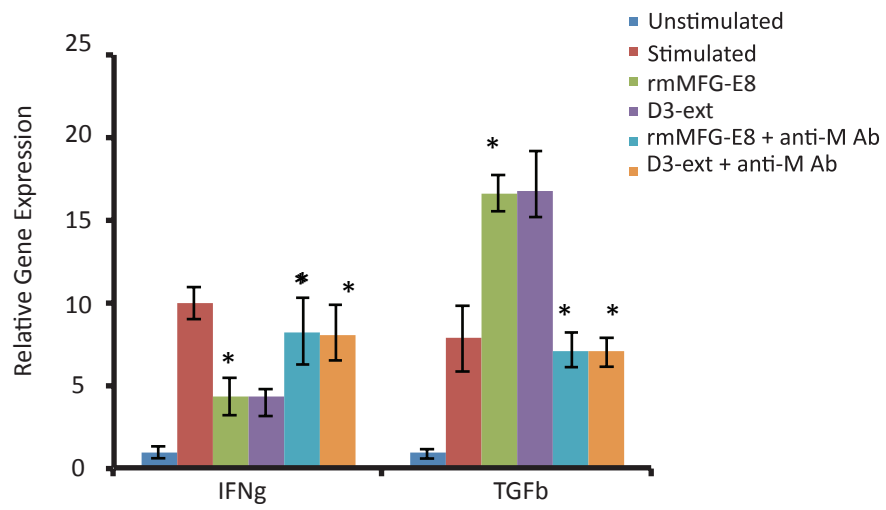
**Figure 15. Gene expression of the signature transcriptional factors for Th1, Th2 and Th17 after treatment with MFG-E8.**

Purified CD3<sup>+</sup> T cells were first primed with anti-CD3/CD28 for 3 days, and then treated with either D3 ESC-derived soluble factors (D3-ext) or rmMFG-E8, synergistically with/without MFG-E8 blocking antibody, followed by PMA stimulation. T cell transcriptional factors (T-bet, ROR $\gamma$ T, GATA3 and Foxp3) were measured by qPCR; endogenous expression of  $\beta$ -actin was used for normalization. mRNA level in unstimulated control was set at 1.0 (baseline) and all other normalized mRNA levels were converted to fold differences in Ct values relative to that value of control. Data represent mean  $\pm$  s.d. of at least three independent studies. (\*  $p < 0.05$ )



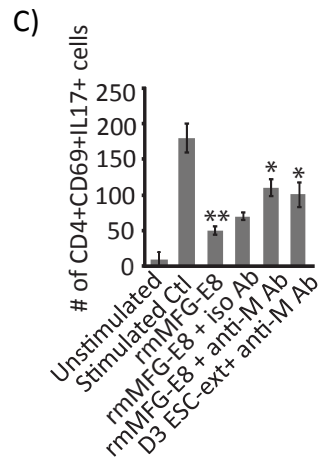
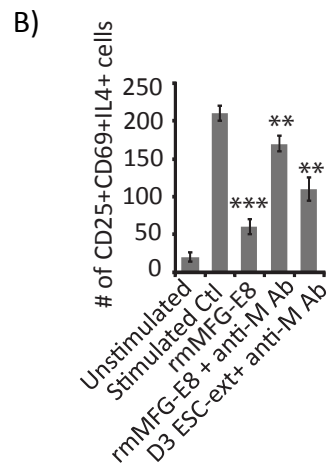
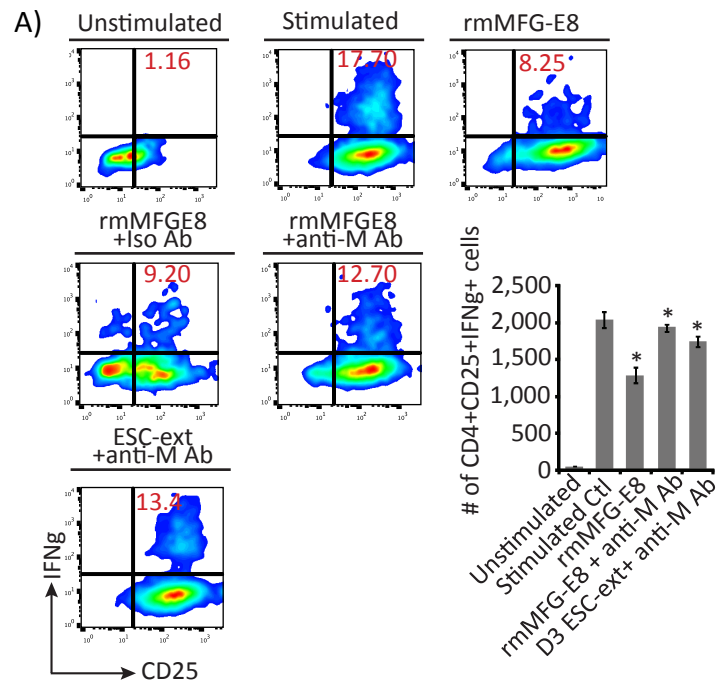
**Figure 16. Gene expressions of Th1 and Treg specific cytokine.**

Purified CD3<sup>+</sup> T cells were first primed with anti-CD3/CD28 for 3 days, and then treated with either D3 ESC-derived soluble factors or rmMFG-E8 and in the presence or absence of MFG-E8 blocking antibody, followed by PMA stimulation. Gene expression of IFN $\gamma$  (Th1) and TGF $\beta$  (Treg) were determined by qPCR. Data represent mean  $\pm$  s.d. of at least three independent studies; basal expression of  $\beta$ -actin was used for normalization. mRNA level in unstimulated control was set at 1.0 (baseline) and all other normalized mRNA levels were converted to fold differences in Ct values relative to that value of control. Abbreviation: MFG-E8+Mab, rmMFG-E8 + anti-MFG-E8 antibody; D3 ESC+Mab, D3 mESC-derived soluble factors + anti-MFG-E8 antibody. (\* $p$ <0.05)



