Embryonic stem cell extracts possess immune modulatory properties that prevent dendritic cell maturation and T cell activation

by:

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Abstract:

Embryonic stem cells (ESC) possess immune privileged properties and have the capacity to modulate immune activation. ESCs can persist across allogeneic immunological barriers, prevent lymphocyte proliferation in mixed lymphocyte reaction (MLR) assays and can promote graft acceptance. However, clinical application of live ESC to treat immunological disorders is not feasible as live ESC can form teratoma in-vivo. In order to harness these properties of ESCs without adverse risk to patients, we hypothesized that ESC derived extracts may retain immune modulatory properties of whole cells and therefore could be used to abrogate allo-immune responses. We found addition of ESC-extracts from human lines H1 and H9, significantly prevented T cell proliferation in allogeneic MLRs. These results were confirmed using murine J1 ESC line. In-vitro studies showed human ESC EXT were able to modulate maturation of human monocyte derived dendritic cells (DC) by suppressing up-regulation of important co-stimulatory and maturation markers CD80, HLA-DR and CD83. In addition, DCs educated in the presence of human ESC extracts significantly lost their ability to stimulate purified allogeneic T cells compared to control extract treated DCs. We also determined that ESC extracts have an independent effect on T cells. ESC extracts prevented T cell proliferation in response to anti CD3/CD28 stimulation. In MLRs, ESC derived factors significantly down-regulated IL-2 and IFN-γ expression, while up-regulating TGF-β and Foxp3 expression. Furthermore, lymphocytes and purified T cells activated with anti-CD3/CD28, ConA and PMA proliferated poorly in the presence of ESC derived factors, while proliferation in response to ionomycin was not affected. Western blot analysis indicated that ESC derived factors prevented PKC-θ phosphorylation without influencing total PKC-θ levels. Moreover, IκB-α degradation was abrogated, confirming absence of PKC-θ activity. Therefore, ESC extracts have potent immune suppressive properties and may have clinical applications in ameliorating transplant rejection and autoimmune conditions.

Dedication:

I dedicate my thesis to the memory of my father Mohibullah Mohib.

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List of Abbreviations:

^{3}H - Tritiated Thymidine

7AAD - 7-Amino-Actinomycin D

AICD - Activation Induced Cell Death

APC - Antigen Presenting Cell

bFGF - Basic Fibroblast Growth Factor

BMP-4 - Bone Morphogenic Factor-4

CFSE - Carboxyfluorescein Diacetate Succinimidyl Ester

ConA - Concanvalin A

CPM - Counts Per Minute

CsA - Cyclosporin A

CTL- Cytotoxic Lymphocyte

CTLA-4 - Cytotoxic Lymphocyte Antigen-4

DAG - Diacylglycerol

DC - Dendritic Cell

ELISA - Enzyme linked Immunosorbant Assay

ESC - Embryonic Stem Cell

EXT - Extract

GM-CSF - Granulocyte Macrophage Colony Stimulating Factor

hESC - Human Embryonic Stem Cell

HLA - Human Leukocyte Antigen

ICM - Inner Cell Mass

IDO - Indoleamine-2,3-dioxygenase

IFN-g - Interferon Gamma

IL-10 - Interleukin-10

IL-12p40 - Interleukin-12-p40

IL-13 - Interleukin-13

IL-17 - Interleukin-17

IL-2 - Interleukin-2

IL-4 - Interleukin-4

IL-5 - Interleukin-5

IONO - Ionomycin

IP_3 - inositol triphosphate

IPS - Induce Pluripotent Stem Cells

ISSCR - International Society for Stem Cells Research

LIF - Leukemia Inhibitory Factor

mAB - Monoclonal Antibody

mDC - Moncyte derive Dendritic Cell

MEF - Mouse Embryonic Fibroblast

mESC - Mouse Embryonic Stem Cell

mHA – Minor Histocompatibility Antigen

MHC - Major Histocompatibility Complex

MLR - Mixed Lymphocyte Reaction

NK - Natural Killer

PBMC - Peripheral Blood Mononuclear Cell

PD-1 - Programmed Death-1

PD-2 - Programmed Death-2

PDL - Programmed Death Ligand

PIP_2 - Phosphatidylinositol 4,5-bisphosphate

PKC-t - Protein Kinase C-theta

PLC-g - Phospholipase C- gamma

PMA - Phorbol 12-myristate 13-Acetate

QPCR - Quantative Polymerase Chain Reaction

SSEA - Stage Specific Embryonic Antigen

TCR - T Cell Receptor

TGF-b - Transforming Growth Factor Beta

Th - T helper

Thre-538 - Threonine 538

TNF-a - Tumour Necrosis Factor alpha

Treg - T regulatory

U - Units

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Chapter 1. Introduction:

Overview:

The isolation of the first embryonic stem cell lines may be one of the most important advancements in modern biology [1-4]. Embryonic stem cells (ESC) are defined by their unique properties of unlimited proliferation (self-renewal) in vitro, ability to differentiate into almost any adult cell type derived from three germ layers (pluripotency) and capacity to contribute to chimera when injected into a developing embryo [1-5]. These properties make ESCs an invaluable tool in developmental and cellular biology and regenerative medicine. As a result, important and otherwise hard to study aspects of development can be elucidated using ESCs [5-12]. Moreover, rare and difficult to isolate populations of adult stem cells can now be generated in large numbers for further molecular and genetic studies [5, 8, 13-16]. Finally, ESCs may provide cures for a variety of degenerative disease through cell replacement therapy [13, 14, 17-20].

The unique properties of human ESCs have led many stem cell researchers to focus their studies on pathways that induce differentiation of ESCs into specific cell types, such as neuronal, hematopoietic, hepatic, and insulin producing pancreatic β-cells [7, 8, 13, 14, 16, 21, 22]. With ongoing research and improvement of differentiation protocols, the clinical use of stem cell based cell replacement therapies is impending. Along with this prospect an important obstacle facing cellular and tissue transplantation will be immune rejection. Undoubtedly, ESC-derived tissues and cells will be recognized as allogeneic cells by the immune system, leading to an inflammatory response. In order to address this inevitable

problem various strategies have been devised which may be able to circumvent immune rejection including the use of clinically established immune suppressive drugs, development of a large stem cell bank that would cover most human leukocyte antigen (HLA) diversity in order to escape immune recognition, somatic nuclear transfer, and finally customized patient specific ESC through induced pluripotent stem cells (IPS) [19, 23-28]. However, the aforementioned strategies may have major limitations that will limit their use in a clinical setting. For example, the use of immune suppressive drugs can result in organ toxicity and suppression of the immune system thereby increasing susceptibility to infection and incidence of cancer [29-32]. On the other hand, creating a stem cell bank may not be feasible since the human HLA genes are one of the most polymorphic genes. At the same time it is currently a formidable task to take into account minor histocompatibility antigens, which contribute to chronic rejection [33-36]. Moreover, ethical issues hinder use of somatic nuclear transfer, as it involves the same process that can be used for human cloning, while the use IPS are plagued by uncertainty about their true stem cell like qualities raising questions about their pluripotency and long term genomic stability [37-39]. Therefore, further strategies aimed at preventing immune rejection of stem cell derived tissues are needed.

In an attempt to decipher the outcome of ESC transplantation, various groups have studied the interaction between ESCs and allo-immune cells. Surprisingly, it was elucidated that unlike their differentiated counterparts, undifferentiated ESC possess immune privileged properties similar to fetal tissues during pregnancy [40-52]. ESCs were capable of surviving across allogeneic barrier

and persisted in immune competent hosts over a prolonged period without inducing immune activation [40, 41, 43, 44]. Hence, undifferentiated ESCs were found to be immunologically inert. Beyond their inability to evoke an immune response and survive in an allogeneic environment, undifferentiated ESCs were also able to actively inhibit or modulate the immune system to a state of reduced responsiveness [40, 41, 43, 44, 52]. ESCs have been shown to inhibit T cell proliferation even in the presence of third party antigen presenting cells, indicating that they may have the capacity to induce a tolerant state [40, 41, 43, 44, 52]. Significantly, rat-ESCs appear to protect a co-transplanted solid organ from immune rejection for a prolonged period of time [43]. Therefore, ESCs possess powerful immune modulatory properties that alter normal immune responses thereby providing protection to the cells themselves as well other allogeneic cells and organs.

Harnessing the immune properties of ESC may have important applications in auto-immunity, transplantation and chronic inflammatory conditions. However, several major obstacles impede clinical application of live ESCs. The greatest risk associated with undifferentiated ESC infusion into humans is the formation of teratoma or ectopic tissue masses [1-4, 24, 25, 27, 53-55]. Most alarming, there exists a direct correlation between the number of ESCs transplanted and the incidence of teratoma occurrence in recipients [44]. Teratomas present a danger to the patient due to a number of causes. This includes aberrant growth that results in local tissue dysfunction, risk of malignant transformation and growth to the point where it becomes unresectable [17, 56, 57]. As such, the ability of ESC to give rise to teratoma continues to be a major issue identified by the International Society for Stem Cell

Research (ISSCR) in the usage of ESC in the clinic [58, 59]. Additional formidable deterrents to ESC-based therapies are associated with logistics that include the high cost of creating specialized facilities, regulatory issues and employing trained personnel for cell preparation. Therefore, ESC therapies may not be feasible at the moment due to serious risk to the patient, logistical difficulties and high cost.

Hypothesis:

In order to overcome the hurdles facing the use of ESC based therapies we hypothesized that *ESC derived extracts will retain immune modulatory properties of whole cells and therefore could be used to abrogate allo-immune responses.*

The purpose of the remainder of this introduction will be to provide more detailed information on the basic properties of ESC, expand on their ability to modulate immune responses and outline the rationale for the use of ESC extracts as an innovative approach in treating immune-based conditions while overcoming the dangerous risk of using whole cells.

Embryonic stem cells are defined by their unique properties:

Embryonic stem cells possess unique properties that differentiate them from other types of stem cells that have been previously defined. This section will describe and highlight the importance of these properties. There are three defining properties of ESCs: 1) ESCs are derived from cells from a pre-implantation embryo, 2) they have unlimited capacity to proliferate in-vitro and self-renew and 3) they can give rise to all three germ layers.

Derivation of embryonic stem cells:

Embryonic stem cells are isolated from the inner cell mass (ICM) of a pre-implantation blastocyst [1-4, 43]. The primary method used to isolate ESC from primates and humans consists of obtaining six-day old blastocysts [3, 4]. Subsequently the ICM of the blastocyst is isolated by what has been termed "immuno-surgery" [3, 4]. The outer cells are removed by the addition of antibodies specific to the trophoectoderm that makes up this layer. After antibody binding, complement is added causing apoptosis of the trophoectoderm cells thereby releasing the ICM [3, 4]. An alternate method used to isolated ESC consists of dissociating the blastocyst without removing the trophoectoderm. With this method the cells need to be separated through sub-culturing techniques in order to obtain ICM cells [1]. A similar method is used for the isolation of mouse ESC-like cells (termed epiblast stem cells) except pre-implantation blastocysts are obtained at day 3.5 instead of day 6 [1, 2]. Regardless of the methods to obtain ICM cells, ESCs are then maintained on mouse embryonic fibroblast (MEF) feeder cells until sufficient numbers are generated [1-4].

In order to determine whether ESCs were isolated from the ICM, several cell surface and intracellular markers can be used. Human and primate ESC are characterized by surface expression of stage specific embryonic antigen-3 (SSEA-3), SSEA-4, TRA-1-60, and TRA-1-81 [3]. Intracellularly, ESCs are characterized by transcription factors Oct-4 and Nanog, alkaline phosphatase and the expression of telomerase [3, 4, 60-62]. Murine ESCs isolation can be validated by expression of Oct-4, Nanog and alkaline phosphatase and SSEA-1 instead of SSEA-3 or SSEA-4 [60-64].

Characterizing the expression of above markers can be used as indicators of the quality of ESC cell lines. Low levels of Oct-4, Nanog or alkaline phosphatase expression in a cell line could have an effect on genetic as well as epigenetic stability and have an enormous impact on long term maintenance and differentiation, therefore affecting its potential application in a clinical setting [60-62, 65-67].

Unlimited capacity to proliferate and self-renew in-vitro:

The capacity of ESCs to proliferate indefinitely and self-renew in-vitro may be one of the most useful and important characteristic of the cells. ESCs remain stable in culture without senescence [1-4]. In contrast, most other isolated adult stem cells and primary cells are hard to maintain for long-term and are often limited to a few passages [68]. For example, mesenchymal stem cells, isolated from bone marrow, can be maintained in culture for about 12 to 15 passages [69] (personal observation). In contrast, ESCs can be maintained in culture for over several years and hundreds of passages [1-4, 70, 71]. ESCs have a rapid cell cycle with the mouse cell cycle lasting 10-16 hours and the human cell cycle lasting 24-36 hours [70, 71]. Following each cell cycle two identical undifferentiated cells arise through symmetric division [72]. Despite, rapid proliferation and prolonged maintenance in culture, ESCs remain genetically stable and tend to retain their karyotype [1-3]. As a result, large numbers of cells can be obtained from a single cell line. This provides an unlimited source of cells for experiments and facilitates reproducibility without the fear of aberrant results due to genetically induced changes.

Self-renewal of ESCs is not inherent in in-vitro culture rather it is contingent on the presence of external factors. Initial studies elucidated that ESCs themselves were unable to support their undifferentiated state and self-renewal[1-3]. As a result the cells were maintained with media conditioned by embryonic carcinoma cells and supporting MEF cells [1]. Various factors and culture conditions have been identified that play important roles in ESCs self-renewal. The most essential factor required for mouse ESCs is the presence of leukemia inhibitory factor (LIF) while human ESCs require basic fibroblast growth factor (bFGF) for their long-term culture [73-79]. The presence of LIF can sustain self-renewal of mouse ESC cells in MEF conditioned media containing serum without MEF cells. Self-renewal of mouse ESCs can be enhanced by the addition of bone morphogenic protein-4 (BMP-4) [75]. The combination of LIF and BMP-4 are able to maintain mouse ESCs even in the absence of serum [75]. Similarly, the addition of Noggin along with bFGF can improve self-renewal of human ESCs [77, 80]. However, co-culture with MEF cells along with either LIF or bFGF remains the optimal condition for self-renewal for both mouse and human ESCs, respectively. The MEF cells are thought to provide a supporting environment as well as secrete various growth factors and cytokines that stabilize ESC self-renewal [1-3, 81]. The presence of these factors and cells supports ESCs self-renewal by maintaining the expression of important transcription factors such as Oct-4, Nanog and Sox2 that retain their undifferentiated state [60, 65, 67, 76, 77, 82].

Evidently self-renewal of ESCs is a characteristic that is very valuable to researchers. The immortal nature of ESCs makes them unique amongst all other stem cell types as well as primary cell types. The capacity of the cells to provide an

indefinite source of cells without changes in karyotype assures reliability and reproducibility of experiments.

ESCs differentiate to cells derived from all 3 germ layers:

Different types of stem cells exhibit varying differentiation capabilities. For example, hematopoitic stem cells are known as multipotent stem cells since they can give rise to only a few cell types [83-85]. Pluripotent stem cells, able to give rise to cells from all three germ-layers but not an organism, were initially isolated from mouse embryos in extra-uterine sites giving rise to embryonic teratocarcinomas [55, 86, 87]. The masses that formed from these embryos were found to have cells with the ability to differentiate into various types of cells [55, 86, 87]. These cells were termed embryonal carcinoma cells and at the time were the only cell types able to differentiate into all three germ layers [55]. However, embryonal carcinoma cells were limited in their differentiation potential [1, 2, 55, 86, 87]. Moreover, they were unstable in culture often containing aneuploidy [88]. Isolation of ESCs ensued as a direct result of these initial studies [1, 2]. Similar to the embryonic carcinoma, ESCs cell were found to differentiate into all three germ layers, however, ESCs were found to have a greater differentiation capacity and remained stable in culture without incidence of aneuploidy [1-3].

The pluripotency of newly isolated ESC line can be tested using several methods. There are two in-vivo methods used to the determine pluripotency. The first method involves injection of ESCs into immune compromised mice. Subsequently, the mice are examined for teratoma formation, which are tumor like masses of differentiated ESCs [1-4]. These masses contain highly organized tissue

segments resembling gut like structures, muscle cells, and neural tubes [1-4]. These teratomas result due to spontaneous differentiation ESCs in the absence of signals that retain their undifferentiated state. The second in-vivo based method used to determine pluripotency involves the introduction of ESCs into pre-implantation embryos [89, 90]. These chimeric embryos are implanted in mice and examined for the contribution of the ESCs to the developing embryo [12, 91, 92]. ESCs have been shown to contribute to most tissues of the embryo with the exception of extra-embryonic endoderm and trophoblast layers [12, 91, 92]. Significantly, ESCs have been found to contribute to germline cells that are then passed on to progeny [93]. It should be noted that this method of pluripotency determination is not used for human ESCs for obvious ethical reasons. The combination of these two in-vivo tests provides a robust examination of true ESC pluripotency thereby giving a good measure of its possible future application for research.

The greatest promise and value of ESC pluripotency lies in the ability of researchers to differentiate cells in-vitro. Therefore, a majority of research has been concentrated on elucidating development pathways and methods to differentiate ESCs into various cell types. The primary in-vitro method used to differentiate ESCs involves passage as small clumps in suspension known as embryoid bodies [94]. Embryoid bodies resulting from cell aggregates form 3 dimensional cell structures that create a favorable environment for differentiation through multi-cellular interaction and communication. During this process, ESCs are grown in the absence of external growth factors such LIF as well as MEF cells which maintain them in an undifferentiated state [73-79]. Without support from growth factors and

MEF, ESCs undergo a default differentiation pathway [76, 79]. However, addition of specific factors to the culture medium facilitates the differentiation of hESCs into the desired cell type. Directed differentiation of ESCs into specific lineages has been a great focus of researchers. By studying various signaling pathways in response to specific stimuli, such as growth factors, cytokines, supporting cells, extracellular matrices, growing conditions, various protocols have been developed to differentiate ESCs into specific cell types such as hepatocytes, neuronal cells, insulin producing β-cells, muscle and hematopoietic cells [3, 5, 7, 13, 14, 16].

The pluripotent nature of ESCs has given researchers an extraordinary tool to answer fundamental questions in developmental biology, genetic, drug discovery and regenerative medicine. Using ESCs, developmental biologists are able to study aspects of embryology and cell development otherwise impossible to study in embryos. As such populations of cells found only at the embryonic stage, like the hemangioblast a precursor to endothelial and hematopoietic cells, can be generated and examined closely [15]. Meanwhile, manipulation of ESCs at the genetic level followed by incorporation into chimeric embryos has had a tremendous impact on biological research as a whole [11]. The creation of transgenic and knock out mice has facilitated the study of countless genes and has significantly advanced our knowledge of their function under steady state as well as diseased conditions. The development of this technique was awarded the 2007 Nobel Prize in Medicine and remains an important tool for many researchers [11]. ESCs also have important applications in pharmacology. Through ESC differentiation pure populations of primary cells can be generated for testing of drug efficacy and toxicity [95]. As a

result, the effects of newly developed drugs can be tested on specific target cells. Finally, the possible future use of ESCs in regenerative medicine represents a great hope. The capacity of ESCs to provide an unlimited source of purified differentiated cells for cell replacement therapy to treat tissue injuries, various forms of genetic and degenerative conditions may provide cures for a large number of patients [13, 14, 17-20]. More significantly, the advent of induced pluripotent stem cells, which creates individually tailored-stem cells, is an enormous step in reducing major complications that may be associated with stem cell transplantation [24, 25, 27].

Embryonic stem cells possess immune privileged properties:

For quite some time it has been known that embryonic and fetal tissues possess immune privilege or tolerance-inducing properties. Owen, Medawar and colleagues were among the first to elaborate on fetal-maternal tolerance [96, 97]. It is apparent that despite expression of both maternal and paternal antigens throughout pregnancy, the maternal immune system does not reject the fetus [97, 98]. Evidently, pregnancy represents an altered state of the immune system. This is validated by remission of autoimmune diseases such as rheumatoid arthritis in a majority of patients [99]. During the term of pregnancy, the severity of these conditions are either decreased or completely eliminated [99]. Numerous studies have established the existence of various factors capable of promoting fetal-maternal tolerance by modulating the maternal immune system. This includes the expression of high levels of FasL, HLA-G, TGF-β, IL-10, indoleamine-2,3-dioxygenase (IDO) as well as increases in specific population of lymphocytes such as T regulatory cells [100-106]. However, pregnancy does not permanently eliminate autoimmune disease as

shortly following delivery of the fetus symptoms often return [107]. Changes induced during pregnancy appear temporary and dependent on the presence of the placenta and fetus to promote the expression of soluble factors and maintenance of pregnancy-specific lymphocytes.

As the prospect of ESC transplantation nears clinical application the possible risk of immune recognition must be addressed. Undoubtedly, differentiated stem cell derived cells will trigger allogeneic responses by the host immune system, leading to possible immune rejection [40, 48, 49, 108]. In order to solve this inevitable problem various strategies have been devised. The strategies that are currently being explored include immune suppression, creating a stem cell bank able to accommodate most HLA diversity, somatic nuclear transfer and reprogramming of somatic cells with defined factors [23-27]. However, as discussed above these strategies have major limitations. Surprisingly, several studies have established that undifferentiated ESCs, unlike their differentiated derivatives, possess immune privilege and modulatory properties similar to fetal tissues [40, 41, 43, 44, 52]. These properties of ESCs may have important implications in the outcome of ESC transplantation and due to resultant immune modulation. The purpose of the following sections will be to provide a brief overview of transplant immunology, describe the immunological phenotype of ESCs and provide evidence for their immune privilege and modulatory properties.

Transplantation immunology:

There are three important immunological elements in transplantation that influence successful engraftment. These include immune activating antigens, antigen presentation and proper co-stimulation of effector T cells.

Immune rejection of allogeneic tissue is initiated by recognition of polymorphisms found in the major histocompatibility complex, minor histocompatibility antigens and ABO blood group antigens [33-36,109, 110]. The strongest stimulus for allogeneic rejection arises from disparity between donor and recipient MHC proteins. Major differences in the MHC-class I and II proteins can result in an intense immune response by the recipient resulting in acute rejection of the donated tissue. Such strong immune attack results due to 10% of T lymphocytes having the capacity to recognize allo-MHC expressed on the transplanted cells [109-111]. As a result, matching of HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 are essential and determine long term survival in kidney transplant recipients [112]. Minor histocompatibility antigens also influence the outcome of organ transplantation. These antigens are thought to consist of small differences in common genes, typically arising as single nucleotide polymorphisms that may have an impact on the recognition of the gene product by T cells upon antigen presentation in the context of MHC-I or MHC-II [113, 114]. In contrast to allogeneic MHC, mHA do not appear to have a major influence on acute rejection of tissues. Instead, mHA variances between donor and recipient are thought to play a part in chronic rejection even in monozygotic twins [35, 36]. ABO blood groups represent a further antigen group that may contribute to transplant rejection [115]. ABO group antigens are commonly known

to significantly impact blood transfusions due to presence of antibodies in the recipient. These antibodies can also have a serious impact on the success of solid organ transplantation [115]. However, ABO type matching has made it easy to avoid this problem and as a result it does not represent a major barrier in solid organ transplantation [115-117]. It is evident that the expression of the above three different antigen groups has the most significant impact on the outcome of transplantation. Assuring that proper precautions are taken in order to match HLA antigens is optimal in order to assure long term graft survival. Similarly, research is now being conducted in typing important mHA that may contribute to chronic organ rejection in order to improve the outcome of transplantation.

Antigen presenting cells (APC) have a central role in transplant rejection. They initiate the activation of the adaptive immune response in one of three ways: direct, indirect and semi-direct antigen presentation [118-122]. Upon organ transplantation, direct presentation occurs when donor derived tissue resident APC migrate to a local lymph node and encounter the recipient's immune cells [123, 124]. This type of antigen presentation leads to a vigorous immune response as the recipient T cells recognize an allogeneic antigen in the context of allogeneic MHC [109, 110, 125, 126]. As a result direct antigen presentation contributes to rapid immune activation and represents a significant hindrance to long-term graft survival [118, 127]. In contrast to direct antigen presentation, indirect antigen presentation is mediated by the recipient's own APC that encounter donor antigen. In this case, antigen from donor organs is taken up by the APC that then migrate to secondary lymphoid organs and present to effector cells [119, 120, 126]. This form of antigen presentation

does not elicit as intense of an immune response and may contribute to chronic rejection of the transplant [120]. Finally, semi-direct antigen presentation represents a hybrid of the first two modes of antigen presentation. Semi-direct antigen presentation occurs when recipient APC interact with donor lymphocytes or tissue and take up whole MHC molecules from the surface of these cells and then present to T cells [121, 122]. In this case, the same APC are capable of both direct and indirect antigen presentation leading to robust stimulation of immune effector cells [121, 122, 124]. Hence, strategies aimed at depleting donor APC and preventing effective antigen presentation are methods used to promote transplant survival.

In addition to antigen presentation, effector T cells require a second signal from the APC also known as co-stimulation in order to become activated. Well-studied co-stimulatory molecules include CD80, CD86, CD40 and OX-40L [128-130]. Classically CD80/86 on APC interact with CD28 expressed on T cells, and represent the most commonly studied secondary activating signal [128, 131-134]. CD40 and OX-40L, also expressed on APC, interact with CD40L and OX-40 on the effector cells respectively [128-130, 135-137]. The expression of these co-stimulatory molecules on APC is contingent on the maturation state of the cells [138, 139]. Immature APC that have not encountered antigen and inflammatory signals lack the expression of co-stimulatory molecules on their surface [140, 141]. In the absence of proper co-stimulation, effector T cells are induced to undergo anergy and are prevented from further activation [140, 141]. Upon antigen encounter and inflammatory signals the expression of co-stimulatory markers are strongly induced on the surface of APCs which subsequently potently activate effector cells [138, 139, 141, 142]. In

addition to co-stimulatory molecules that positively impact effector cell activation there exist also co-stimulatory molecules that can provide negative signals to the effector cell which can induce anergy and cell death. Specifically, molecules such as cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1) expressed on effectors deliver an inhibitory signal when interacting with B7.1/B7.2 and programmed death ligand-1 (PDL-1) respectively on APC [143-145]. A large focus of the transplant field has been deciphering the role of different co-stimulatory molecules in rejection. As such various strategies have been developed that either hinder APC maturation, prevent positive co-stimulatory signals or enhance negative co-stimluatory signals. For example, negative co-stimulatory molecules have been successfully employed, like soluble CLTA-4 Ig, at improving the outcome of allogeneic transplantation [146-149]. Therefore, co-stimulatory signals play a major role in organ transplantation and may represent a key in promoting long-term graft survival.

Embryonic stem cells possess a unique immunologic phenotype that facilitate evasion of the immune system:

The immunological phenotype of ESC has been proposed to allow the cells to escape immune recognition and rejection. The main mechanism that may facilitate the evasion of ESCs from the immune system is the lack HLA and co-stimulatory expression [42-45]. Undifferentiated ESCs in culture, do not express either MHC I or II on their surface [42-45]. However, MHC I but not MHC II expression can be induced on ESC upon treatment with IFN-γ. Notably, in comparison to HeLa cells, levels of MHC I on ESC remain minute in the presence of IFN-γ [42, 45]. Similarly, ESC

differentiation results in the relative increase in expression of MHC I, however MHC II expression still remains absent [42, 45]. The absence of co-stimulatory molecules on ESCs represents a further factor that may allow immune escape of the cells. Examination of undifferentiated ESC in culture has revealed that the cells are completely deficient in the expression of co-stimulatory molecules including the expression of CD80, CD86 and CD40 [42-45]. Moreover, treatment of the cells with IFN-γ does not induce the expression of any co-stimulatory molecules [41-43, 45]. Even differentiation of the cells does not promote co-stimulatory molecule expression unless the cells are specifically directed towards a hematopoietic cell type [10]. Additionally, microarray analysis indicates ESCs do not express various other genes that could facilitate immune activation such as adhesion molecules, cytokines and chemokines [41, 48]. As a result, T cells are unable to recognize undifferentiated ESC as foreign and are not provided with co-stimulatory signals to cause rejection. Interestingly, several studies have elucidated that these properties are very similar for mouse, rat and human ESCs [42-44].

The absence of MHC and co-stimulatory expression give ESCs an immunological inert phenotype. Consistent with this notion, several studies have carried out in-vitro and in-vivo studies examining the capacity of ESCs to survive in allogeneic and even xenogeneic conditions. The pioneering study carried out by Fandrich et al. demonstrated that several rat ESC lines were able to survive across allogeneic barrier for up to 25 weeks, whereas the recipient rapidly rejected control bone marrow cells from the same strains as the ESCs [43]. Follow up studies using mouse and human ESCs confirmed the ability of cells to survive across allogeneic

barrier [41, 42, 44, 45, 47]. Both mouse and human ESCs were shown to escape immune attack using in-vitro and in-vivo assays [40-42, 44, 45, 48, 52]. In-vitro assays elucidated that ESCs have the capacity to escape cytotoxic T lymphocyte (CTL) and natural killer (NK) cell mediated lysis [40, 42]. Significantly, ESCs were resistant to lysis from CTL and NK cells even when the cells were pre-activated and IFN-γ added to the killing assay[40, 42]. In vivo studies carried out with both mouse and human ESC further validated these results. Mouse ESCs injected into the portal vein of allogeneic recipients had the capacity to form teratoma in these mice [44]. Moreover, the authors found a direct correlation between increasing ESC number and the frequency of teratoma formation [44]. Amazingly, another study demonstrated that mouse ESCs survived when injected into sheep and gave rise to cardiomyocytes in infracted hearts [47]. Interestingly, improved results of cardiac function, were obtained in groups where ESCs were injected into the heart without immune suppression [47]. Similarly, experiments involving injection of human ESC found that they avoid rejection when injected under the kidney capsule of a mouse trimera model reconstituted with allogeneic human peripheral blood mononuclear cells (PBMC) [41]. Once again, control cells were rejected by the PBMC while ESCs were able to form teratomas [41]. Human ESCs also have the capacity to survive in normal mouse hosts indicating that these cells are able to evade xenogeneic immune rejection to some degree [45]. It is apparent from these studies that ESCs possess immune privileged properties that facilitate their survival across allogeneic and xenogeic barriers. However, several studies have found ESC immune privilege to be limited, as differentiation results in immune activation and subsequent rejection [46, 48, 49, 150, 151].

It is possible that these differences are due to specific models used to determine immunogenicity. Nevertheless, most papers agree that ESCs are more likely to encounter immunological tolerance compared to adult tissues and organs [40, 41, 43-50, 150].

Embryonic stem cells have the capacity to modulate immune system:

The fact that stem cells do not induce a detectable immune response following transplantation may be attributed to an immunological inert phenotype, however this does not exclude the possibility that the cells also actively influence immune activation. This section will provide evidence supporting the capacity of ESC to modulate the immune system and the possible factors that may be involved.

The immune modulatory properties of ESC were discovered through both in-vitro and in-vivo assays. Initial studies examining ESC immunogencity also led to observations that the cells may actively play a role in altering immune responses. ESCs used as third party cells in mixed lymphocyte reaction (MLR) assays elucidated that the cells were able to inhibit responder cell proliferation in the presence of stimulator allogeneic lymphocytes [40-42, 44, 45]. Moreover, ESCs were able to inhibit T cell proliferation in response to the strong activation signal of direct antigen presentation by allogeneic DCs [44, 45]. Further assays were able to establish that ESCs were able to affect both T cells and DCs [44, 45]. In vivo studies established that the presence of ESCs were associated with an increased number of regulatory cells and lower number of effector cells producing inflammatory cytokines [40, 52]. In a separate study, mouse ESC conditioned media was shown to inhibit proper maturation of DC in response to LPS [44]. Most significantly, rat ESC injected into the

portal vein of allogeneic hosts were able to facilitate the acceptance of syngeneic heart transplants in an allogeneic host without the use of immune suppressive drugs [43]. These studies present compelling evidence that ESC indeed possess immune modulatory properties. Determining the factors and mechanisms used by ESC that promote immune evasion may have important clinical applications.

The factors and mechanisms attributed to ESC mediated immune modulation have not been extensively studied. However, some already well known factors have been described to contribute to ESC immune modulatory properties. Fandrich et al. were able to determine that rat ESC expressed high levels of Fas ligand (Fas-L) and that this may play a role in overcoming T cells [43]. In contrast, studies with mouse ESCs elucidated that TGF-β contributed to the survival of the cells [44]. However, examination of human ESCs for FasL and TGF-β were found to be negative [5, 42, 45]. In contrast, human ESCs were found to lack expression of ligands for NK activating receptors that contributed to their ability to evade NK cell lysis [42]. More recently, two papers have described the expression of arginase-I and HLA-G and their contribution to modulating the immune system [50, 52]. In an attempt to further identify possible factors, several investigators have carried out screening of a wide array of immune related genes known to have immune inhibitory effects or tolerance inducing properties. Drukker et al. looked at the expression of over 300 genes, including cytokines and their receptors by microarray and found most genes in human ESCs were absent [41]. Among these 300 genes, expression of IL-10 and TGF-β genes was undetectable [41]. Using mouse ESCs, another independent study examined 87 immunologically relevant genes using Taqman assay and obtained

similar results. The study found that IL-10, TGF-β1, indoleamine 2,3-dioxygenase, arginase-1 and 2 do not contribute to immune privilege of mouse ESCs [48]. However, the study was able to establish a positive correlation between ESC survival and TGF-β2 expression [48]. Moreover, we recently reviewed a list of highly abundant proteins found in hESCs available in our lab using quantitative mass spectrometry and have not found any known candidate factors such as CTLA-4, PDL-1, PDL-2, PGE-2, and HLA-G. The above-mentioned studies have provided some clues on how ESCs modulate the immune system based on known mechanisms. The fact that ESCs have the capacity to affect multiple lymphoid cell types makes it evident that the cells modulate the immune system through a number of different mechanisms. Hence, there is an exciting opportunity for the discovery of novel factors and mechanisms that contribute to ESC mediated immune modulation.

Rationale:

The use of ESC derived extracts for immune modulation:

Current evidence indicates that ESCs possess powerful immune modulatory properties. 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 risk of teratoma formation [3, 54, 55]. Experimental evidence indicates the incidence of teratoma formation is directly linked with increasing number of ESCs transplanted [44]. In mice, to overcome the immune system a minimum of 5 x 10^6 ESC are required (teratoma formation in 30% of recipients), with 20 x 10^6 being the optimal number (teratoma formation in 90% of recipients),

whereas 1.0 x 10^6 were found to be completely ineffective in overcoming the immune system (teratoma formation in 0% of recipients) [44]. This very real risk has recently been brought to the forefront of clinical application as a Russian group conducting clinical trials with embryonic neural stem cells found donor derived tumors in the recipients brain and spinal cord [17]. In response to these findings the International Society for Stem Cell Research has re-iterated their apprehension for clinical trials and have emphasized their guidelines that include stringent quality control measures and safety and efficacy requirements prior to proceeding to human trials [58, 59]. As a result, the use of undifferentiated ESCs and their differentiated counterparts may not be feasible presently or for the foreseeable future.

As an alternative to the use of live ESC to promote immune modulation, we have proposed that ESC derived extracts may retain the immune modulatory properties of whole cells and could be used to overcome aberrant immune responses. There are several arguments that may provide grounds for the use of ESC extracts instead of live cells. To begin with, evidence suggests that ESCs do not require cell contact to modulate the activity of immune cells since their ability to inhibit T cell cytokine production can be observed across a transwell [52]. Moreover, ESC conditioned media have been shown to inhibit DC maturation [44]. Hence, it may be argued that some of the effects of ESC immune modulation may be mediated through soluble factors. Moreover, extracts derived from the placenta and amniotic membrane have been shown to decrease contact hypersensitivity and inhibit macrophage activity [152, 153]. This supports the notion that embryonic tissues and

cells may retain their immune modulatory efficacy in the extract form. Notably, ESCs would provide a consistent and reliable source of extracts compared to placental extracts that would have to be donated following pregnancy. Finally, even if safety can be assured for live ESCs, the cells have a tendency to localize in a specific area in-vivo [40, 46, 151]. As such, ESCs would exert their immune modulatory effects in a localized area. In contrast, the use of soluble extracts would allow a wider effect which may be required for most immune based conditions. Therefore, use of ESC extracts may represent an innovative approach in providing an unlimited source of immune modulatory factors for the treatment of aberrant or unwanted immune based conditions.

In order to determine whether ESC derived extracts retain the immune modulatory effects of whole cells, I have examined the impact of the extracts on DCs and T cells. As such, I designed my experiments to elucidate the phenotype, activation and function of the cells. Here I present my work and the resulting data that further contributes to the understanding of the immune modulatory properties of ESCs.

Chapter 2. Materials and Methods:

Cells and mice.

Human ESC lines H1 and H9 were obtained from Wicell. The CA1 cell line was a kind gift from Dr. Nagy (University of Toronto, Toronto Ontario, Canada). Mouse ESC D3 and J1 (129/J background) cells were kind gifts from Dr. Qiao Li and Dr. Michael Rudnicki, respectively (University of Ottawa, Ottawa, Ontario, Canada). Mouse ESC C57BL/6 (B6) cell line was obtained from ATCC. Mouse strains C57BL/6, B6C3F1, Balb/c and CD1 (10 to 12 weeks old) were obtained from Charles River Laboratories (Montreal, Canada). All hESC lines were used with the approval of the local Ethics Board and the Stem Cell Oversight Committee of the Canadian Institutes for Health Research. Animals were maintained at the University of Ottawa (Ottawa Ontario, Canada) in accordance with the Canadian Council on Animal Care guidelines under protocols approved by the Animal Use Subcommittee at our Institution.

Preparation of embryonic stem cell extracts.

Human ESC:

Human ESC lines H1, H9, and CA1 were cultured on plates coated with Matrigel (BD Biosciences Canada Inc., Mississauga ON) in mouse embryonic fibroblast conditioned medium supplemented with 8.0ng/mL of human bFGF, (Invitrogen Canada Inc., Burlington ON), throughout the culture period hESC were grown under MEF free conditions, and were incubated at 37°C with 5.0% CO_2.

Upon reaching confluence, the cells were harvested by treatment with collagenase IV (Invitrogen) followed by cell dissociation buffer (Invitrogen) to obtain a single cell suspension. Subsequently, hESCs were washed twice with ice cold PBS and centrifuged at 400g for 6 minutes at 4°C. After washing, the cells were re-suspended in lysis buffer, 50mM HEPES, 50mNaCl, 1.0mM EDTA, 1.0mM DTT, 50mM L-arginine, pH 8.2. The lysis buffer was supplemented with pan protease inhibitors at 1:100, (4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin, and aprotinin) dissolved in DMSO, Sigma Aldrich Canada Ltd, Oakvile ON). At this point the cells were incubated for 30 minutes on ice and sonicated until complete lysis of the cells was achieved. The sonicated cells were centrifuged at 15000g for 15 minutes at 4°C to remove cell membrane, mitochondrial and nuclear fractions. The soluble cell free fraction was separated from the insoluble fraction and both stored at -80°C. Protein concentration was determined using Bio-Rad Protein assay kit (Bio-Rad Laboratories Ltd. Mississauga ON).

Mouse ESC:

Mouse ESC lines B6, D3, and J1 were grown on mitomycin C treated MEF cells in Dulbecco's modified eagle medium (DMEM) containing 4.0mM L-glutamine, 1.0% non-essential amino acids, 0.10μM 2-ME, $1.0x10^2$ units of Penicillin, $1.0x10^2$ units of Streptomycin and 15% FBS (Invitrogen Inc.) supplemented with $1.0x10^3$ units/mL of LIF (Millipore Canada Ltd., Etobicoke ON) and incubated at 37°C with 5.0% CO_2. Subsequently, the cells were cultured on 0.10% gelatin coated plates for

two passages in order to eliminate MEF cells. Cell extraction was carried out as described for hESCs.

Mixed lymphocyte reaction (MLR) assays.