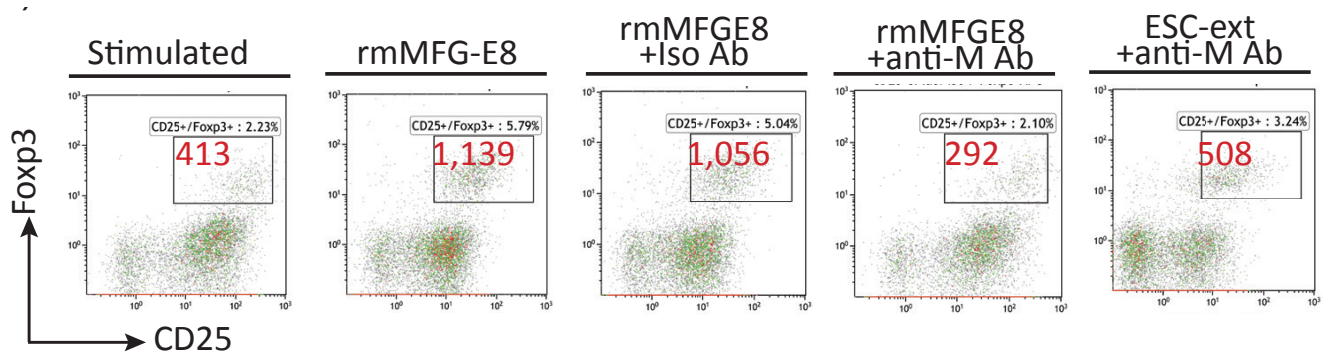
**Figure 17. MFG-E8 in ESCs is capable of suppressing Th1, Th2 and Th17 differentiation.**

To determine whether rmMFG-E8 could suppress IFN $\gamma$ , IL4 and IL-17 production, primed CD3<sup>+</sup> C57BL/6 T cells were treated with rmMFG-E8 (0.05 $\mu$ g/mL for 24hr) alone or in the presence of isotype antibody (0.5 $\mu$ g/mL) or anti-MFGE-E8 antibodies (0.5 $\mu$ g/mL), or D3 mESC-derived soluble factors with anti-MFG-E8 antibody (0.5 $\mu$ g/mL). Following a 48hr stimulation with plate-bound anti-CD3/anti-CD28 antibodies, cell was stained with CD4, CD25 or CD69 for surface marker and A) IFN $\gamma$  or B) IL4 or C) IL-17 for intracellular cytokine, and then analyzed by flow cytometry. Histograms are shown for the mean  $\pm$  s.d. of the absolute number of A) IFN $\gamma$  or B) IL4 or C) IL-17 producing T cells of three independent experiments (\*p<0.05, \*\*p<0.01, \*\*\*p<0.005). Abbreviation: Stimulated Ctl, stimulated control; MFG-E8 + iso Ab, rmMFG-E8 + isotype antibody; MFG-E8 + anti-M Ab, rmMFG-E8 + anti-MFG-E8 antibody; D3 ESC + anti-M Ab, D3 mESC-derived soluble factors + anti-MFG-E8 antibody.



**Figure 18. MFG-E8 in ESCs is capable of enhancing Treg differentiation.**

Under the similar treatment and stimulation conditions as above, T cells were stained with CD4, CD25 surface markers and Foxp3 intracellularly to assess the changes of Treg population. Dot plots represent one of three independent studies, and the red numbers represent absolute number of cells that gated within. Abbreviation: M+iso, rmMFG-E8 + isotype antibody; M+Mab, rmMFG-E8 + anti-MFG-E8 antibody; D3+Mab, D3 mESC-derived factors + MFG-E8 blocking antibody.



augmented numbers of IFN $\gamma$  (Figure 17A), IL4 (Figure 17B) and IL17 (Figure 17C) secreting cells.

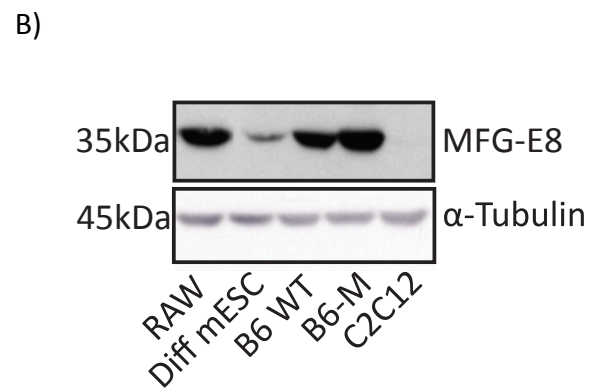
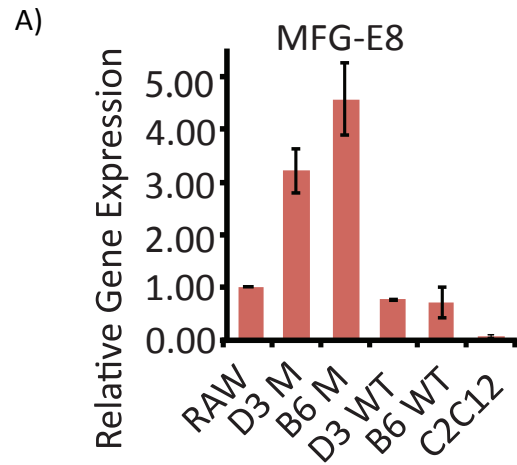
Furthermore, a significant up-regulation of Foxp3 and TGF $\beta$  gene expression were detected (Figure 15 and Figure 16, respectively). Through direct examination of changes in Treg cell population, I observed that CD4+CD25+Foxp3+ Treg differentiation was markedly promoted by treating purified T cells with rmMFG-E8 (Figure 18). Of note, insofar as MFG-E8 was neutralized by blocking Ab in ESCs, the elevated Treg population induced by mESC-derived factors was withdrawn (Figure 18). These results collectively show that ESC-produced MFG-E8 favorably enhances Treg cell differentiation, suppresses Th1 function, and prevents the development of Th2 and Th17.