Human MLR:

Healthy volunteers' donor blood was collected in heparin-coated tubes (BD Biosciences Inc.) and mixed 1:1 with Ca^{+} and Mg^{+} free PBS. Subsequently, the blood was gently layered on top of Ficoll Paque (BD Biosciences Inc.) and tubes were spun at 400g for 30 minutes at room temperature with the brakes off. PBMCs were isolated from the buffy coat and washed 3 times with PBS. One-way MLR were carried out with 1.0 x 10^5 PBMC responder and stimulator cells in 96 well U bottom plates using RPMI media (10% FBS, 1.0x10^2 units of Penicillin, 1.0x10^2 units of Streptomycin, 2.0mM L-glutamine). The stimulator cells were pre-treated with 50μg/mL of mitomycin C for 40 minutes at 37°C prior to MLR. The cells were allowed to proliferate for 5 days and 1.0μCi tritiated thymidine (^{3}H) (GE-Amersham Canada Inc., Baie D'Urfe Quebec) was added to the culture for an additional 16 to 18 hours. The cells were harvested onto 96 well filters-mats (Wallac Inc., Turku Finland) using a TomTec harvester. Tritium uptake was determined by liquid scintillation using a Wallac 1450 Microbeta Plus liquid scintillation counter (Wallac Inc.). Results are displayed as counts per minute (CPM) of triplicate wells ± SD.

Mouse MLR:

Mouse spleens were removed aseptically and gently homogenized with the frosted ends of two sterile microscope slides and passed through a 45μm mesh filter. The cells were washed twice with PBS and red blood cells were removed by Ficoll centrifugation or ACK red blood cell lysis buffer (Cederlane Laboratories Ltd. Burlington ON). One-way MLR were carried out with 1.0 x 10^5 splenocytes from both responder and stimulator cells in 96 well U bottom plates. Stimulator cells were pre-treated with 50μg/mL of mitomycin C for 40 minutes at 37°C prior to MLR. The cells were allowed to proliferate for 3 days and tritium uptake was determined as described for human MLR. Results are displayed as counts per minute (CPM) of triplicate wells ± SD.

Monocyte to dendritic cell maturation.

Monocytes were isolated from human peripheral blood by CD14 negative selection using magnetic labeling based kits (StemCell Technologies Inc., Vancouver, BC) according to manufacturer protocols. PBMC were suspended at 5.0 x10^7 cells in PBS containing 2.0% FBS and 1.0mM EDTA in a 5ml polystyrene tube. At this point 50μl/ml of enrichment antibody was added to the tube, mouse IgG1 antibodies against CD2, CD3, CD16, CD19, CD20, CD56, CD66b, CD123, glycophorin A, mixed and incubated at 4°C for 10 minutes. Subsequently, the tube received 50μl/ml of magnetic particles, followed by mixing and incubation at 4°C for 5 minutes. The cells were then topped up to 2.5ml with PBS and inserted into the magnet for 2.5 minutes. The cells were transferred to a new tube in one swift motion

to obtain the purified monocytes. The cells were washed twice with PBS before being used for further experiments (Purity was >90% for CD14 marker).

Five hundred thousand purified monocytes were cultured in 24 well plates with 0.50ml of complete RPMI medium containing $5.0x10^2$ units/mL of granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) for 6 days to differentiate monocytes to immature DCs. On day 0, the cells also received either 0.15mg/mL (final concentration) of H1 hESC extract, L-132 fibroblast control extract, or equivalent volume of vehicle. Media was changed on days 2, 4, and 6 by removing 0.20ml of spent media and adding 0.30ml new medium with fresh GM-CSF and IL-4. Along with the media change, the cells received 0.075mg/mL of hESC extract, L-132 extract or vehicle. On day 6, in addition to GM-CSF and IL-4, the cells also received 20ng/ml of tumour necrosis factor-α (TNF-α) to induce maturation.

Phagocytosis assay of human monocyte and dendritic cells.

Monocyte to dendritic cell maturation was carried out as described in the previous section. Cells were harvested on day 6 or day 8 by vigorous pipetting followed by scraping with the pipette tip. The cells were washed twice with PBS and re-suspended in Eppendorf tubes with PBS containing 1.0% FBS. At this point the cells received 1.0mg/mL of dextran-FITC beads (40,000 MW) (Sigma Aldrich, Canada Ltd.) and placed at 37°C or on ice as a control for 90 minutes. At the end of incubation period the cells were washed twice with PBS containing 1.0% FBS and 0.10% sodium azide to inhibit further phagocytosis. Subsequently, the cells were

analyzed with Beckman Coulter FC500 flow cytometer (Beckman Coulter Canada Inc. Mississauga ON).

Allogeneic T Cell proliferation induced by hESC extract-treated DCs.

Purified human T cells were obtained by positive selection using a magnetic labeling kit against CD3 (StemCell Technologies Inc.) according to manufacturer instructions. PBMC were suspended at 1.0 x 10^8 cells/ml in PBS containing 2.0% FBS and 1.0mM EDTA and 1.0 x10^2μl/ml of positive selection coctail was added to the cells. The cells were mixed and incubated at room temperature for 15 minutes. Next, the cells received 50μg/ml of magnetic particles, mixed and incubated at room temperature for 10 minutes (mouse IgG1 against CD3). The cells were transferred to a new tube in one swift motion to remove all CD3 negative cells. At this point, the tube was removed from the magnet and resuspended in PBS. The tube containing the cells were once again inserted into the magnet and the above procedure was repeated to further eliminate any contaminating cells. The cells were washed twice with PBS before being used for further experiments (Purity was >95% for CD3 marker). Subsequently, 1.0 x 10^5 purified $CD3^+$ cells were incubated with hESC extract-treated or fibroblast extract-treated DCs for 3 days. DCs were treated with 50μg/mL of mitomycin C for 40min prior to incubation with T cells. On day 3, 1.0μCi of tritium was added to the culture for an additional 16 to 18 hours and the cells were harvested and tritium uptake was determined as described for human MLR.

Quantitative PCR assays.

RNA was isolated using Qiagen RNeasy Mini Kit (Qiagen Canada Inc. Mississauga ON) according to manufacturer instructions. Subsequently, 500ng of RNA was used to synthesize cDNA using Qiagen QuantiTech Reverse Transcription kit (Qiagen Canda Inc.). RNA was mixed with 2.0μl of gDNA wipeout and topped up to 14μl with RNase free water. The samples were incubated at 42°C for 2 minutes and transferred to ice. At this point, the tubes received 1.0μl of Reverse transcriptase, 4.0μl of Reverse transcriptase buffer and 1.0μl of RT primer mix. The samples were incubated at 42°C for 15 minutes. For QPCR, 2.0μl of cDNA from each tube was used carry out with iQ SYBR Green Supermix (Bio-Rad Laboratories Ltd.) and My iQ-iCycler (Bio-Rad Laboratories Inc.) with an initial hot start for 90 seconds at 94°C followed by 40 cycles set for 10 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C. The primers used were as follows; human TGF-β forward primer 5'GCAACAATTCCTGGCGATACC, reverse 5' AGTTCTTCTCCGTGGCTGA and IL-10 forward primer 5'CACCGGACTCCTTTAACAACAA and reverse primer 5'GAGATGCCTCAGCAGAGTG. Detection of MLR cytokines were performed with mouse IL-2 forward CAGGATGGAGAATTACAGGAACCT, IL-2 reverse 5' TTTCAATTCTGTGGCCTGCTT, IFN-γ forward 5' GAAAATCCTGCAGAGCCAGA, IFN-γ reverse 5' TGAGCTCATTGAATGCTTGG, TGF-β forward 5' GTGCTCGCTTTGTACAACAGC, TGF-β reverse 5' TTACCAAGGTAACGCCAGG, Foxp3 forward 5' CGAAAGTGGCAGAGAGGTATTGA, Foxp3 reverse 5' ACTGTCTTCCAAGTCTCGTCTGAA, Tbet forward and Tbet reverse. Gene

expression levels were normalized to β-actin and fold change was compared to relative gene expression by responder cells alone.

TGF-β neutralization of hESC extract.

Pan anti TGF-β antibody (R&D Systems Inc.) was added to hESC extract at a concentration of 20μg/mL. The extracts were incubated with the antibody for two hours at 4°C. The extracts were subsequently used in one-way MLR and compared to extract treated with isotype antibody.

Flow cytometry analysis of human dendritic cells.

Fluorophore–conjugated antibodies against CD80, CD83, CD86, and HLA-DR (BD Bioscience Inc.) were used. Cells were washed with PBS and incubated in 10% human serum for 15 minutes for the blocking step. Subsequently the cells were stained with the indicated antibodies for 30 minutes on ice and washed twice prior to analysis with Beckman Coulter FC500 flow cytometer (Beckman Coulter Canada Inc.).

Quantitative cytokine detection of human monocytes and dendritic cells.

IL-12p40 ELISA assay:

Supernatants during monocyte to dendritic cell differentiation and maturation were collected on day 6 and day 8 and stored at -20°C. Interleukin-12p40 (IL-12p40) was detected using two separate monoclonal antibodies (mAb) specific for different epitopes. Plates were coated overnight at 4°C with the first antibody (R&D

Systems Inc., Minneapolis MN), 4.0μg/mL, in coating buffer (0.040 M Na_2CO_3, 0.060 M $NaHCO_3$, pH 9.6). The plates were washed 6 times with washing buffer (0.050% Tween 20 in PBS). Blocking was carried out with 10% FCS in PBS for 2 hours at room temperature followed by 6 washes. Samples were diluted 1:5 in RPMI and 100μl was added to each well. Incubation was allowed to proceed for two hours at room temperature and the plates were washed 6 times. The second biotinylated mAb anti-IL-12p40 antibody (Biosource-Invitrogen Canada Inc., Burlington ON) was added at 0.35μg/mL for two hours at room temperature. Once again the plates were washed 6 times and streptavidin peroxidase conjugate (1.0μg/mL in PBS containing 10% FCS, Jackson ImmunoResearch Laboratories Inc., West Grove PA) was added for 30 minutes. The plates were washed 6 times and *o*-phenylenediamine (Sigma-Aldrich) was added for 30 minutes at room temperature in the dark. The reaction was stopped using 1.0N HCl and absorbance was measured at 450nm. Sensitivity of assay was 16 pg/mL. Standard curve was prepared with rIL-12p40 (R&D Systems Inc.).

Quantative FlowCytomix analysis of IL-10 and TGF-β produced by human monocytes and dendritic cells:

Supernatants from monocyte to dendritic cell differentiation and maturation were collected on day 6 and day 8 and stored at -20°C. Subsequently 25μl of supernatant from each indicated treatments was used to detect interleukin-10 (IL-10) or transforming growth factor-beta (TGF-β) using detection kits (Bender Medsystems Inc., San Diego CA) according to manufacturers' instructions. Briefly, samples were mixed with beads coated with anti IL-10 and anti TGF-β as well as

biotin-conjugated to a second antibody against the respective cytokines for 2 hours at room temperature. Subsequently, the samples were extensively washed with a provided buffer. At this point streptavidin-PE was added to the samples for 1 hour at room temperature. Once again the samples were washed extensively. Detection was carried out using Beckman Coulter FC500 flow cytometer. Flowcytomix Pro 2.3 software was used to analyse results. Assay sensitivity was1.9pg/mL for IL-10 and 10pg/mL for TGF-β.

Mouse splenocyte/CD3+ T cells CFSE staining and activation.

Spenocytes were harvested as described above. Purified CD3+ T cells were obtained by negative selection using a magnetic labeling kit (StemCell Technologies Inc.) according to manufacturer instructions. Splenocytes were suspended at 1.0 x 10^8 cells/ml in PBS containing 2.0% FBS and 1mM EDTA and 50μl/ml of enrichment cocktail was added (mouse IgG1 against CD11b, CD19, CD49b, TER119). The cells were mixed and incubated at 4°C for 15 minutes. Next, the cells received 1.0 x 10^2 μl/ml of biotin selection cocktail and incubated at at 4°C for 15 minutes. The cells then received 50μl/ml of magnetic particles, mixed and at 4°C for 15 minutes. The cells were topped up to 2.5ml and inserted into the magnet. Incubation was allowed to proceed for 5 minutes. The cells were transferred to a new tube in one swift motion to obtain the purified CD3+ cells. The cells were washed twice with PBS before being used for further experiments (Purity was 92% for CD3 marker).

Isolated splenocytes/CD3+ T cells were suspended in serum free RPMI media at $1.0x10^6$ cells/mL and stained with 5.0μM of carboxyfluorescein diacetate succinimidyl ester (CFSE) (Simga Aldrich Inc.) or CellTrace Violet 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 media (10% FBS, 2mM L-glutamine, 1.0×10^2U penicillin, 1.0×10^2U streptomycin, 1.0mM Non-essential amino acids, 50μM 2-mercapto-ethanol). Cells were stimulated either with soluble 1.2μg/ml of anti-CD3 and 0.50μg/ml of anti-CD28 (eBioscience Inc., San Diego CA) (unless otherwise stated), or concanavalin A (ConA), or phorbol 12-myristate 13-acetate (PMA) and ionomycin (IONO) in the presence or absence of ESC-derived factors. Proliferation was allowed to proceed for 2-3 days and CFSE dilution was analyzed by Beckman Coulter Cyan flow cytometer.

Examination of T cell activation markers.

T cell activation was examined using CD3, CD4, CD8, CD25, CD44 and CD69 (eBioscience Inc.). CD3+ T cells were stimulated with plate bound anti-CD3 and anti-CD28 (eBioscience Inc.) in the presence or absence of ESC-derived factors and allowed to proliferate for the indicated times. Cells were harvested and washed with PBS. Next, blocking was carried out with 10% rat serum for 15 minutes on ice. At the end of the incubation period, antibodies were added to the cells according to manufacturer recommendation and the cells were incubated for 30 minutes. Cells were analyzed by Beckman Coulter Cyan flow cytometer. Data were

analyzed by gating on CD3 positive cells followed by examination of activation markers CD25, CD44 and CD69 on CD4 and CD8 separately.

Cell death assay of mouse Cells.

Mouse splenocytes were stimulated with 1.2μg/ml of anti-CD3 and 0.50μg/ml of anti-CD28 (eBioscience Inc.) in the presence or absence of ESC-derived factors 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 re-suspended in Annexin-V buffer and stained with 5.0μl of Annexin V-PE for 30 minutes (BD Biosciences Inc. Mississauga, ON). Cells also received 5μl of 7-amino-actinomycin D (7AAD) for the last 10 minutes of incubation. Cell death was determined by using Beckman Coulter Cyan flow cytometer.

Western blot assays.

Two million splenocytes were-pretreated with ESC-derived factors or vehicle control overnight in 0.50ml of RPMI medium in 48 well plates. The next day, cells were stimulated with either 50ng/ml of PMA or 1.2μg/ml of anti-CD3 and 0.50μg/ml of anti-CD28 for the indicated periods. Cells were harvested and lysed immediately in 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]) and an equivalent volume of Laemmli Sample buffer (Bio-Rad Laboratories Ltd.) was added to the samples. Samples were boiled for 5 minutes and ran on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were blocked with 5% powder milk (w/v) or 5% BSA (w/v) in TBS-T for 1 hour. Membranes were probed with rabbit anti-mouse phospho-

PKC-θ (Thre538) and total PKC-θ antibodies at 1:500 (Santa Cruz Biotechnology Inc. Santa Cruz, CA) overnight. Subsequently, membranes were washed 3 times with TBS-T for 15 minutes and were probed with goat anti-rabbit secondary HRP conjugated antibody. Again, membranes were washed 3 times with TBS-T. At this point, the bands were visualized with Amersham ECL Plus western blot detection systems (GE Healthcare Biosciences Corp. Piscataway, NJ). A similar procedure was carried out for pAKT (1:3000), IκB-α (1:500) and pPLC-γ (1:1000) (Cell Signaling Technology Inc. Danvers, MA). Blots were visualized using CHEMI GENIUS^2 Bio Imaging Systems.

Intracellular cytokine and transcription factor staining.

Two million splenocytes were pre-treated with ESC-derived factors or vehicle control overnight in 0.50ml of RPMI media in 48 well plates. The next day, the cells were stimulated either with anti-CD3/CD28 or PMA and ionomycin for 6 hours. After the first 1-2 hours of stimulation cells were also treated with Protein Transport inhibitor cocktail (eBioscience Inc.). At the end of the incubation period cells were harvested and washed twice. Subsequently the cells were incubated with 10% rat serum for 15 minutes and stained for surface markers CD4, CD8 and CD25. Staining was carried out for 30 minutes followed by two washes with PBS. The cells were fixed and permeabilized using the Foxp3 Fixation/Permeabilization Concentrate kit according to manufacturer's instructions (eBioscience Inc.). Following permeabilization, the cells once again received blocking solution for 15 minutes at room temperature. At this point, cells were stained for intracellular IFN-γ and Foxp3 (eBioscience Inc.) for 1 hour at room temperature. The cells were

washed three times with PBS and analyzed by Beckman Coulter Cyan flow cytometer.

Statistical Analysis

Statistical significance was determined using a Student's *t*-test, ANOVA or chi-square wherever appropriate. Results were considered significant when $P < 0.05$.

Chapter 3. The effect of ESC extracts on dendritic cell maturation.

ESC extracts retain the immune modulatory properties of intact ESCs.

The primary objective was to establish whether ESC extracts retain the immune modulatory properties of live cells. In order to test the efficacy of ESC extracts during allo-immune responses, MLR was utilized. MLR assays were chosen as they represent a generalized immune activation assay in response to allo-antigen that encompasses APCs as well as effector cells. In addition, live ESCs derived from humans and mice have been shown to inhibit PBMC or splenocyte proliferation in one-way allogeneic MLR [44, 45]. For MLR assays, human PBMCs were isolated from two healthy donors using Ficoll-Paque gradient centrifugation. Subsequently, one set of cells were treated with mitomycin C to prevent them from proliferating, thereby serving as stimulatory cells. PBMC from the second donor served as responder cells and therefore any proliferation measured in this assay could be attributed exclusively to these cells (Figure 1). MLRs were performed by incubating responders and stimulators in the presence of increasing concentrations of human ESC extract (hESC EXT), volume matched vehicle control, or control human fibroblast cells extract (Control EXT). The addition of soluble hESC extract to the MLR significantly suppressed PBMC proliferation compared to vehicle control ($p = 0.0005$, Figure 2a). Notably, inhibition of proliferation was enhanced with increasing concentrations of hESC extract. In contrast, control fibroblast extract did

Figure 1. One-way mixed lymphocyte reaction assays.

Peripheral blood mononuclear cells were isolated from two healthy volunteer donors using Ficoll gradient centrifugation. One set of PBMCs was inactivated with mitomycin C to be used as stimulator cells. Subsequently, responder and stimulator cells were mixed together 1:1 and either treated with vehicle control, ESC extracts or control extracts. A similar procedure was used for mouse splenocytes isolated from various mouse strains.

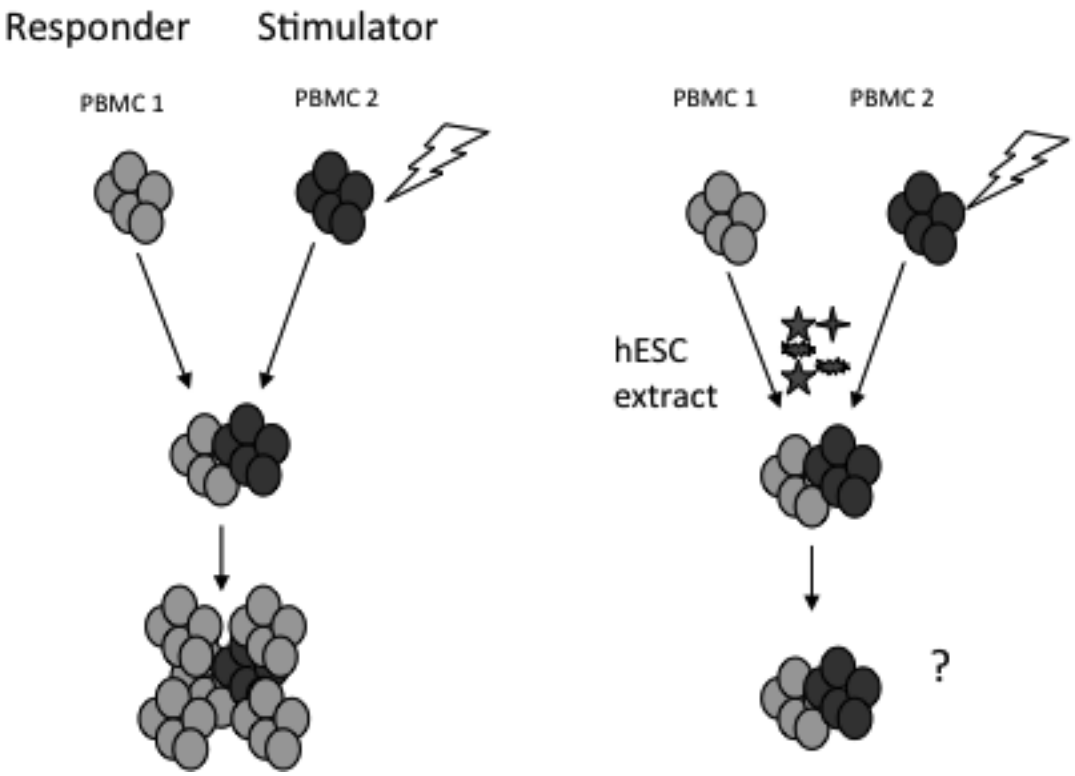
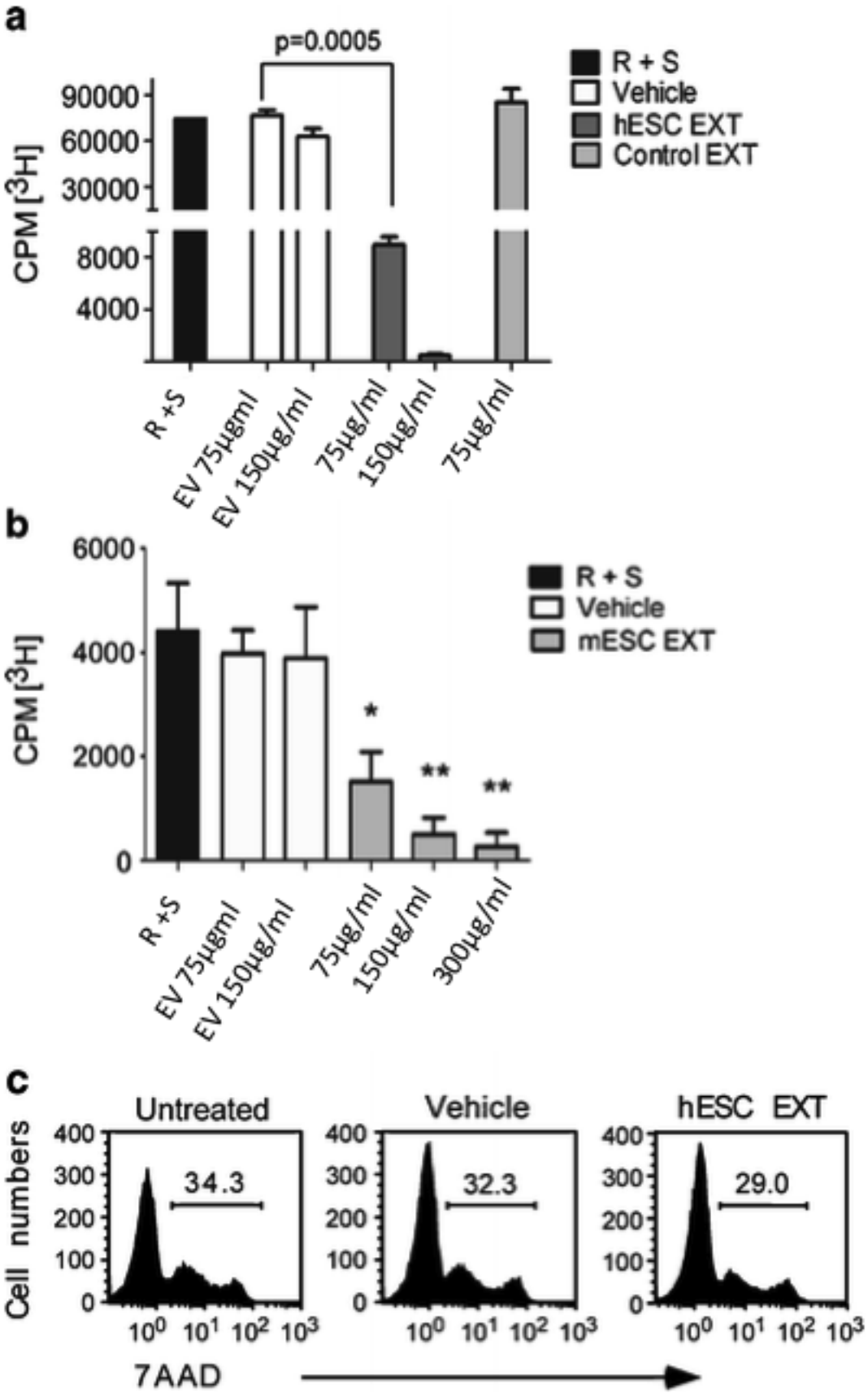

Responder
Stimulator
PBMC 1
PBMC 2
PBMC 1
PBMC 2
hESC
extract
?

Figure 2. ESC-extracts inhibit allogeneic PBMC proliferation in mixed lymphocyte reactions.

A. One way mixed lymphocyte reactions (MLR) were carried out with 1 x 10^5 PMBC obtained from healthy volunteers. One set of donor cells was treated with 50μg/mL of mitomycin C to serve as stimulators while the second set of donor cells were used as responders. MLRs were carried out in the presence of increasing amounts of hESC extracts (EXT) or control fibroblast EXT (Control EXT) or equivalent volume (EV) of vehicle, and compared to untreated cultures (R + S). Tritiated thymidine was added on day 5 and the cells were cultured for an additional 16 to18 hours. Results are displayed as counts per minute (CPM) of triplicate wells ± SD. Results are representative of at least 5 separate experiments.

B. Cellular extracts prepared from B6 murine ESCs were used in one-way MLR. One hundred thousand C57BL/6 splenocytes were cultured together with 1 x 10^5 mitomycin-treated B6C3F1 splenocytes. Tritiated thymidine was added on day 3 of incubation and the cells were allowed to proliferate for an additional 16 to 18 hours. Cell proliferation is displayed as mean counts per minute (CPM) of triplicate wells ± SD. Results are representative of at least 5 separate experiments.

C. The inhibitory effect of hESC-EXT on PBMC proliferation in MLR is not due to cell death. Human PBMCs cultured as in **(A)** were harvested on day 6, washed with PBS, stained with 7AAD for 30 minutes and analyzed by flow cytometry. Results are representative of 3 separate experiments.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p=0.0005
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Vehicle
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Control EXT
CPM [3H]
90000
60000
30000
8000
4000
R +S
EV 75µgml
EV 150µg/ml
75µg/ml
150µg/ml
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b
R + S
Vehicle
mESC EXT
CPM [3H]
6000
4000
2000
0
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R +S
EV 75µgml
EV 150µg/ml
75µg/ml
150µg/ml
300µg/ml
c
Untreated
Vehicle
hESC EXT
Cell numbers
400
300
200
100
0
34.3
32.3
29.0
10^0 10^1 10^2 10^3
7AAD

not have an inhibitory effect on MLR and even induced proliferation of PBMC (Figure 2a). In addition to fibroblast extracts, Jurkat T cell extracts and primary PBMC extracts were tested for inhibitory properties on MLR proliferation. Both Jurkat and PBMC extracts were unable to inhibit MLR proliferation (Figure 3a). These results were reproduced using murine ESC extracts. One-way allogeneic MLR were carried out with B6 splenocytes (responders) and B6C3F1 splenocytes (stimulators). Similar to hESC extracts, mouse ESC extracts (mESC EXT) were able to prevent proliferation of splenocytes in a dose dependent manner compared to vehicle controls ($p = 0.01$, Figure 2b). Once again control extracts did not have an inhibitory effect on MLR proliferation of responders cells but rather caused enhanced proliferation (Figure 3b). Hence, these findings indicate that cellular extracts from both human and mouse ESCs retain the immune modulatory properties of live cells. Importantly, the above results were reproducible using different hESC lines (H1, H9, and CA1) and different mESC lines (J1, D3 and B6) as well as a combinations of different PBMCs prepared from various healthy donors and mouse strains. These results suggest that the immune modulatory properties of ESC extracts are consistent across different species.

The strong inhibitory effect of ESC extracts observed on MLR proliferation raised the immediate question on whether this effect was due to cell death. I performed cell death assays and found that the inhibitory effect was not due to cell death since PBMCs treated with hESC extracts on day 6 of one-way MLR showed a similar amount of dead cells compared to those treated with vehicle control (Figure 2c).

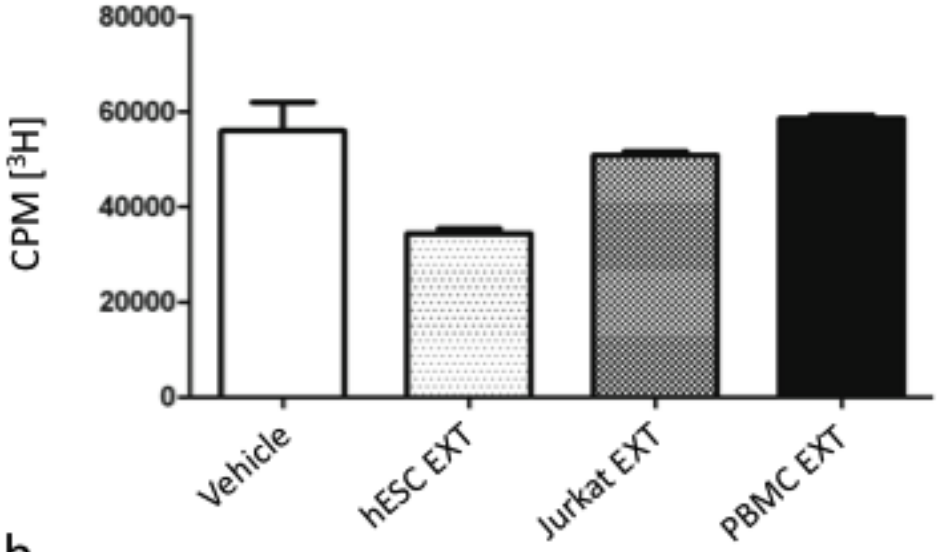
Figure 3. Control extracts do not have an inhibitory effect on allogeneic PBMC proliferation in MLR.

MLR were performed to determine whether extracts derived from non-embryonic cell types have the capacity to inhibit allo-immune activation.

A. Extracts derived from Jurkat cells, a human T cell line, and primary PBMCs obtained from a healthy individual were tested for inhibitory effects on MLR proliferation and compared to human ESC-extracts. All extracts were used at a concentration of 75μg/ml. MLR were carried out as described in Figure 2. Tritiated thymidine was added on day 5 and the cells were cultured for an additional 16 to18 hours. Cell proliferation is displayed as counts per minute (CPM) of triplicate wells ± SD. Results are representative of at least 3 different experiments.

B. Extracts were derived from C2C12, a mouse muscle stem cell pre-cursor, and examined for its ability to inhibit mouse MLR proliferation. MLR was carried out as described in Figure 2. Tritiated thymidine was added on day 3 of incubation and the cells were allowed to proliferate for an additional 16 to 18 hours. Cell proliferation is displayed as mean counts per minute (CPM) of triplicate wells ± SD. Results are representative of at least 4 separate experiments

a


80000
60000
40000
20000
0
CPM [3H]
Vehicle
hESC EXT
Jurkat EXT
PBMC EXT

b

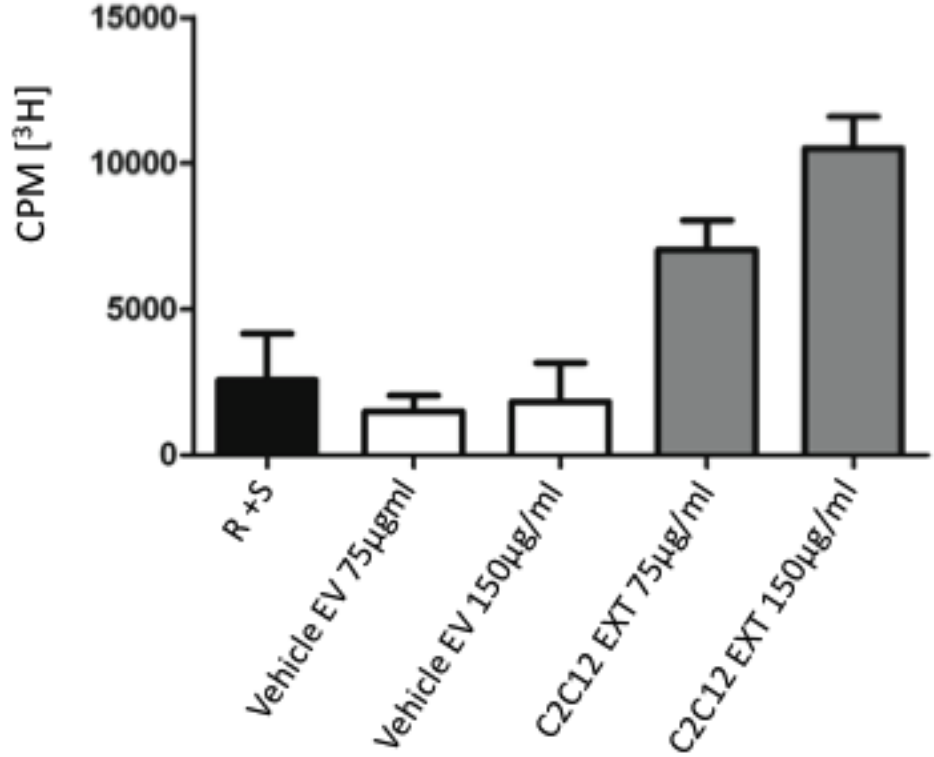

15000
10000
5000
0
CPM [3H]
R +S
Vehicle EV 75µgml
Vehicle EV 150µg/ml
C2C12 EXT 75µg/ml
C2C12 EXT 150µg/ml

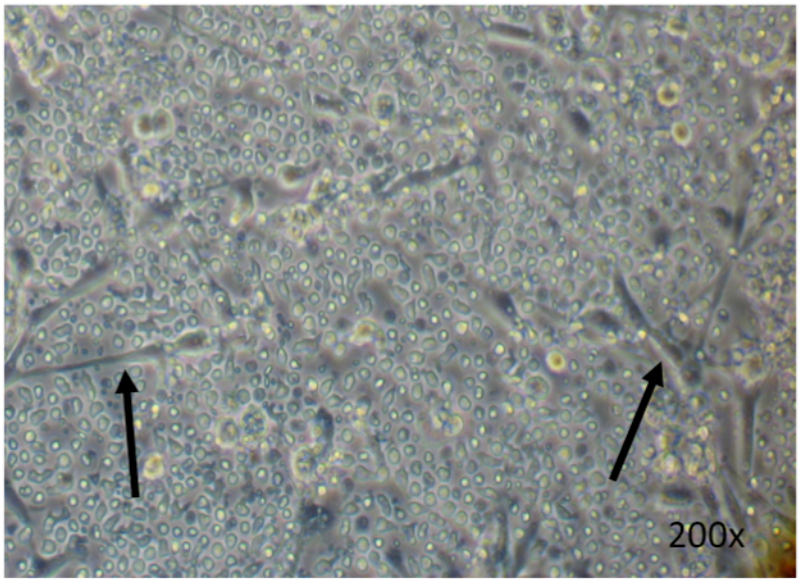
Figure 4. Treatment of MLR with hESC extracts induced changes in morphology.

A. Vehicle treated MLR. Arrows indicated elongated fibroblast cells types that represent dendritic cells. These cells were observed at a high frequency in the vehicle treated cultures.

B. hESC treated MLR. Results shows a notably decreased number of cells with an elongated phenotype when compared to vehicle control.

MLR were performed as described in Figure 1 and 2 above with 2.0×10^6 in 24 well plates. Images were taken on day 6 of MLR proliferation (Zeiss Invertoskop 40C magnification 200x).

a



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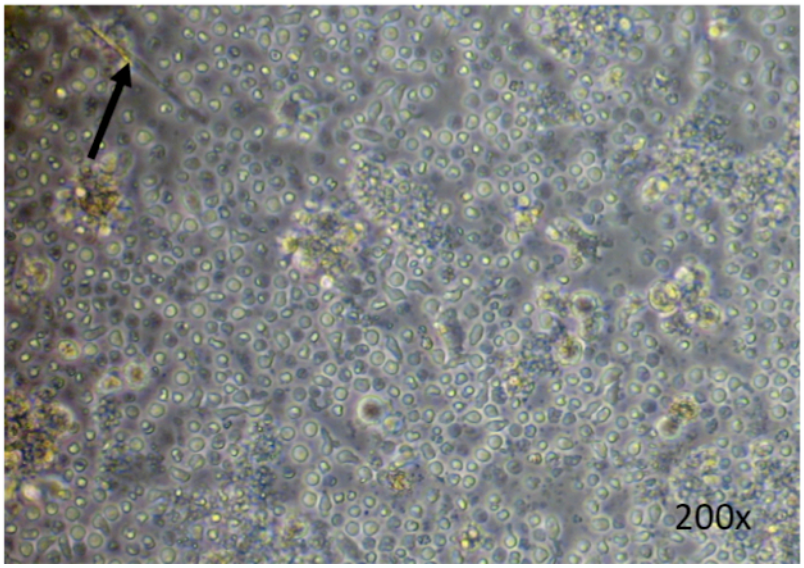


Interestingly, while carrying out these initial studies I observed slight differences in cell morphology in the wells treated with ESC extract compared to vehicle or untreated wells throughout the assay period. Specifically, cells with morphology resembling DC after the first few days were observed in untreated and vehicle treated wells while these cells were absent from the ESC extract treated wells (Figure 4a,b). It was reasoned that these cells were dendritic/monocyte cells that had undergone maturation in the untreated and vehicle treated wells whereas they were negatively affected in the ESC treated wells. As a result, I examined the impact of ESC extracts directly on DC maturation.

Determining the effect of hESC extracts on monocyte-derived dendritic cells maturation and function.

Dendritic cells are professional antigen presenting cells that play an important role in activating the adaptive immune system. The maturation state of a DC at the time of antigen presentation can have an enormous impact on whether naive T cells become either activated or undergo anergy [140, 141, 154]. In an immature state, DCs are in an antigen-processing mode and express low levels of MHC II and co-stimulatory molecules CD80/CD86 [138, 155]. As a result, immature DCs are relatively poor antigen presenters and do not provide proper costimulation to T cells [156-159]. This interaction of immature DCs and naïve T cells has been shown to result in anergy and apoptosis [140, 141, 154]. Upon receiving activating signals, DCs undergo a maturation process and become potent APCs [138]. At this mature stage, both antigen-presenting molecules and co-stimulatory molecules are up-regulated and as a result the cells effectively activate naïve T cells [140, 141, 154]. Hence, DCs can play a

pivotal role in inducing a strong immune response or tolerance towards a pathogen depending on their maturation state. As discussed in the introduction, antigen presentation by donor and recipient DCs represents a critical factor in transplant rejection [140, 141, 148]. Strategies aimed at preventing DCs maturation following transplantation have been shown to prolong graft survival. In order to examine the impact of ESC extracts on DCs, experiments were designed to assess the cells activation state, function and ability to activate allogeneic T cells.

Immature DC represent only a small fraction of cells in peripheral blood and are sparsely distributed in various organs of the body serving as sentinels for infection [160]. As a result, harvesting sufficient numbers of immature primary cells directly from individual donors is not easily attainable. An established method to study DC maturation involves isolation of monocytes from PBMC and subsequent differentiation of the cells to immature DC using GM-CSFand IL-4 [161, 162]. Monocytes were isolated from the peripheral blood of healthy volunteer donors by CD14 negative selection in an effort to avoid activation of the cells. Subsequently, the cells were cultured with GM-CSF and IL-4 for 8 days to induce differentiation of the cells to mature DCs. Media was changed on days 2 and 4 by removing spent media and adding fresh cytokines. On day 6, in addition to receiving IL-4 and GM-CSF, the cells also received TNF-α in order to induce maturation (Figure 5). Simultaneously, monocytes were treated with either vehicle control, hESC extracts or fibroblast extracts on days 0, 2, 4, and 6 (Figure 5). On day 8, the cells were examined for maturation markers CD80, CD86, CD83 and HLA-DR. Microcospic examination of the cells throughout the culture period revealed that vehicle treated monocytes

Figure 5. Schematic diagram of monocyte to dendritic cell differentiation.