#### **(5) Immunomodulation of ESC-produced MFG-E8 *in vivo***

Teratoma formation assay is an important tool for monitoring pluripotency and evaluating the immunogenicity of ESCs and/or iPSCs *in vivo* (32, 80). Of particular noteworthy, study by Zhao et al. documented that allogeneic ESCs failed to generate teratomas by rapid T cell-mediated immune rejection (81). By utilizing this technique, *in vivo* experiments were designed to examine whether up-regulation of MFG-E8 in undifferentiated ESCs would offer protection against immune rejection, allowing for the survival of ESCs across allogeneic barriers. I transfected wild-type B6 ESCs with a lentiviral vector carrying MFG-E8 and a GFP marker. The level of protein up-regulation was controlled to achieve a 2 to 4 fold increase in MFG-E8 expression (herein denoted as B6-M ESCs), confirmed with both qPCR and western blotting (Figure 19A and Figure 19B). Fluorescent microscopy further validated the success of the transfection process with efficiency of approximately 92%, marked by the presence of strong GFP signals in B6-M ESCs (Figure 20). *In vivo* transplantations were carried out into four groups: B6 Wild type ESCs (B6-WT) into either syngeneic or allogeneic mice and B6-M ESCs into either syngeneic or allogeneic mice (Figure 21). Both B6-WT and B6-M ESCs successfully formed teratomas in syngeneic C57BL/6 mice (Figure 22A), and histological

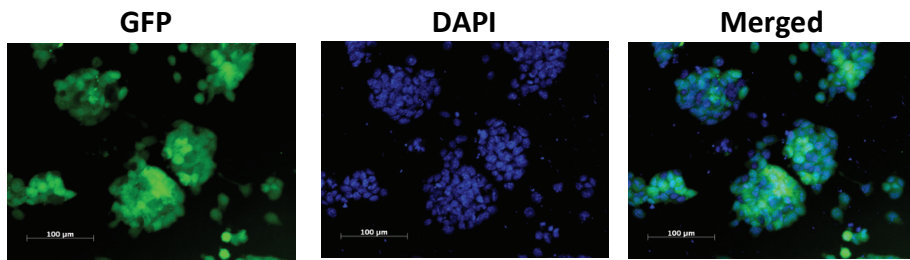
**Figure 19. Assessment of the level of up-regulated MFG-E8 in B6-M ESCs after transduction.**

Wild type B6 mESCs (B6-WT) were transfected with a lentiviral vector carrying a vector with MFG-E8 and GFP. Drug selection was done over 2 week to establish a stable B6 mESC line (B6-M) with up-regulated MFG-E8 expression. qPCR (A) and western blot (B) were performed to assess level of MFG-E8 up-regulation. Basal expression of  $\beta$ -actin was used as normalization control for qPCR, and  $\alpha$ -tubulin for western blot. In qPCR results, mRNA level in RAW cells was set at 1.0 (baseline) and all other normalized mRNA levels were converted to fold of differences in Ct values relative to that value. Data represent mean  $\pm$  s.d. of at least three independent studies. Western blotting was one representative of three biological repeats.



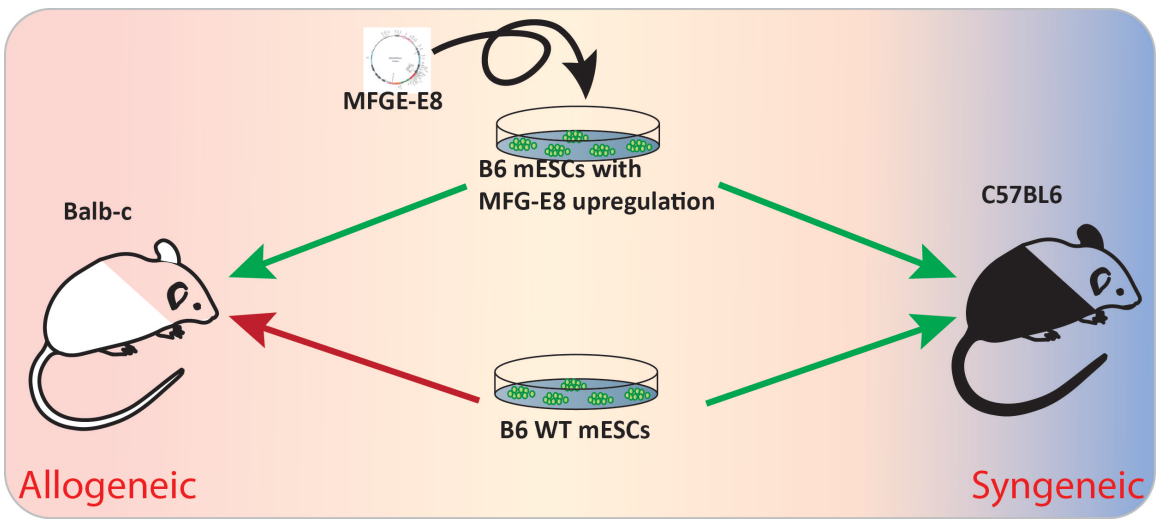
**Figure 20. Validation of MFG-E8 transduction by fluorescence microscopy.**