Half a million monocytes were cultured in the presence of 500U/mL of GM-CSF and IL-4 (G4) in order to induce them to differentiate into dendritic cells. The cells also received either 0.15mg/mL (final concentration) of hESC extracts (hESC EXT), L-132 fibroblast extracts (Control EXT), or equivalent volume of vehicle on day 0. Fresh media were added every 2 days containing fresh cytokines and 0.075mg/mL of hESC EXT, L-132 EXT or vehicle on day 2, 4, and 6. On day 6, the cells also received 20ng/mL of TNF-α in order to induce dendritic cell maturation.

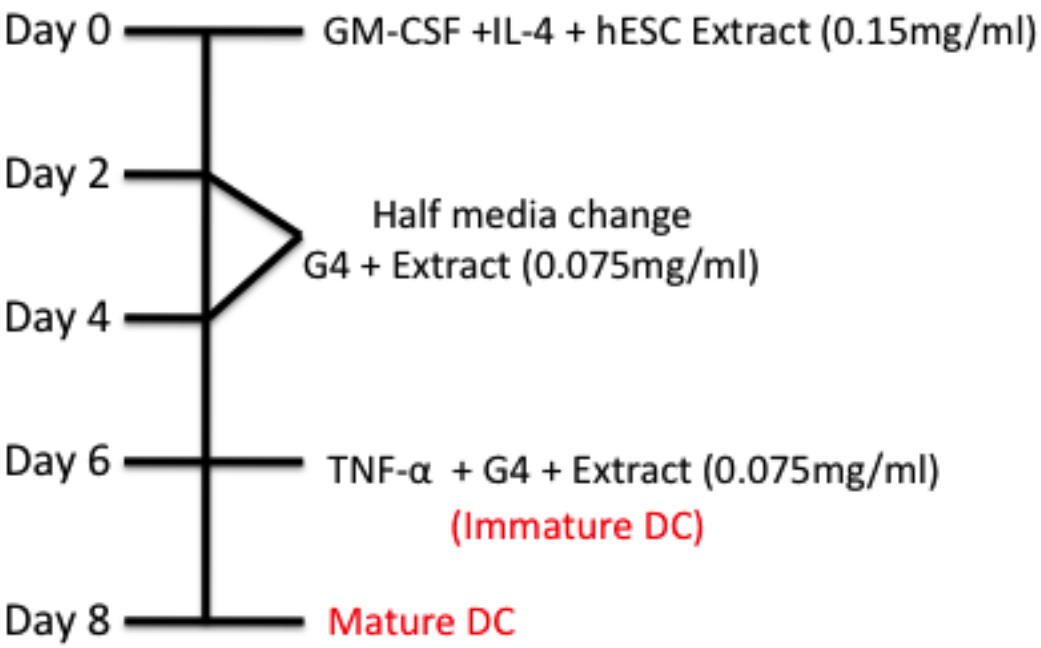

Day 0
GM-CSF +IL-4 + hESC Extract (0.15mg/ml)
Day 2
Half media change
G4 + Extract (0.075mg/ml)
Day 4
Day 6
TNF-α + G4 + Extract (0.075mg/ml)
(Immature DC)
Day 8
Mature DC

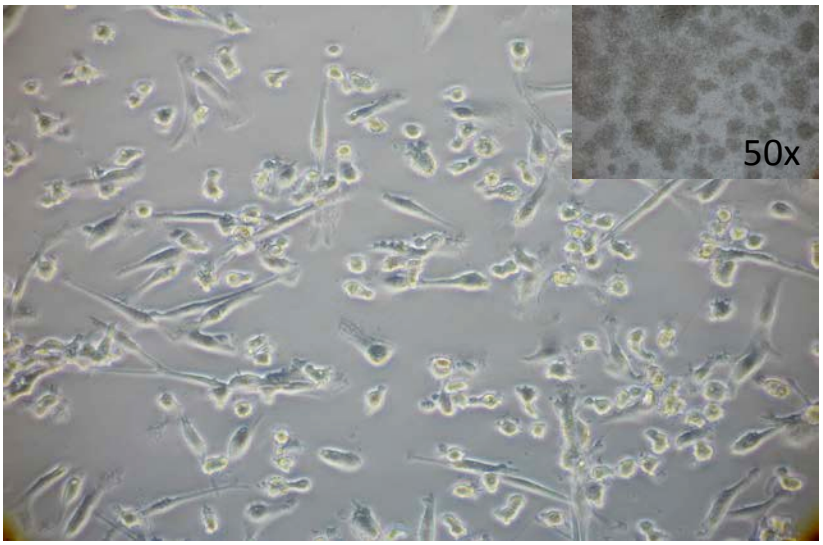
Figure 6. hESC treatment of monocytes inhibits morphological transition to a dendritic cell phenotype.

A. Monocytes were treated with vehicle control throughout culture conditions. Cells transitioned from a rounded phenotype to more elongated cells with some having discernable dendrites.

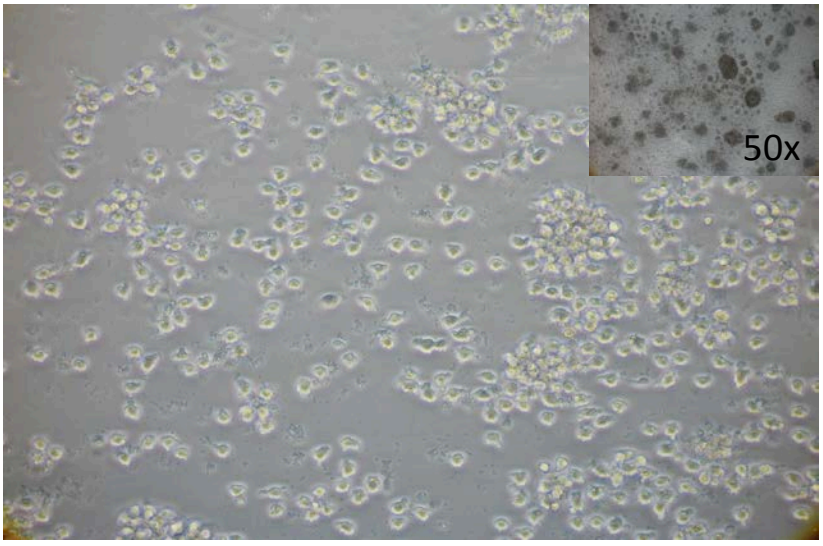
B. Monocytes were treated with hESC extracts throughout culture conditions. Cells were found to remain in a more rounded phenotype with very few taken on an elongated shape.

Images were taken on day 7 of culture following TNF-α additions to the cultures. Main images were taken at 200x magnification and inset with 50x magnification. Results are representative of at least 4 different experiments (Zeiss Invertoskop 40C).

a



b



developed into elongated fibroblast like cells while hESC extracts treated cells retained a more rounded shape as observed in MLR assays (Figure 6a,b). Examination of the cells by flow cytometry showed that treatment with vehicle and fibroblast-extracts resulted in the up-regulation of DC maturation markers CD80, HLA-DR, CD83, and CD86 (Figure 7a-d). In contrast, DC treated with hESC extracts did not up-regulate these maturation markers to the same extent as controls (Figure 7a-c). However, we found hESC extracts did not inhibit the surface expression of CD86, and in some cases even slightly increased CD86 expression (Figure 7d). Interestingly, these findings were found to be similar to the results obtained with mesenchymal stromal cells (MSC). MSC have been shown to inhibit DC maturation by preventing upregulation of CD80, CD83 and HLA-DR. However, MSCs were also unable to prevent up-regulation of CD86 on DCs [163]. Collectively, these results indicate that hESC-extract treated DCs remain in an immature state.

hESC extract-treated DCs Retain Phagocytic Function after TNF-α Treatment.

The ability to take up and process antigen presents an important function of immature dendritic cells. Tissue resident immature dendritic cells are highly phagocytic and process a large amount of antigen [139]. Upon maturation, their phagocytic function is down regulated and the cells become potent antigen presenters [123, 142]. In an attempt to decipher the effect of hESC extracts on DC function, we examined phagocytosis on both day 6 and 8, representing immature and mature states of DC respectively. DCs treated with vehicle, hESC extracts and control extracts and incubated with FITC labelled dextran beads on day 6 all

Figure 7. hESC-extracts inhibit monocyte-derived dendritic cell maturation.

Moncytes were cultured as described in Figure 5 and examined by flow cytometry for the surface expression of DC maturation markers.

A. CD80

B. HLA-DR

C. CD83

D. CD86

Grey lines represent controls and filled histograms represent treated mDCs. Values within plots indicate mean fluorescent intensity. Results are representative of at least 3 separate experiments.

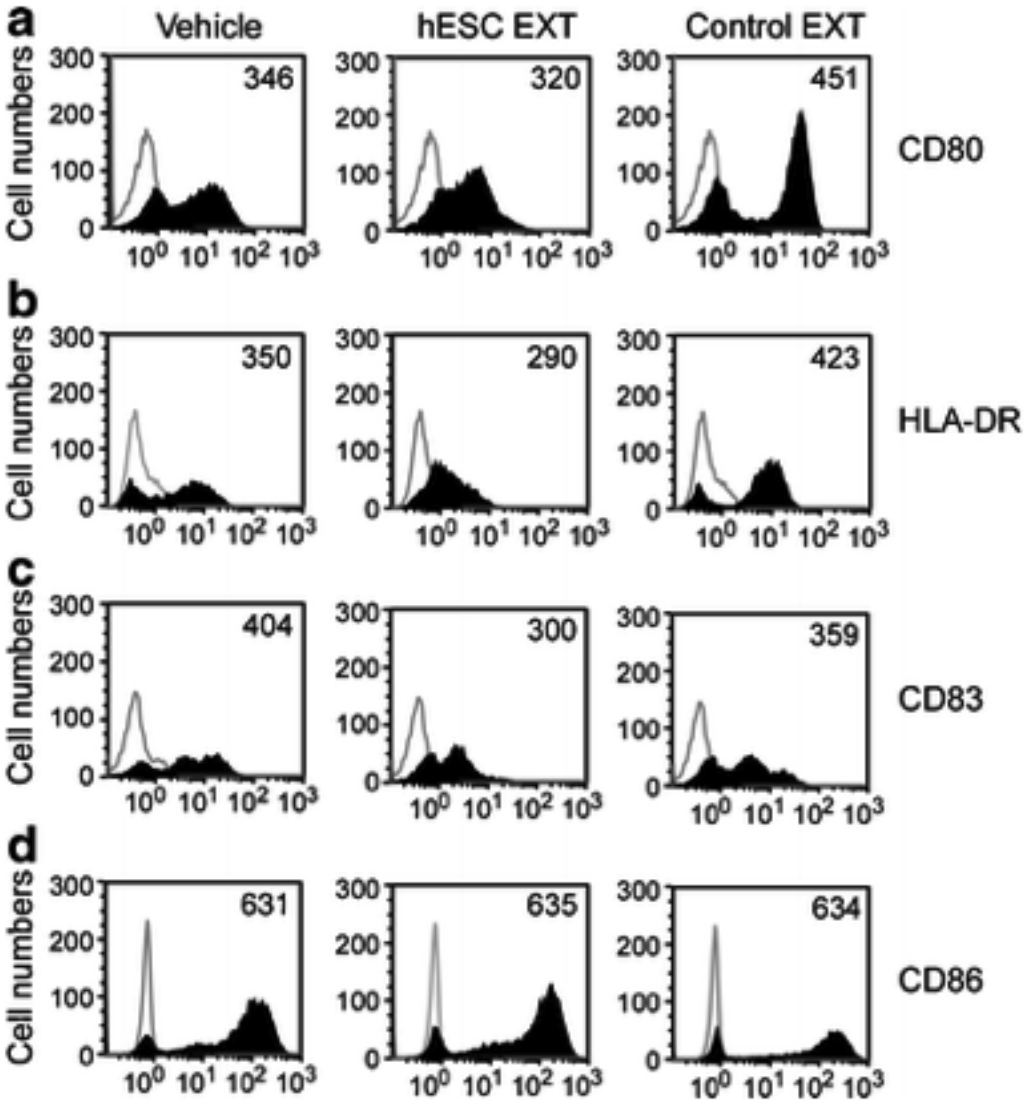

a
Vehicle
hESC EXT
Control EXT
Cell numbers
346
320
451
CD80
b
350
290
423
HLA-DR
c
404
300
359
CD83
d
631
635
634
CD86
0
100
200
300
10^0 10^1 10^2 10^3

Figure 8. hESC extract-treated mDCs retain greater phagocytic function following maturation.

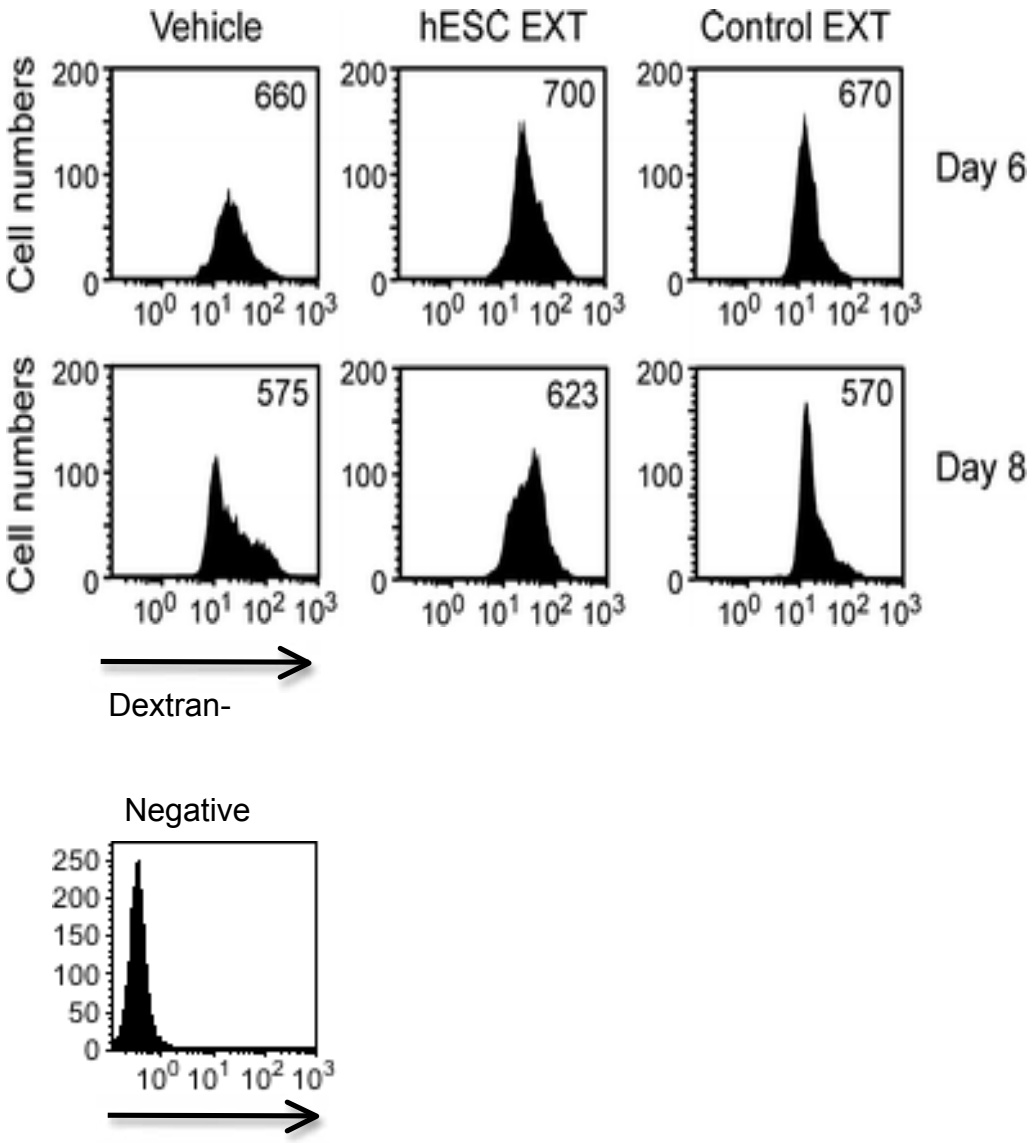
Immature and TNF-α matured mDCs were cultured and collected on

A. Day 6

B. Day 8

The cells were washed twice with PBS containing 1% FBS and incubated with 1mg/mL of dextran-FITC beads at 37°C or on ice for 90 minutes. Subsequently, the cells were washed twice with PBS containing 1% FBS and 0.1% sodium azide and analyzed by flow cytometry. Values within plots indicate mean fluorescent intensity. Results are representative of at least 3 separate experiments.

Figure re-printed with permission from Humana Press.


Vehicle
hESC EXT
Control EXT
Cell numbers
660
700
670
Day 6
575
623
570
Day 8
Dextran-
Negative

exhibited high phagocytic function, indicative of immature DCs (Figure 8). However, analysis of phagocytosis on day 8, TNF-α treatment for 2 days, revealed a much greater phagocytic function for DCs treated with hESC extracts compared to controls (Figure 8). These data further corroborate the finding in Figures 6 and 7 and suggest that hESC extracts cause DC to remain in an immature state even after induction of maturation with TNF-α.

hESC extract-treated DCs Secrete Lower Levels of IL-12p40.

In order to further substantiate the above findings cytokine production by DCs was investigated. Cytokine production by DCs has an important impact on the type of immune response elicited. For instance DC production of IL-12 induces a Th1 response whereas the production of IL-10 leads to a Th2 response and suppresses a Th1 response [164, 165]. Similarly, production of TGF-β in combination with IL-6 results in a Th17 response while TGF-β alone can lead to T regulatory cell development [166, 167]. To begin with, secretion of IL-12p40, a monomer known to make functional IL-12 and IL-23, was examined [168]. As such, supernatants were collected on day 6 and day 8 of monocyte culture and the level of IL-12p40 was determined by quantitative ELISA assays. On day 6 of culture, very little or no IL-12p40 was detected in any of the supernatants (Figure 9a). However, upon maturation with TNF-α (day 8 supernatants), monocyte treated with either vehicle or control extracts throughout the culture period were found to secrete high levels of IL-12p40. In contrast, supernatants from monocyte cultures treated with hESC

Figure 9. hESC extract-treated mDCs secrete lower levels of IL-12p40 following maturation.

Supernatants from mDCs treated with vehicle, hESC EXT, and fibroblast EXT (as described in Figure 5) were collected on day 6 and day 8.

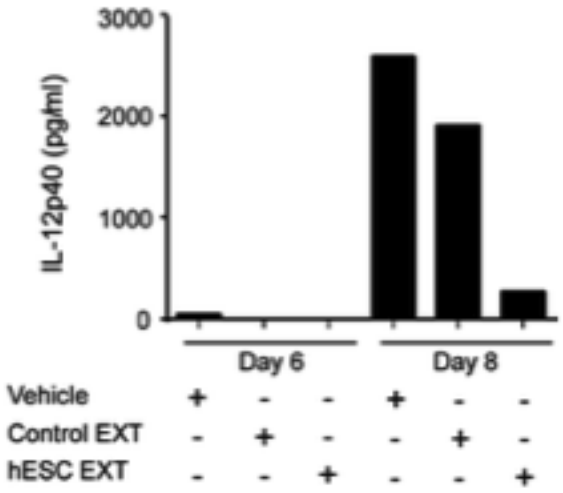
A. IL-12p40 levels were measured by ELISA assays.

B. TGF-β levels were determined by Flow Cytomix assays.

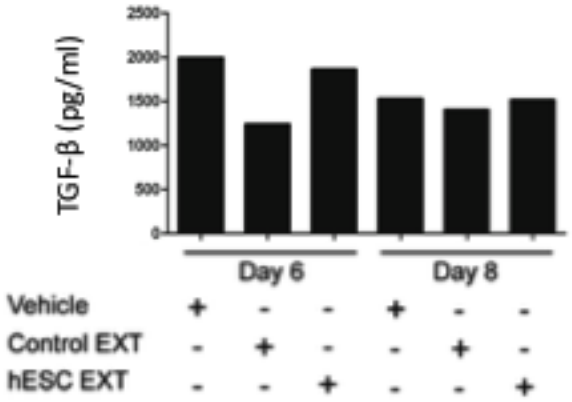
C. IL-10 levels were determined by Flow Cytomix assays.