Wild type B6 mESC (B6-WT) was transfected with a lentiviral vector carrying a vector with MFG-E8 and GFP. Drug selection was done over 2 week to establish a stable B6 mESC line (B6-M) with up-regulated MFG-E8 expression. Established B6-M ESCs were cultured on cover slides, and later fixed with 10% formalin for fluorescence microscope analysis. Cell nuclei were stained with DAPI. Transfection efficiency was calculated by counting the number of cells positive for both GFP and DAPI signals. Figures are representative of three biological repeats.



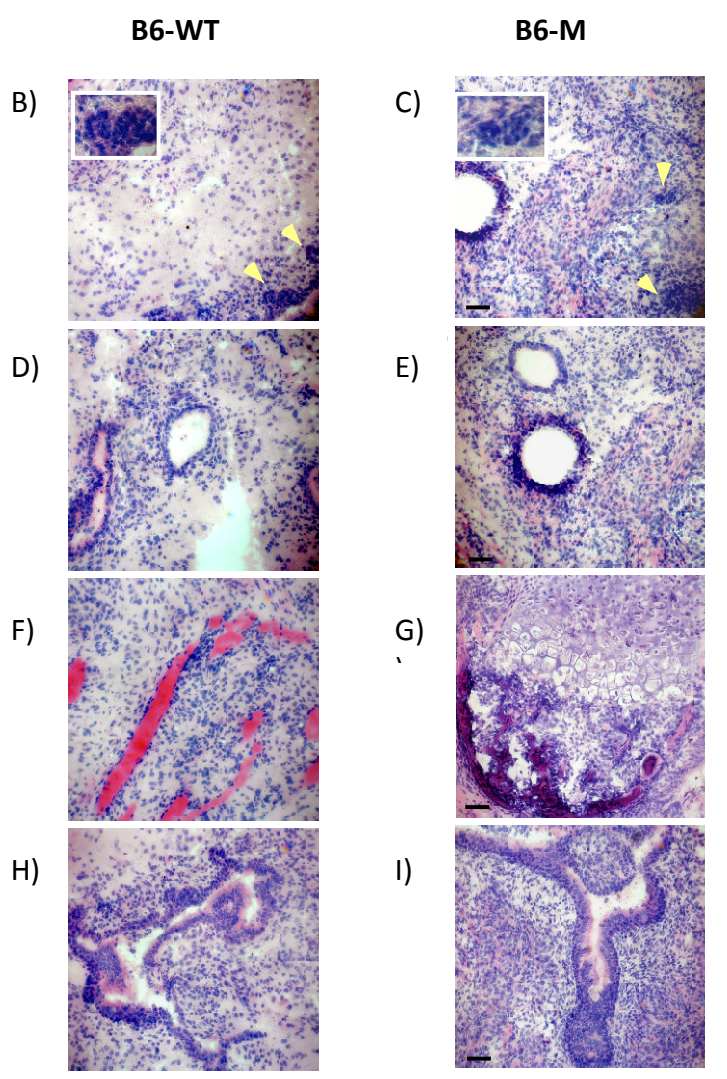
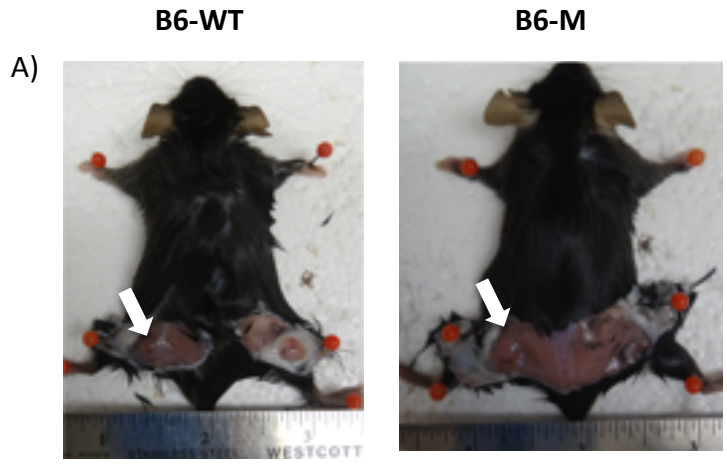
**Figure 21. Schematic of *in vivo* experimental design**

*In vivo* studies were carried out into four groups: B6-WT ESCs into either syngeneic or allogeneic mice, as well B6-M ESCs into syngeneic and allogeneic mice. B6 ESCs were isolated from embryos of C57BL/6 mice. Inoculation of both B6-WT and B6-M ESCs into C57BL/6 mice was a syngeneic engraftment. In contrast, B6-WT and B6-M were allogeneic to Balb/c mice.



**Figure 22. Teratoma formation of syngeneic B6-WT and B6-M ESCs in C57BL/6 mice.**

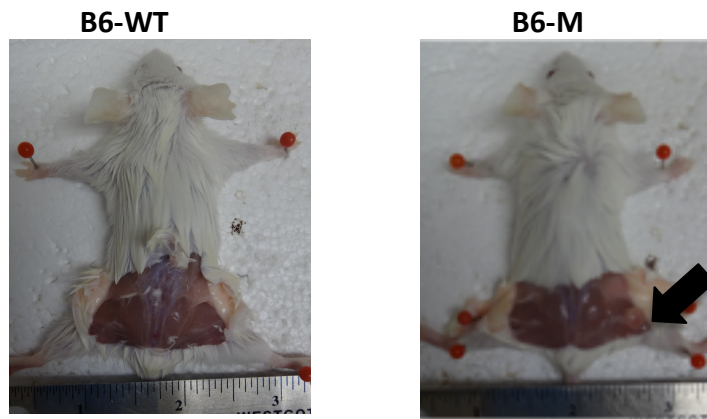
A) Both B6-WT and B6-M ESC lines formed teratomas in syngeneic C57BL/6 mice after 4 weeks of implant into flank regions. B) Tumors were embedded in OCT gel, snap-frozen in liquid nitrogen, and stored at -80°C. Cryostat sections (10µm) were fixed in cold acetone and examined histologically after hematoxylin and eosin staining. B6-WT and B6-M formed teratomas were composed of tissues from all three germ layers, including ectoderm ([B, C] neural rosettes and glial tissue, [D, E] cystic epithelium), mesoderm ([F] striated muscle, [G] cartilage and osteoid island) and endoderm ([H, I] glandular epithelium). (n=5)



**Figure 23. Implantation of allogeneic B6-WT and B6-M in Balb/c mice.**

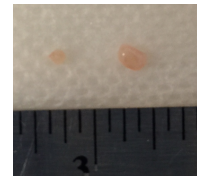
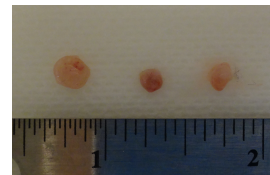
A) *In vivo*, whereas no detected teratoma was formed after transplantation of B6-WT ESCs into Balb/c mice, 5 teratomas were generated with B6-M ESCs in 9 Balb/c mice. B) Summary of teratoma formation by B6-M ESCs in Balb/c mice 4-6 weeks after implantation. No teratoma by B6-WT was detected, but 5 teratomas were formed by B6-M ESCs. (n=9)

A)



B)

	Frequency of Teratoma Formation
<b>B6-WT</b>	0/9
<b>B6-M</b>	5/9



analysis revealed components of all three germ layers (Figure 22B-I). All of the allogeneic recipients, however, vigorously rejected B6-WT ESCs engraftment before the formation of detectable teratomas (Figure 23). In contrast, with up-regulation of MFG-E8 in B6 ESCs, allogeneic implants of B6-M ESCs were able to be tolerated and successfully engrafted in 5 of 9 recipients (Figure 23). These findings further strengthen my proposition that MFG-E8 is, indeed, a key contributor in ESCs that induces immunosuppression.

## **Chapter 4. DISCUSSION**

Many complex mechanisms associated with ESC-mediated immunosuppression are not well understood, and no report has yet identified a critical immune suppressive mediator molecule produced by ESCs. In this study, I have demonstrated a novel functional role of MFG-E8 in ESCs for T cell immunomodulation. I showed that undifferentiated but not differentiated ESCs expresses a high level of MFG-E8. Combinational uses of rmMFG-E8 and specific MFG-E8 or its receptor blockade confirm that ESC-produced MFG-E8 is of crucial importance in suppressing T effector cell activation and proliferation, as well as PKC $\theta$  being the central signaling kinase in MFG-E8 mediated immunomodulation of T cells. MFG-E8-induced inhibition on PKC $\theta$  selectively restrains Th1 function and suppresses Th2 and Th17 differentiation, but promotes Treg cell fate. Finally, teratoma formation assay confirms that up-regulation of MFG-E8 enables successful engraftment of allogeneic ESCs.

### **(1) MFG-E8 anti-inflammatory properties and signaling**

As an evidence of direct functional effects on T cells other than its conventional role in phagocytosis, I provided the first proof of principle that MFG-E8 produced by ESCs is involved in regulating T cell immune responses. It has been established that, through enhancing the phagocytic potentials of apoptotic cells, MFG-E8 could facilitate an immune-tolerant state (49, 64). Direct functions of MFG-E8 on T cells, however, have yet been investigated. Researches have shown that T cells selectively up-regulate integrin,  $\alpha_v\beta_3/\alpha_v\beta_5$ , on their surface when they encounter activation agents: a viral challenge or PMA treatment (82, 83). Therefore, I reasoned that engagement of MFG-E8 to its integrin receptor on activated T cell could be a key mechanism to control the level of T cell responses in ESC-mediated immunomodulation. By comparing these findings with current observations, I proposed a link between MFG-E8 and cellular immunity. High MFG-E8 expression in undifferentiated ESCs suppresses T cell activation while the loss of

MFG-E8 expression along the course of differentiation suffices to restore normal T cell responses and facilitates rejection of differentiated cells. Taken together, sustaining the immune privileged status of undifferentiated ESCs entails high levels of MFG-E8 expression.

In the absence of apoptotic cells, MFG-E8 generates anti-inflammatory effects in innate immune cells by utilizing STAT3 pathway, which then down-regulates NF $\kappa$ B activation (84). Examination of NF $\kappa$ B activity in T cells did not show differences in the presence or absence of rmMFG-E8 (data not shown). Instead, I observed that PKC $\theta$  phosphorylation was greatly affected. Several studies have indicated a role of MFG-E8 in down-regulating TNF $\alpha$  production via the STAT pathway (85). With TNF $\alpha$  production, PKC $\theta$  is released from restraint and recruited directly to immunology synapses, favoring T effector cell fates but suppressing Treg development (86). Additionally, a study of MFG-E8-mediated tissue regeneration reported that MFG-E8 transduces its downstream signaling for mucosal healing by means of activating intracellular PKC $\epsilon$  in epithelial cells (87). Provided the premise of extensively similar structures shared among novel PKC family proteins (88), it is indicative of diversified signaling complexes of MFG-E8 in each cell type. By using rMFG-E8 and specific MFG-E8 blockade in ESC-derived soluble factors, my elucidation of MFG-E8 mechanistic function on inhibiting PKC $\theta$  is substantiated. Hence, to regulate T cell responses, signal from ESC-produced MFG-E8 would culminate in the inhibition of PKC $\theta$  activity.