Results are representative of 3 separate experiments

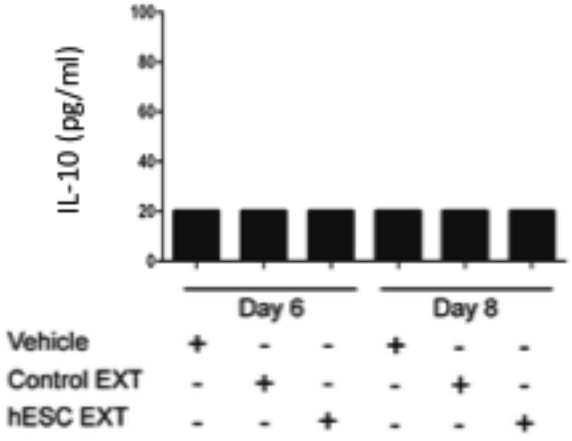
a


IL-12p40 (pg/ml)
3000
2000
1000
0
Day 6
Day 8
Vehicle + - - + - -
Control EXT - + - - + -
hESC EXT - - + - - +

b


TGF-β (pg/ml)
2500
2000
1500
1000
500
0
Day 6
Day 8
Vehicle + - - + - -
Control EXT - + - - + -
hESC EXT - - + - - +

c


IL-10 (pg/ml)
100
80
60
40
20
0
Day 6
Day 8
Vehicle + - - + - -
Control EXT - + - - + -
hESC EXT - - + - - +

extracts contained 8 to 10-fold less IL-12p40 (Figure 9a). On the other hand, no major differences were found when TGF-β and IL-10 were examined by quantative flow cytomix assays (Figure 9b,c). The results indicated that hESC extracts causes a decrease in IL-12p40 secretion by DCs.

IL-10 and TGF-β Do Not Contribute to hESC extract-mediated Immune Modulation.

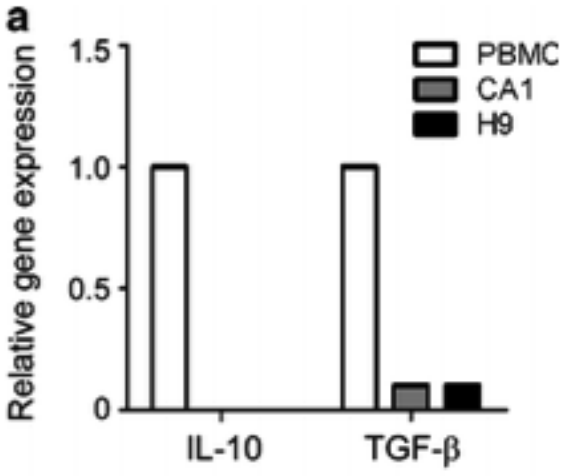
To ensure that hESC extracts do not contain immune modulating cytokines several publicly available microarray databases were analyzed. These analyses revealed an absence of expression of most cytokines. Moreover, we determined the expression of IL-10 and TGF-β by sensitive QPCR assays because these two cytokines are well known for their inhibitory effects on immune activation [169, 170]. Examination of hESC lines CA1 and H9 by QPCR revealed a complete absence of IL-10 gene expression and only minor expression of TGF-β mRNA (Figure 10a). To completely rule out the role of TGF-β in hESC extract-mediated immune modulation, hESC extracts were treated with 20μg/mL of pan TGF-β neutralizing antibodies. Subsequently, MLR was carried out using TGF-β neutralized and non-neutralized hESC extracts. The results indicated that neutralizing TGF-β antibodies did not have an impact on the hESC extract-mediated MLR inhibition (Figure 10b). Therefore, hESC extract-mediated immune modulation of MLR and DC maturation cannot be attributed to IL-10 and TGF-β and possibly involve other known and novel factors. Our results corroborate previous findings observed by Drukker et al. who

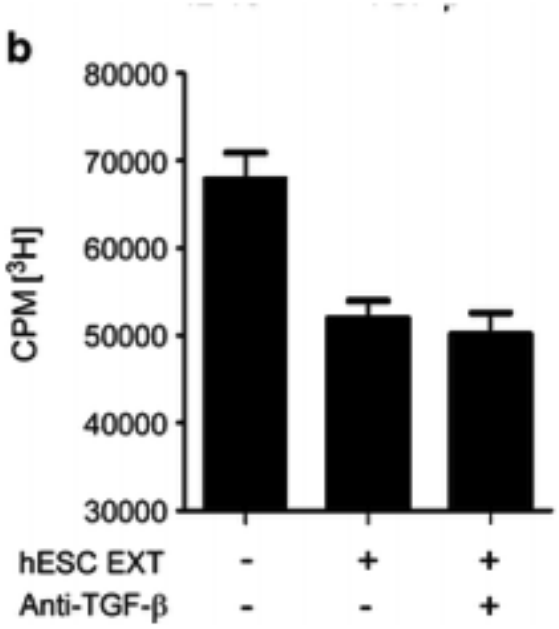
Figure 10. IL-10 and TGF-β do not contribute to hESC extract-mediated immune modulation.

A. Relative mRNA expression of IL-10 and TGF-β by hESCs as measured by QPCR.

B. One-way allogeneic MLRs were carried out in the presence of hESC-EXT that have been treated with isotype control antibody or with a neutralizing antibody against TGF-β. Proliferation was allowed to proceed for 5 days and tritiated thymidine was added for an additional 16 to 18 hours. Cell proliferation is displayed as mean counts per minute (CPM) of triplicate wells ± SD.

Results are representative of 3 separate experiments.


a
Relative gene expression
1.5
1.0
0.5
0
PBMC
CA1
H9
IL-10
TGF-β


b
CPM [3H]
80000
70000
60000
50000
40000
30000
hESC EXT - + +
Anti-TGF-β - - +

studied over 300 immune related genes using microarrays and found negligible expression of IL-10 and TGF-β genes in hESCs.

hESC extract-treated DCs Are Poor Stimulators of Allogeneic T Cells.

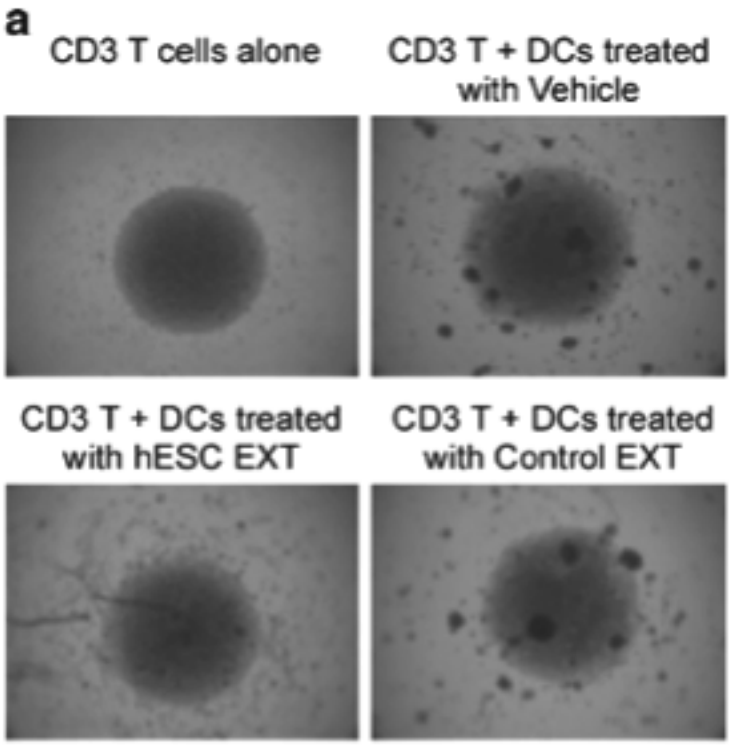
The maturation state of DCs at the time of antigen presentation has an enormous impact on T cell activation. In an immature state, DCs remain in an antigen-processing mode and express low levels of HLA-DR and co-stimulatory molecules. As a result, they are relatively poor T cell activators [140, 141, 154,156-159]. The above data indicates that hESC derived extracts may have an impact on DC maturation. As a result we tested their ability to activate allogeneic T cells. To determine whether hESC extracts-treated DCs influence effective T cell activation, TNF-α matured DCs were incubated with purified allogeneic T cells. Microscopic inspection of wells of the different treatment groups revealed development of clusters in wells containing vehicle and control extract treated DCs after one day of stimulation, indicative of T cell proliferation (Figure 11a). However, no cluster formation was observed in wells which received hESC extract treated DCs that were found to be similar to unstimulated T cells (Figure 11a). Expectedly, quantitation of T cell proliferation incubated with DCs treated with either vehicle or fibroblast-extracts showed a strong response to the allogeneic DCs (Figure 11b). In contrast, T cells incubated with hESC extract-treated DCs proliferated significanlty less when compared to controls ($p = 0.007$, Figure 10b). Moreover, addition of exogenous IL-12p40 to hESC treated DC did not restore the ability of hESC extract treated DCs to stimulate T cells to the

Figure 11. hESC extract-treated mDCs are poor stimulators of allogeneic T cells. Monocytes incubated with either vehicle, hESC EXT, or fibroblast EXT and matured with TNF-α were treated with mitomycin C and cultured with 1 x 10^5 purified CD3+ allogeneic T cells at a ratio of 1:50.

A. Images depict indicated treatments 24 hours after incubation (50x, Zeiss Invertoskop 40C).

B. Proliferation of CD3+ T cells in response to allogeneic mDC stimulation. Proliferation was allowed to proceed for 3 days and tritiated thymidine was added for an additional 16 to 18 hours. Cell proliferation is displayed as mean counts per minute (CPM) of triplicate wells ± SD.

Results are representative of 3 separate experiments.


a
CD3 T cells alone
CD3 T + DCs treated with Vehicle
CD3 T + DCs treated with hESC EXT
CD3 T + DCs treated with Control EXT

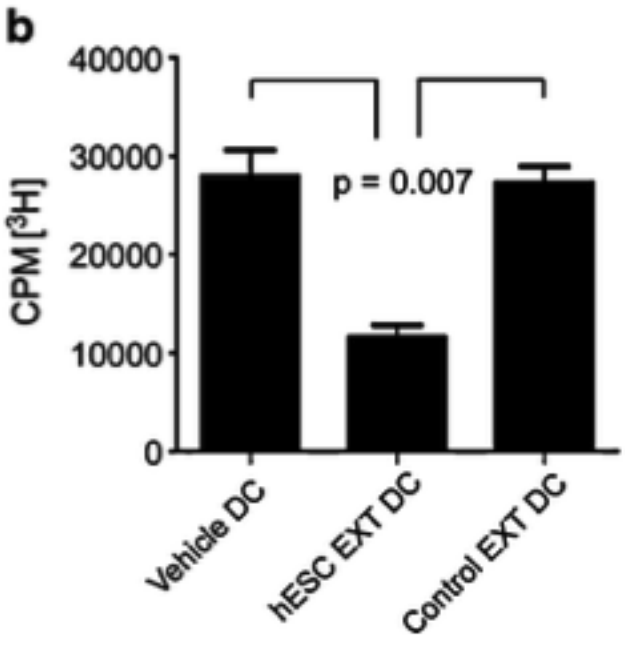
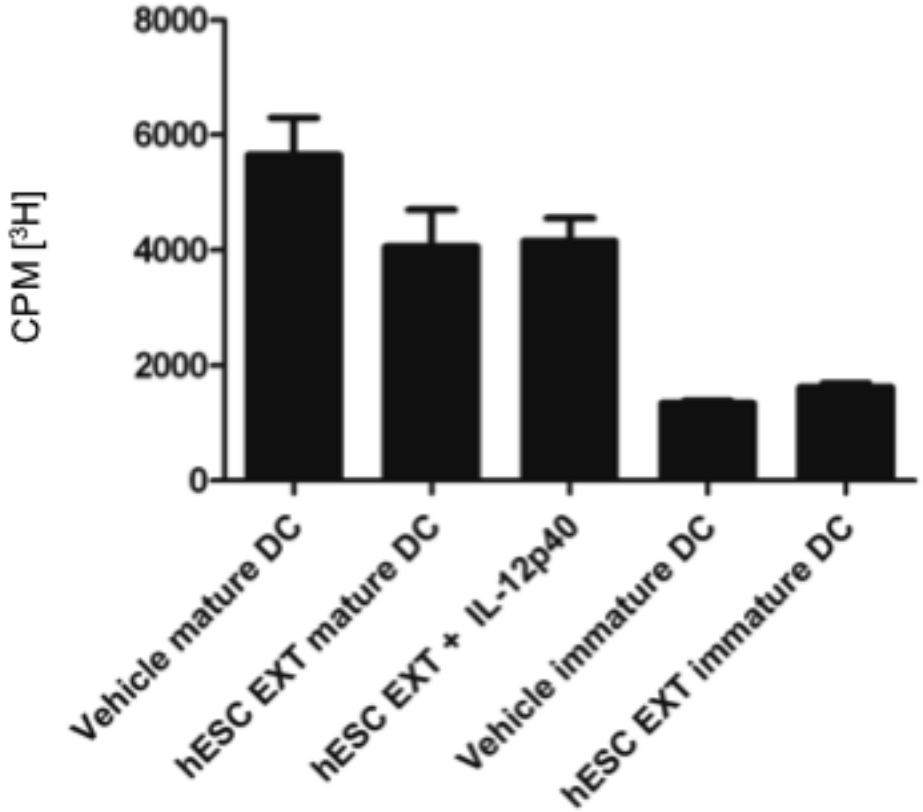

b
40000
30000
20000
10000
0
CPM [3H]
p = 0.007
Vehicle DC
hESC EXT DC
Control EXT DC

Figure 12. hESC extract-treated mDCs are poor stimulators of allogeneic T cells.

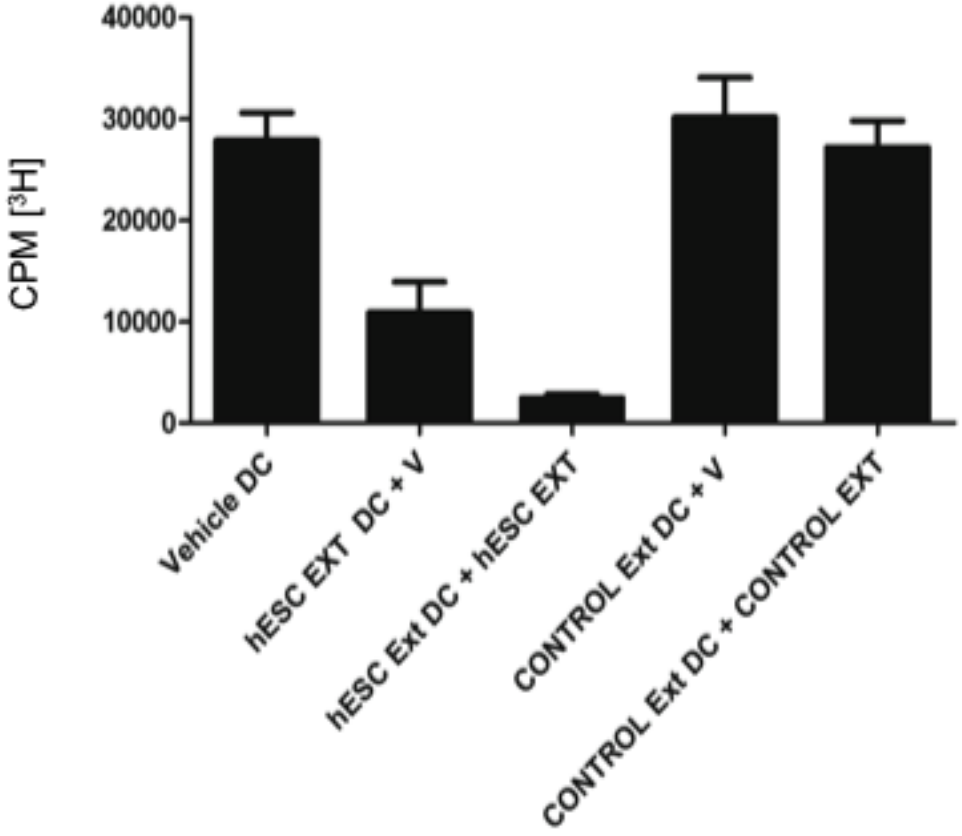
mDCs incubated with either vehicle, hESC EXT, hESC EXT + IL-12p40 and were induced to undergo maturation to compare their ability to stimulate allogeneic T cells. Alternatively, vehicle and hESC EXT were also examined at the immature stage for their ability to induce allogeneic T cell proliferation. mDCs were treated with mitomycin C and cultured with 1 x 10^5 purified CD3+ allogeneic T cells at a ratio of 1:50. Proliferation was allowed to proceed for 3 days and tritiated thymidine was added for an additional 16 to 18 hours. Cell proliferation is displayed as mean counts per minute (CPM) of triplicate wells ± SD.


8000
6000
4000
2000
0
CPM [3H]
Vehicle mature DC
hESC EXT mature DC
hESC EXT + IL-12p40
Vehicle immature DC
hESC EXT immature DC

same extent as vehicle treated DC (Figure 12). Interestingly, immature DCs, obtained on day 6 of the maturation process, treated with vehicle or hESC extracts induced T cell proliferation to the same extent as controls (Figure 12). Hence, hESC extracts impair DC maturation and render them as poor stimulators of allogeneic T cells. It may also be concluded that hESC extracts may impact DCs at the point when maturation signals are provided. This is reflected by the fact that difference in allogeneic T cell stimulation between hESC extract treated and control DCs was only observed when mature DCs were used (Figure 11 and 12). In our attempts to determine the ability of DCs to stimulate allogeneic T cells we also observed that additional extracts added to the cultures may have a direct impact on T cell proliferation. When matured hESC extracts treated DCs were further supplemented with additional hESC extract during T cell allo-stimulation, we observed an enhancement in the inhibition of allogeneic T cell proliferation (Figure 13). In contrast, additional control fibroblast extract added to fibroblast extract treated DCs did not have any effect on T cell proliferation (Figure 13). Moreover, vehicle added to hESC extract treated or control fibroblast treated DCs did not have an impact on T cell proliferation (Figure 13). As a result, we reasoned that hESC extracts might also independently inhibit T cell proliferation. Therefore I examined the impact of ESC extracts on T cell activation and function in Chapter 4.

Figure 13. Further addition of hESC extract to hESC EXT treated mDCs during allogeneic stimulation enhances inhibition of T cell proliferation. Monocytes incubated with either vehicle, hESC EXT, or fibroblast EXT throughout monocyte to dendritic cells and matured with TNF-α. The cells were washed with PBS and were treated with mitomycin C and cultured with 1 x 10^5 purified CD3+ allogeneic T cells at a ratio of 1:50. At this time additional vehicle, hESC EXT or control were added to each respective mDC cultures.

Results are representative of 3 separate experiments.


40000
30000
20000
10000
0
CPM [3H]
Vehicle DC
hESC EXT DC + V
hESC Ext DC + hESC EXT
CONTROL Ext DC + V
CONTROL Ext DC + CONTROL EXT

Chapter 4. The impact of ESC extracts on T cell activation and effector function.

T cells represent an important component of the effector arm of the adaptive immune system as they play a crucial role in clearing infection and eliminating cancerous cells. T cells also play a major role in organ rejection and development of graft versus host disease (GVHD). Several subsets of T helper cells (Th) cells have been identified based on the cytokine profile of CD4 T cells. Th1 cells are identified as CD4 T cells that elicit a cytotoxic response through induction of IFN-γ and are involved in clearance of viral infections [171]. Th2 cells are CD4 T cells that enhance a humoral response over a Th1 cytotoxic response by producing IL-4, IL-10, IL-5 and IL-13 [171]. Th17 cells are also CD4 cells shown to secrete IL-17 that has been shown to contribute to clearance of extracellular pathogens and has been implicated in auto-immunity [172, 173]. On the other hand, T regulatory (Treg) cells, which arise naturally and can be peripherally induced by TGF-β, are identified by intracellular expression of transcription factor Foxp3 and high CD25 expression [167, 174]. Treg cells have the capacity to suppress the other effector subtypes and can contribute to a state of immunological tolerance [174]. This section examines the effect of ESC extracts on T cell activation and function.

It should be noted that at this point most of the work examining T cells was done with murine ESC and splenocytes. In consultation with my supervisor we reasoned that this was the best course since murine ESC can be expanded to

larger cultures more rapidly and easily than hESC. Murine ESC are also being more cost effective than hESC which require expensive serum, media as well as conditioning of the media by MEF cells. Moreover, using mouse splenocytes from genetically defined strains would give more consistent results than PBMC collected from various individuals.

ESC extracts inhibit T cell proliferation in response to anti-CD3/anti-CD28 and Concanavalin A.

In order to specifically determine the impact of ESC extracts on T cells, mouse B6 splenocytes were stimulated with anti-CD3 and anti-CD28 in the presence of increasing concentrations of mESC extracts. Similar to the results observed in the MLR assays, mESC extracts prevented T cell proliferation in a dose dependent manner. Conversely, vehicle control did not affect T cell proliferation, while mouse muscle stem cell precursor-derived factors (C2C12 cell line) enhanced T cell proliferation even further (Figure 14a). These results were replicated using two different mESC cell lines derived from C57BL/6 mice (B6 ESC) and 129/J mice (D3 ESC). Notably, human ESC extracts cell were also found to inhibit T cell proliferation in a similar manner. hESC extracts strongly prevented purified T cells stimulated with concanavalin A (Figure 14b). Hence it can be concluded that ESC extracts indeed have an independent inhibitory effect on T cell proliferation.

ESC extracts do not enhance activation induced cell death.

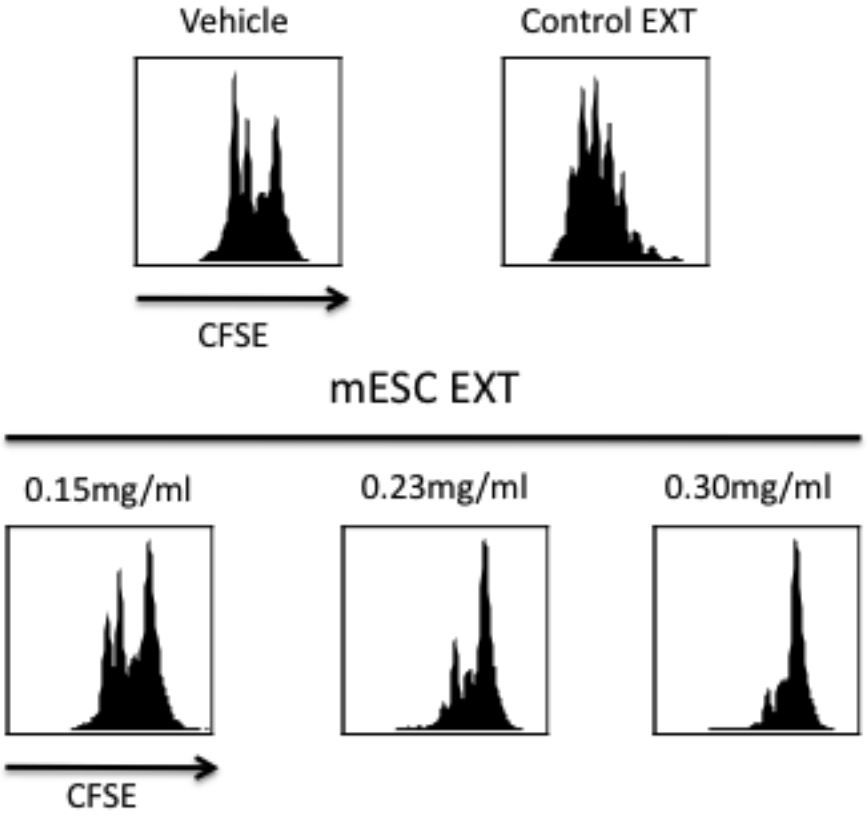
To elucidate whether T cell inhibition is due to activation induced cell death (AICD) by mESC extracts, we examined T cell apoptosis and necrosis using

Figure 14. ESC extracts directly inhibit T cell proliferation in response to anti-CD3/anti-CD28 stimulation.

A. C57BL/6 splenocytes were labeled with CFSE and activated with anti-CD3/anti-CD28 in RPMI media. Increasing concentration of mESC extracts (mESC EXT) was added to B6 splenocytes stimulated with anti-CD3/anti-CD28 in RPMI media. Splenocytes were also treated with vehicle control or control C2C12 extracts (Control EXT). After 48 hours the cells were analyzed by flow cytometry for proliferation. Results are representative of 4 separate experiments.

B. Purified human CD3+ T cells were stimulated with 30μg/ml of ConA in the presence of hESC or vehicle control. Tritiated thymidine was added on day 3 and the cells were cultured for an additional 16 to18 hours. Results are displayed as counts per minute (CPM) of triplicate wells ± SD. Results are representative of at least 3 separate experiments.

a


Vehicle
Control EXT
CFSE
mESC EXT
0.15mg/ml
0.23mg/ml
0.30mg/ml
CFSE

b

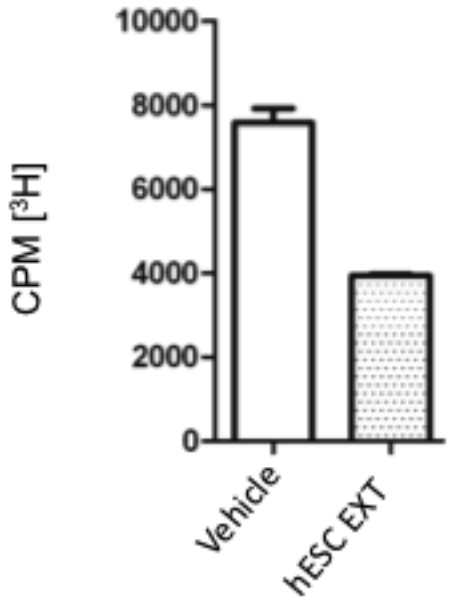
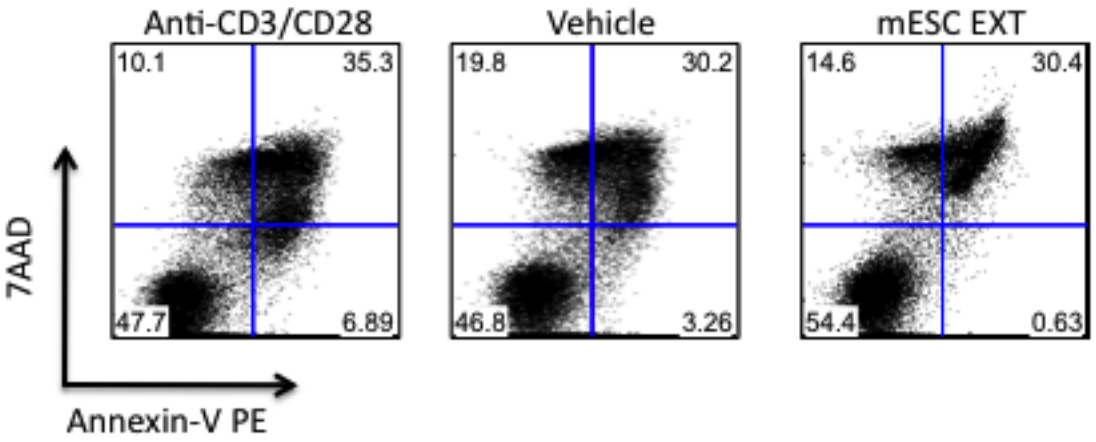

10000
8000
6000
4000
2000
0
CPM [3H]
Vehicle
hESC EXT

Figure 15. mESC extracts do not enhance T cell death. C57BL/6 splenocytes were stimulated with anti-CD3/anti-CD28 antibodies in the presence of ESC extracts for 3 days. 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. Results are representative of 4 separate experiments.


Anti-CD3/CD28
Vehicle
mESC EXT
10.1
35.3
47.7
6.89
19.8
30.2
46.8
3.26
14.6
30.4
54.4
0.63
7AAD
Annexin-V PE

Annexin V and 7AAD respectively. Splenocytes stimulated with anti-CD3 and anti-CD28 and treated with mESC extracts were found to have the same number of CD3+ dead cells as control treatments (Figure 15). These data suggests that ESC-derived factors inhibit T cell proliferation without inducing T cell death following activation.

ESC extracts modulate T cell activation markers on CD4 and CD8 T cells.

Next we assessed the impact of mESC extracts on T cell activation. CD3+ T cells were negatively selected from splenocytes and stimulated with anti-CD3 and anti-CD28. Subsequently, T cells were examined for surface expression of CD25, CD44 and CD69 at the specified time points. These markers are important for T cell activation and subsequent proliferation and function [175-178]. We found that mESC extracts have the capacity to markedly decrease the surface expression of CD25 on both CD4 and CD8 T cells (Figure 16a and 17a). Similarly, ESC-derived factors were also able to noticeably reduce the surface expression of CD44 and CD69 on both T cell subsets (Figure 16b,c and 17b,c). As a result, it can be concluded that mESC extracts inhibit T cell proliferation by decreasing surface expression of important markers that are necessary for proper activation and subsequent proliferation.

Figure 16. mESC extracts inhibit upregulation 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-derived factors or vehicle control. CD4 positive T cells were examined for the following activation markers by flow cytometry;

A. CD25 after 24 hours of stimulation.

B. CD69 expression after 6 hours of stimulation.

C. CD44 expression after 24hours of stimulation.

Results are representative of at least 3 separate experiments.

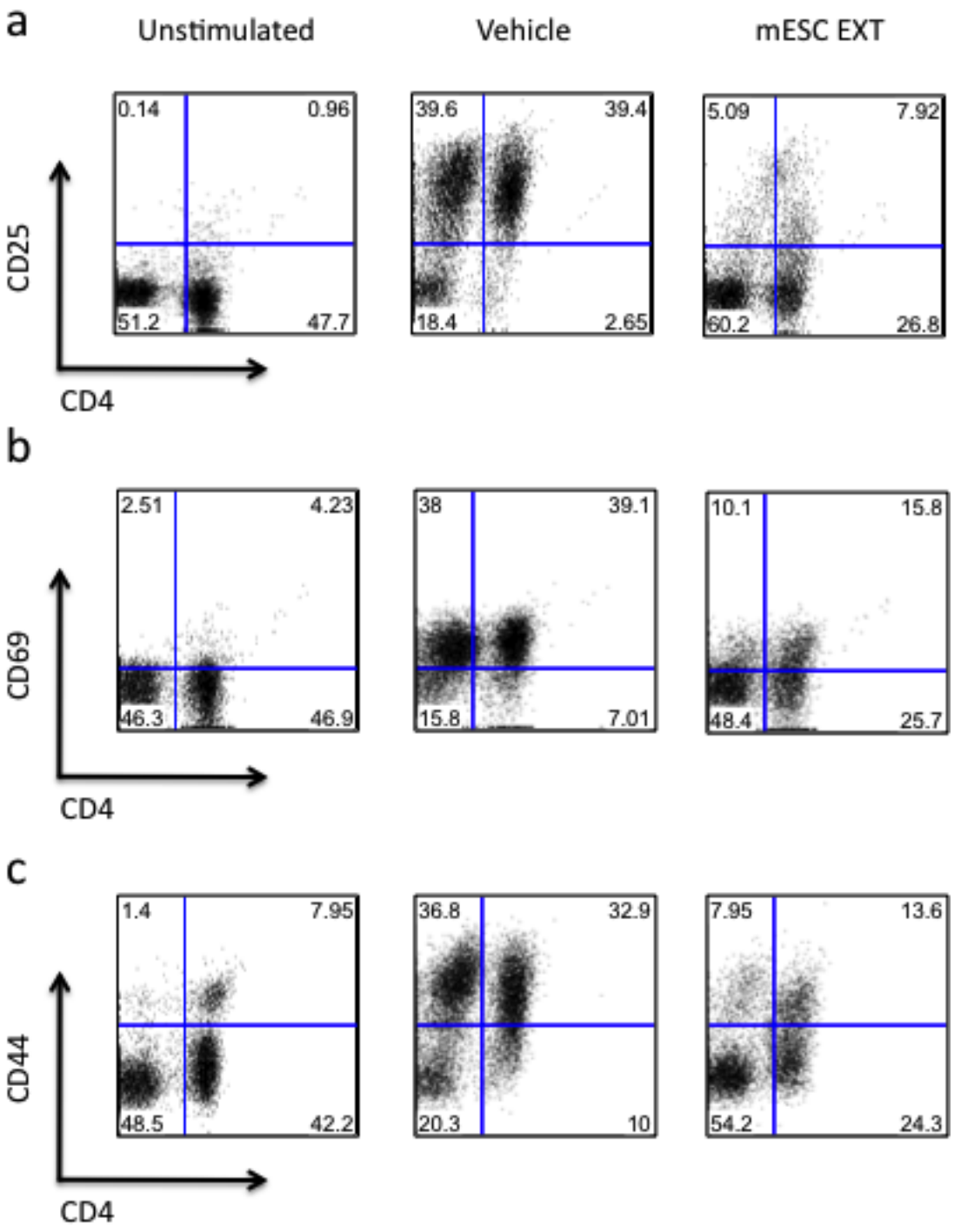

a
Unstimulated
Vehicle
mESC EXT
CD25
CD4
0.14
0.96
51.2
47.7
39.6
39.4
18.4
2.65
5.09
7.92
60.2
26.8
b
CD69
CD4
2.51
4.23
46.3
46.9
38
39.1
15.8
7.01
10.1
15.8
48.4
25.7
c
CD44
CD4
1.4
7.95
48.5
42.2
36.8
32.9
20.3
10
7.95
13.6
54.2
24.3

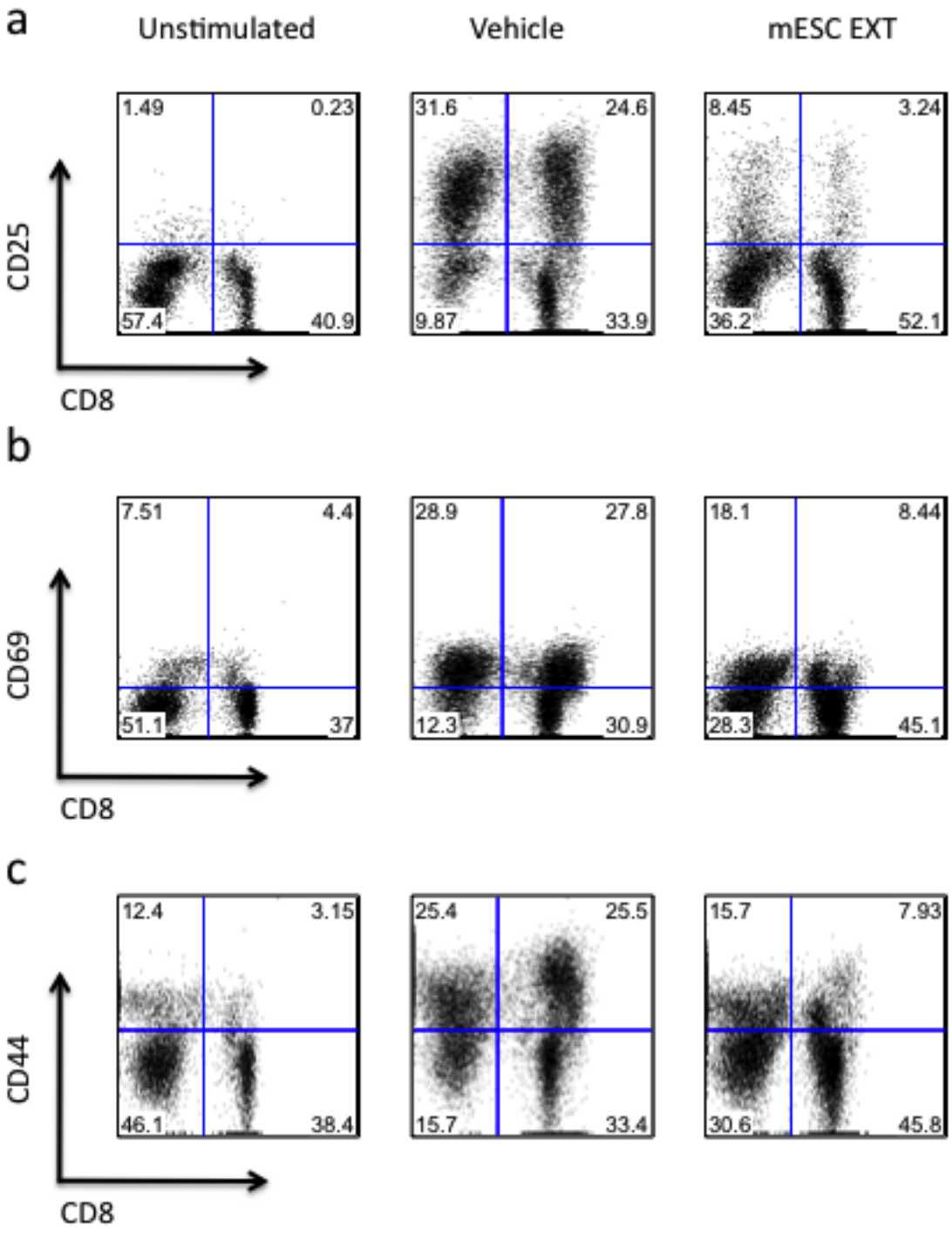
Figure 17. mESC extracts inhibit upregulation of activation markers CD25, CD44 and CD69 on CD8 T cells. Negatively isolated C57BL/6 CD3+ T cells were stimulated with plate bound anti-CD3/anti-CD28 antibodies in the presence of ESC-derived factors or vehicle control. CD8 positive T cells were examined for the following activation markers by flow cytometry;

A. CD25 after 24 hours of stimulation.

B. CD69 expression after 6 hours of stimulation.

C. CD44 expression after 24hours of stimulation.

Results are representative of at least 3 separate experiments.


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

mESC extracts skew T cell cytokine production towards a T regulatory profile.

The ability of ESC-derived factors to affect proper T cell activation led us to examine T cell effector function in response to alloantigen. We performed one-way MLR assays and examined the expression of various cytokines and transcription factors by quantitative PCR at several time points. We found that MLR treated with ESC extracts had significantly lower expression levels of IL-2 and IFN-γ after 8 hours of stimulation compared to controls (Figure 18a,b). In contrast, we observed significantly higher expression of TGF-β and Foxp3 expression in the same samples by 24 hours (Figure 18c,e). However, Tbet expression remained unchanged (Figure 18d). These results indicate that ESC-derived factors may favour development of Treg cells over Th1 cells in response to alloantigen.

To further confirm that the shift in cytokine and transcription factors was specifically induced in T helper subsets, IFN-γ and Foxp3 expression was examined by intracellular staining. Splenocytes were stimulated with anti-CD3 and anti-CD28 or PMA/Ionomycin in the presence of mESC extracts or controls and subsequently analyzed by flow cytometry at the indicated time points. We gated on CD4 and CD8 T cell subsets and found that mESC extracts reduced IFN-γ production in CD8 T cells compared to controls (Figure 19a) but not in CD4 cells at this time point (Figure 19b). Next, the frequency of T regulatory cells was determined based on the combined expression of CD4, CD25 and Foxp3. Treatment of splenocytes with mESC extracts resulted in greater number of CD4+, CD25+ and Foxp3+ cells compared to controls (Figure 20). Therefore, these data support the mRNA results

Figure 18. ESC extracts modulate T helper responses during an allogeneic immune response.

A one way mixed lymphocyte reaction was performed, C57BL/6 splenocytes were used as responders and CD1 splenocytes as stimulators in the presence of mESC extracts or vehicle control. Cells were harvested at the indicated time points and total RNA was isolated. Subsequently, cDNA was synthesized and used to carry out QPCR to examine the expression of cytokines and master regulator transcription factors of T helper cells.

A. IL-2 expression

B. IFN-γ expression

C. TGF-β expression

D. T-bet expression

E. Foxp3 expression.

Relative gene expression is compared to responders alone that were used as baseline. 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. Data collected by Bodour Al-Khamees. Results are representative of at least 3 separate experiments.