## **(2) MFG-E8 mediated PKC $\theta$ inhibition on T cell polarization**

The dominant function of PKC $\theta$ , a serine/threonine kinase, under physiological condition is to effectuate T cell activation, proliferation, and differentiation. Peripheral T cells isolated from PKC $\theta$  knockout (KO) mouse lines show impaired proliferation and increased susceptibility towards parasitic infections (73, 89). Here, T cells under MFG-E8-induced PKC $\theta$  inhibition mimicked such phenotype of PKC $\theta$  KO T cells thus far described. I observed that, by impeding PKC $\theta$  activation, ESC-derived MFG-E8 greatly

dampened Th1 function and severely impaired Th2 and Th17 development. Smeets *et al.* suggested that PMA alone stimulated a Th2 phenotype dependent solely on PKC $\theta$ , but PKC $\theta$  and Lck had a synergistic effect in inducing Th1 responses (90). Additional studies by Lee *et al.* further illustrated that synergistic inhibition of both PKC $\theta$  and Akt was crucial in completely blocking Th1 differentiation, whereas defects in PKC $\theta$  only suppressed Th2 but not Th1 differentiation (91). Unaffected T-bet gene expression but suppressed Th1 function detected by IFN $\gamma$  production seems conflicting, but these issues pertain uniquely to PKC $\theta$ . One possible explanation may be due to the multiple collaborative pathways in controlling Th1 differentiation, and this is supported by the notion whereby PKC $\theta$  is differentially required for each T cell subset differentiation (92–94).

Duality of PKC $\theta$  in Th17 and Treg differentiation is better understood. Kwon *et al.* demonstrated that PKC $\theta$  integrates signals received from TCR and Th17 priming cytokines to promote Th17 differentiation (74). In contrast, PKC $\theta$  inhibition drives Treg functions (93). Of noteworthy, in MFG-E8 associated cancer researches, the loss of MFG-E8 function using specific blocking antibody has been reported to increase tumor destruction and decrease Treg activity (67, 95). In my present thesis, I found that promotion of Treg but inhibition of Th17 differentiation by ESC-derived MFG-E8 was facilitated via a PKC $\theta$  dependent manner. All in all, arresting T effectors cell function and proliferation while promoting Treg differentiation is imperative to the potent immunosuppressive effects of ESCs. Through pinpointing a specific mediator, my study provides a new mechanistic approach of ESC-derived MFG-E8 through inhibiting PKC $\theta$  activity to attenuate T cell responses.

### **(3) MFG-E8 up-regulation in view of allogeneic transplants**

Using teratoma formation assay, *in vivo* experiments were designed to model the process of allogeneic transplants of ESCs. Without applying any immunosuppressive agents, allogeneic transplants would be rapidly rejected predominately facilitated by

activation of recipient T cells responding to allogeneic MHC-peptide complex either directly on allografts or indirectly through recipient APCs (96–98). Although ESCs carry low levels of MHCs on their surfaces, allogeneic ESCs still trigger significant and progressive cellular immune responses causing cell loss following transplant. Studies have shown that *In vivo* differentiation of ESCs induces progression of host responses coinciding with increasing expression of MHC and specific differentiation markers, and therefore renders differentiated cells become increasingly immunogenic (99). Reduction of graft size in allogeneic ESC transplants would start 2 week after injection with progressive T cells infiltration (100, 101). As reflected in my *in vivo* studies, implantations of B6 ESCs into syngeneic recipients were perfectly tolerated and led to the generation of teratomas, whereas allogeneic Balb/c recipient mice rapidly rejected B6-WT ESCs without forming any detectable teratomas. Importantly, the up-regulation of MFG-E8 expression enabled B6-M ESCs to contained immune rejection to a tolerable state and ensured formation of teratomas in 5 of 9 mice, which further amplify the potent T cell immunosuppressive functions of MFG-E8 as proved *in vitro*.

#### **(4) Future Directions**

Data in current studies of ESC-mediated immunomodulation strongly suggests a shift towards an anti-inflammatory/tolerant profile in response to ESC treatment. Though the involvement of multiple surface molecules in such actions is conceivable and better studied, the main proposed effects of soluble factor, MFG-E8, in ESCs here were the inhibition of pro-inflammatory T cell activation and the induction of anti-inflammatory Treg phenotype. In addition to its known functions in innate immune cells, MFG-E8 produced by ESC might signal to not only newly recruited T cells (paracrine) but also localized innate immune cells in an autocrine loop to mediate and propagate the immunomodulation. While these findings open new insights, they also raise several questions as needed to be addressed in future studies.

First of all, MFG-E8 and its integrin receptor signaling pathway have not been well studied in T cells. I have explored that the mode of operation for MFG-E8 in T cells

is to inhibit PKC $\theta$  activity, and thereby limiting T cell responses. It would be critical to map out the direct downstream molecules and investigate the mechanisms of how integrin  $\alpha$ V $\beta$ 3/ $\alpha$ V $\beta$ 5 mediates inhibition on PKC $\theta$ . Secondly, though MFG-E8 alone mediates immunosuppressive functions, it may require combinational uses of minimal immunomodulatory agents to facilitate the acceptance of allogeneic grafts completely. As I observed partial restraints on Th1 function but not complete suppression of their differentiation, introduction of CTLA-4 agonist or nondepleting antibodies for CD4 and CD8 might generate additive beneficial effects on readily establishment of tolerance. Moreover, concern over whether ESCs are more prone towards NK cells recognition due to inherent low MHC expression is another challenge faced by regenerative medicine. “Missing-self” theory states that, with down-regulated MHC molecules, cells would evade T cell surveillance, but render them targets for NK cell recognition and killing. However, Giuliani *et al.* reported disruption of NK cell immunological synapses and their lysis machinery by human embryonic-derived MSCs and iPS-derived MSCs (102). Therefore, investigating these unidentified inhibitory mechanisms that ESCs might employ to escape NK cell recognition would be another direction to pursue. Finally, the direct use of undifferentiated ESCs in tissue repair may not be clinical feasible. Hence, investigation on whether sustaining elevated MFG-E8 in differentiated cells could protect the grafts for immune rejection should be carried out.

### **(5) Concluding remarks**

In short, a novel immunosuppressive mechanism on T cells by ESC-produced MFG-E8 has been elucidated in this thesis, through which ESCs negatively regulate T effector cells and restore the balance of Treg. Further exploration of the beneficial effects of sustained MFG-E8 expression in differentiated cells to overcome immune barrier and establish transplant tolerance should be pursued. Therefore, via MFG-E8, ESCs are potential mean for modulation of immune responses and induction of tolerance and thus offer solutions to autoimmune diseases and induction of tolerance.

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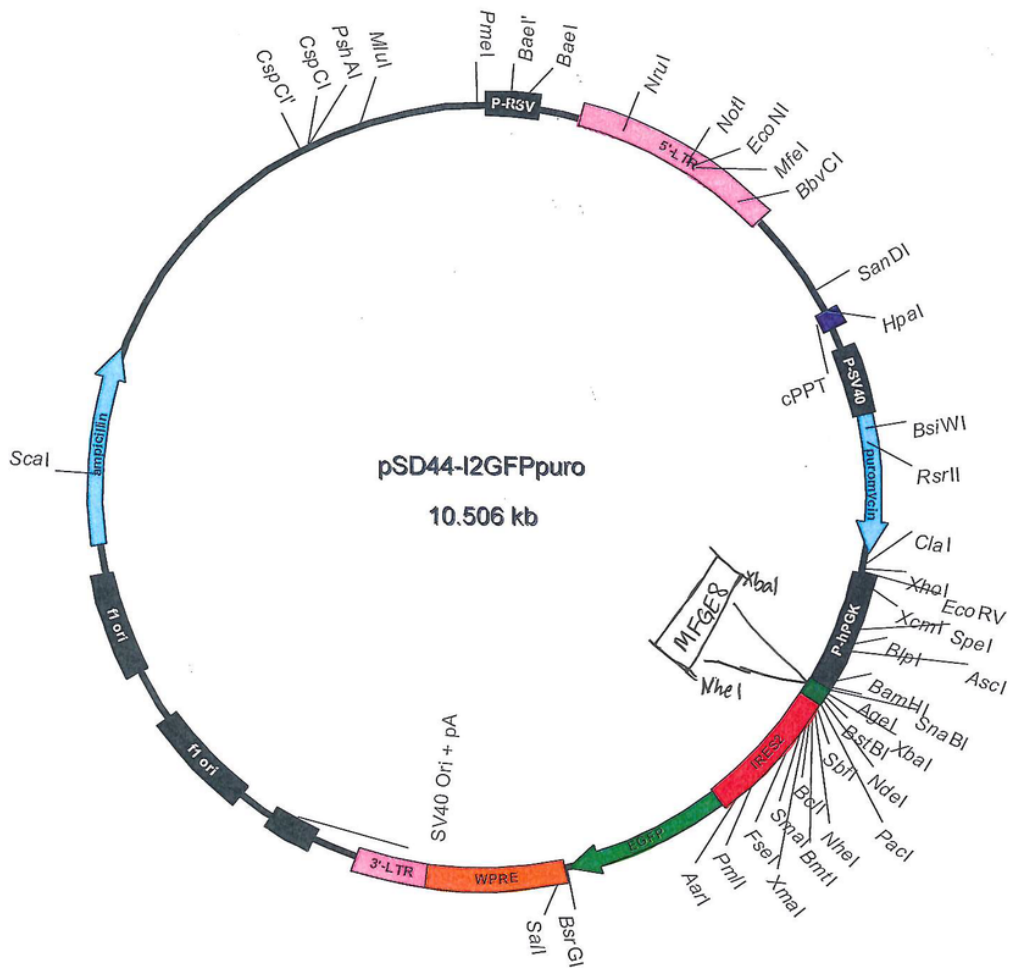
## **Chapter 6. Contributions of Collaborator**

Preliminary data described in the result section were completed by a previous lab member, Bodour AlKhamees, all of which have been included in her thesis in 2012.

Chapter 7. Appendix

MFG-E8 plasmid map

Plasmid #46847 psd44-iGFP-MFGE8-long



## Chapter 8. Curriculum Vitae

### Education

M.Sc. **University of Ottawa.**

H.BSc. **University of Toronto.** Majors in Immunology and Biochemistry

### Research Experience

- **Master of Science in University of Ottawa** **Sept 2012-Recent**
- **Research assistant in Academy of Military Medical Sciences** **Jun. 2011-Aug. 2011**
  - ✓ Participate in a summer research project for the investigation of pharmacodynamics and toxicities of a new cancer drug.
  - ✓ Capable of independently identify problems in the experiments and troubleshooting, and help to implement improvements for experimental designs. Also actively involving in the post-laboratory data analysis processes.
- **Laboratory assistant in YWTK Biotechnology Research institute** **Jun. 2010-Aug. 2010**
  - ✓ Assist in the animal maintenance tasks. Help technicians to do intraperitoneal injection and intragastric administration
  - ✓ Help in the laboratory data collection, input and analyses in YWTK Biotechnology Research institute; highly trained with statistical skills.

### Publications

1. Jia D, Yang W, Li L, Liu H, Tan Y, Ooi S, et al.  $\beta$ -Catenin and NF- $\kappa$ B co-activation triggered by TLR3 stimulation facilitates stem cell-like phenotypes in breast cancer. *Cell Death Differ.* 2014 Sep 26: 1-13. (Impact Factor of 8.385)
2. Tan Y, Ooi S, Wang L. Immunogenicity and tumorigenicity of pluripotent stem cells and their derivatives: genetic and epigenetic perspectives. *Curr Stem Cell Res Ther.* 2014; Jan 9(1): 63-72. (Impact Factor of 2.861)

### Teaching Experience

- Supervising the honor project of a undergraduate student Sept, 2014-ongoing
- Teaching a undergraduate student for basic cell culture techniques, western blotting and flow cytometry Oct, 2013-Feb, 2014
- Mentoring a high school student for a project in national science competition Jan, 2014-Feb, 2014

### **Awards/Scholarship**

- John and Annie Crichton Memorial Award (University of Toronto) Sept, 2010
- Bessie Griffith Cosens Scholarship (University of Toronto) Sept, 2011

### **Community Experience**

- **The Ottawa Hospital general campus** Nov 2013-Recent weekly
  - ✓ Attended the training for radiation therapy.
  - ✓ Assisting in the patient filings and basic administration at reception.
  - ✓ Directing patients into exam rooms.
  - ✓ Actively involved in close patient contacts for comforts and supports.
  - ✓ Helping nurses with patient treatments and filings.
- **Ottawa Regional Cancer Foundation**
  - ✓ Nordic Walk Oct 19<sup>th</sup>, 2014
- **Dancing with Easter Seals** Oct 17<sup>th</sup>, 2014
- **Children's Hospital of Eastern Ontario**
  - ✓ CN cycle May 4<sup>th</sup>, 2014
  - ✓ CHEO BBQ Jun 14<sup>th</sup>, 2014
- **Multiple Sclerosis Society of Canada**
  - ✓ MS walk Apr 27<sup>th</sup>, 2014
  - ✓ 2014 Tamarack Ottawa Race Week May 25<sup>th</sup>, 2014
- **Heart and Stroke Foundation**
  - ✓ Kick for Heart Cardiothon Feb 2<sup>nd</sup>, 2014
  - ✓ Loblaw's Heart Table Feb 21<sup>st</sup>, 2014
  - ✓ Xerox hockey for heart in memory of Bill Kitchen Apr 25<sup>th</sup>, 2014

- **Editor in Production Board of FootPrint Magazine** Sept 2011-Jun 2012
  - ✓ Proficient with graphic design skills using Adobe Illustrator, as well create page designs and layouts using InDesign for digital and paper print magazine. Efficiently coordinate with all boards in the magazine to achieve most desirable goals.
- **Volunteering for SEAS center** Sept. 2007-Mar 2008
  - ✓ Organizing festival events, developed excellent communication acuity and client service skills by volunteering at SEAS center.
  - ✓ Strong team working ability
- **Cashier at Tim Hortons** Oct. 2008-May.2009

### **M. Sc Research Project**

Many aspects of immunosuppressive functions in embryonic stem cells (ESCs) have been reported to potentiate immune surveillance evasion and immune response inhibition, but a defined immunomodulatory component in ESCs is largely unknown and underlying mechanisms remained poorly understood. Based on proteomic database, we report here that milk fat globule epidermal growth factor 8 (MFG-E8) is a key component in ESCs to suppress T cell activation and regulate T cell polarization. MFG-E8 is enriched in undifferentiated ESCs but diminished in differentiated ESCs. Neutralizing ESC-derived MFG-E8 substantially ameliorated the suppressive effect of ESCs on T effector cell activation after anti-CD3/CD28 stimulation. Conversely, MFG-E8 in undifferentiated ESCs is capable of up-regulating T regulatory cells. Using recombinant MFG-E8, specific antibody blockage and rescue experiments, we verified the above findings. Furthermore, we provide the first evidence that MFG-E8 suppresses T cell activation and regulates T cell polarization through inhibiting PKC- $\theta$  phosphorylation. Identification of an important immunoregulatory component in ESCs and reveal of its underlying mechanisms will greatly facilitate stem cell-based therapies.

### **During my master's, I also have**

- Attended and successful presented my experimental achievements in annual OISB symposium.
- Participated in human iPS cell reprogramming workshop, and obtained the opportunities to familiarized and trained for human fibroblasts and CD34+ peripheral blood cells reprogramming.

- Designed Ottawa Institute of System Biology (OISB) logo

**Extracurricular profile/Interests**

- Excellent proficiency in three languages: English, Mandarin and Cantonese. Additional elementary proficiency in Japanese.
- An accomplished piano player with more than fifteen years performance experiences.
- Had received numerous prizes for calligraphy.
- A passionate contemporary dancer that has been participated in numbers of dance performance, as well that actively involves in the choreography designs.