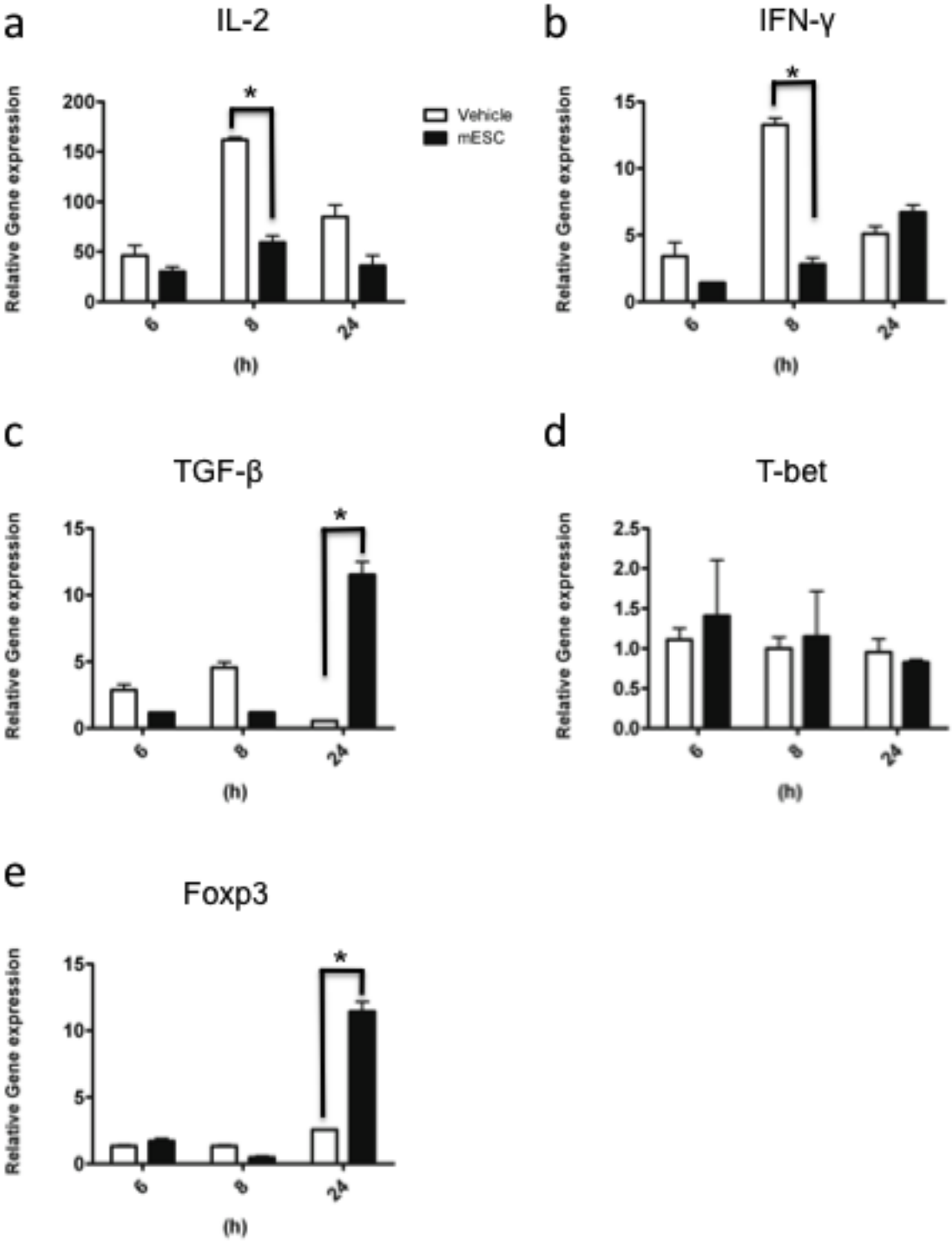

a
IL-2
Relative Gene expression
200
150
100
50
0
*
6
8
24
(h)
Vehicle
mESC
b
IFN-γ
Relative Gene expression
15
10
5
0
*
6
8
24
(h)
c
TGF-β
Relative Gene expression
15
10
5
0
*
6
8
24
(h)
d
T-bet
Relative Gene expression
2.5
2.0
1.5
1.0
0.5
0.0
6
8
24
(h)
e
Foxp3
Relative Gene expression
15
10
5
0
*
6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

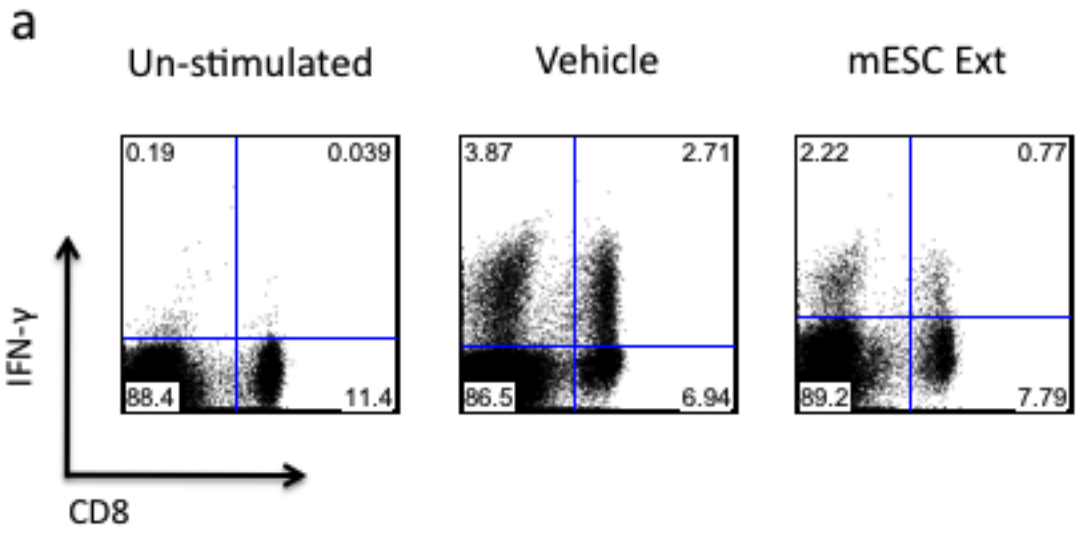
Figure 19. ESC extracts decrease IFN-γ positive CD8 T cells.

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. After washing, the cells were fixed, permeabilized and stained for intracellular IFN-γ.

A. CD8 T cells

B. CD4 T cells

Results are representative of at least 3 separate experiments.


a
Un-stimulated
Vehicle
mESC Ext
0.19
0.039
88.4
11.4
3.87
2.71
86.5
6.94
2.22
0.77
89.2
7.79
IFN-γ
CD8

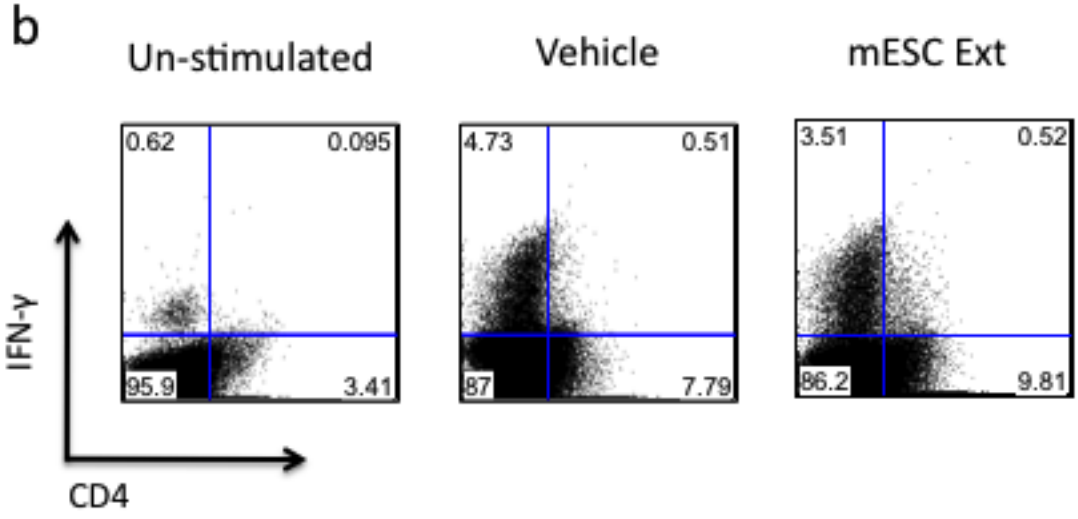
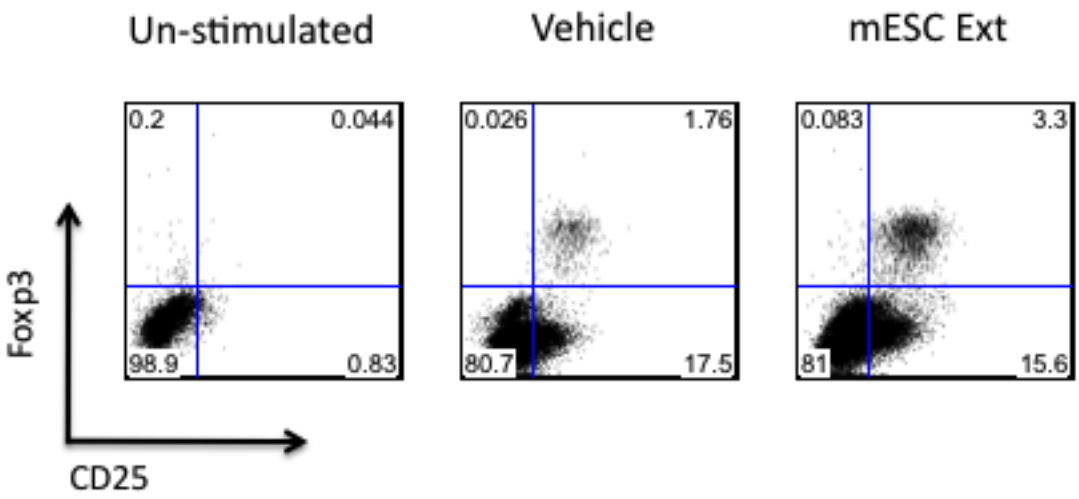

b
Un-stimulated
Vehicle
mESC Ext
0.62
0.095
95.9
3.41
4.73
0.51
87
7.79
3.51
0.52
86.2
9.81
IFN-γ
CD4

Figure 20. ESC extracts favour T regulatory cell development.

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.


Un-stimulated
Vehicle
mESC Ext
0.2
0.044
98.9
0.83
0.026
1.76
80.7
17.5
0.083
3.3
81
15.6
Foxp3
CD25

described in Figure 18 and it can be concluded that mESC extracts favour the development of Treg cells over Th1 cells.

mESC extracts may skew T cells responses by preventing PKC-θ phosphorylation.

The mechanisms underlying ESC-mediated T cell inhibition remain poorly understood. Several studies have described already known mechanisms of immune modulation, including TGF-β, to be involved in mouse ESCs and FasL in rat ESCs. More recently, two papers have described that intact human ESCs are able to directly inhibit T cell proliferation through the expression of the enzyme arginase-1 and HLA-G [50, 52]. However, in our assays we supplement the cytoplasmic lysates with excess amounts of L-arginine to prevent protein-protein aggregation and examination of our own cell lines by quantitative mass spectrometry has not revealed HLA-G expression. It is very likely that ESCs may have multiple properties that affect T cell activation and proliferation.

In an attempt to delineate alternate mechanisms, purified T cells and PBMC were stimulated with several mitogens that mimic activation of major signaling pathways in T cells. Signaling through the T cell receptor (TCR) results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). Subsequently, IP_3 and DAG propagate the TCR signal by increasing intracellular calcium and phosphorylation of PKC-theta (PKC-θ) that in turn activate transcription factors NFAT and NFκB/AP1, respectively [179]. The

combined effect of the two pathways in addition to co-stimulation results in T cell activation [179]. We determined that stimulation of T cells with phorbol-1-3-merystate (PMA), an analog of DAG which directly activates PKC-θ, could be strongly inhibited by ESC extracts (Figure 21a) [180, 181]. Interestingly, ESC extracts could not prevent proliferation of the cells in response to ionomycin, which causes calcium influx and activates NFAT (Figure 21b) [182]. These results indicated that ESC-extracts might inhibit proper T cell activation and proliferation by inhibiting PKC-θ activation.

To directly establish the impact of ESC extracts on PKC-θ activation, splenocytes were stimulated with PMA in the presence and absence of mESC extracts. Phosphorylation of PKC-θ at Threonine-538 (Thre-538), reflective of its activity was examined by western blot [183]. Splenocytes treated with mESC extracts had little or no phosphorylation of PKC-θ whereas control splenocytes showed strong phosporylation in response to PMA (Figure 22a). Notably, mESC extracts did not have an effect on total PKC-θ levels, as they remained constant at all time points similar to controls (Figure 22). To determine whether the absence of phosphorylation at Thr-538 was indeed reflective of PKC-θ activity we examined down stream targets of the kinase. PKC-θ is known to form a complex with CARMA-1, Bcl-10 and MALT-1 to induce NFκB translocation to the nucleus by causing degradation of IκB [82]. As a result we examined IκB-α degradation in purified T cells that had been activated with PMA. Consistent with above results, we found that IκB-α levels were stable or only slightly degraded in cells that were treated with mESC extracts whereas IκB-α was visibly degraded in controls (Figure 22b). Hence,

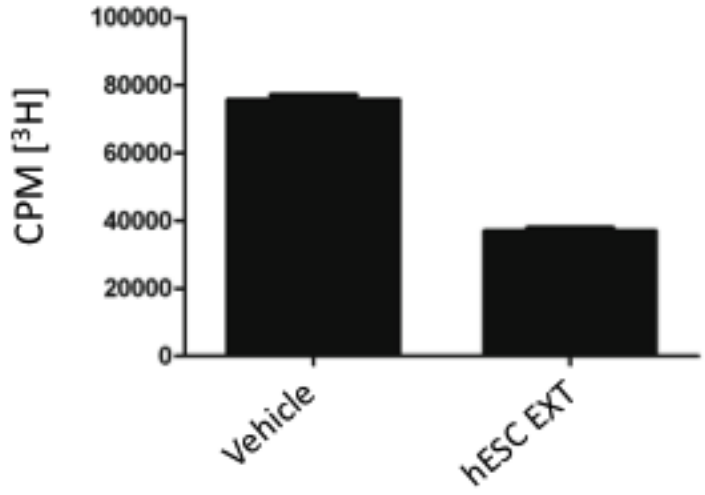
Figure 21. ESC extract inhibit T cell proliferation in response to PMA but not ionomycin.

A. T cells were purified from PBMC and stimulated with 50ng/ml of PMA in the presence of vehicle control or hESC-extracts.

B. Whole PBMC were stimulated with 200μM of ionomycin in the presence of vehicle control or hESC extracts.

Tritiated thymidine was added on day 3 and the cells were cultured for an additional 16 to18 hours. Results are displayed as counts per minute (CPM) of triplicate wells ± SD. Results are representative of at least 3 separate experiments.

a


100000
80000
60000
40000
20000
0
CPM [3H]
Vehicle
hESC EXT

b

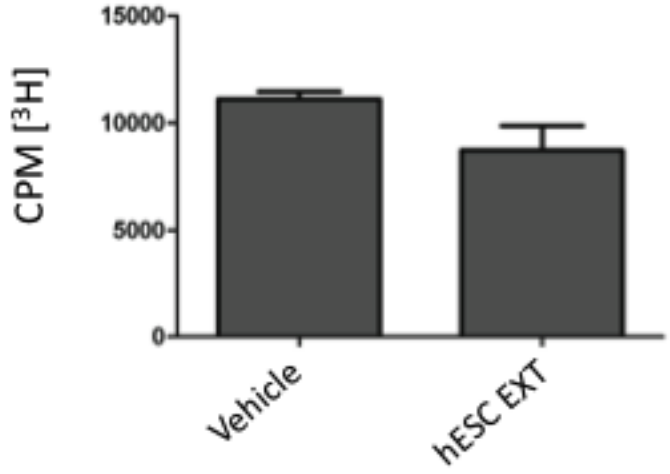

15000
10000
5000
0
CPM [3H]
Vehicle
hESC EXT

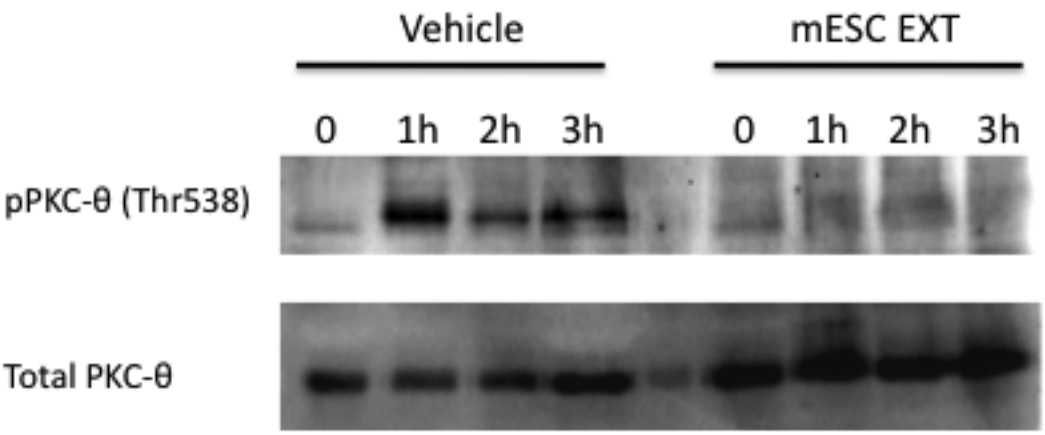
Figure 22. mESC extracts inhibit PMA mediated PKC-θ phosphorylation in T cells.

A. C57BL/6 splenocytes were pre-treated over night with ESC extracts and stimulated with 50ng/ml of PMA for the indicated periods of time. Subsequently, the cells were harvested and lysed. Lysates were examined by western blotting for PKC-θ phosphorylation (Thre 538), and total PKC-θ.

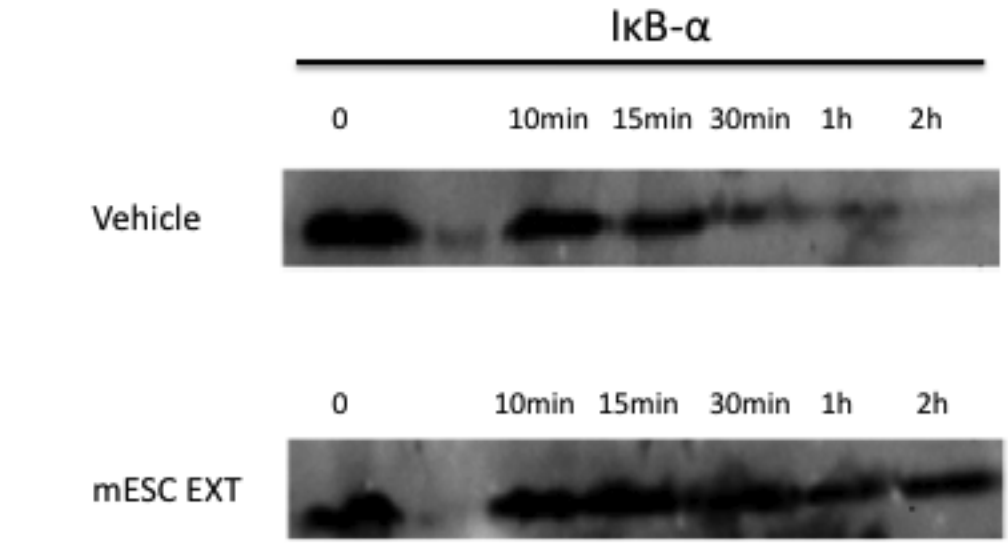
B. C57BL/6 CD3+ T cells were pre-treated over night with ESC extracts and stimulated with 50ng/ml of PMA for the indicated periods of time. Subsequently, the cells were harvested and lysed. Lysates were examined by western blotting for IκB-α degradation.

Results are representative of 4 separate experiments.

a


Vehicle
mESC EXT
0
1h
2h
3h
0
1h
2h
3h
pPKC-θ (Thr538)
Total PKC-θ

b


IκB-α
0
10min
15min
30min
1h
2h
Vehicle
0
10min
15min
30min
1h
2h
mESC EXT

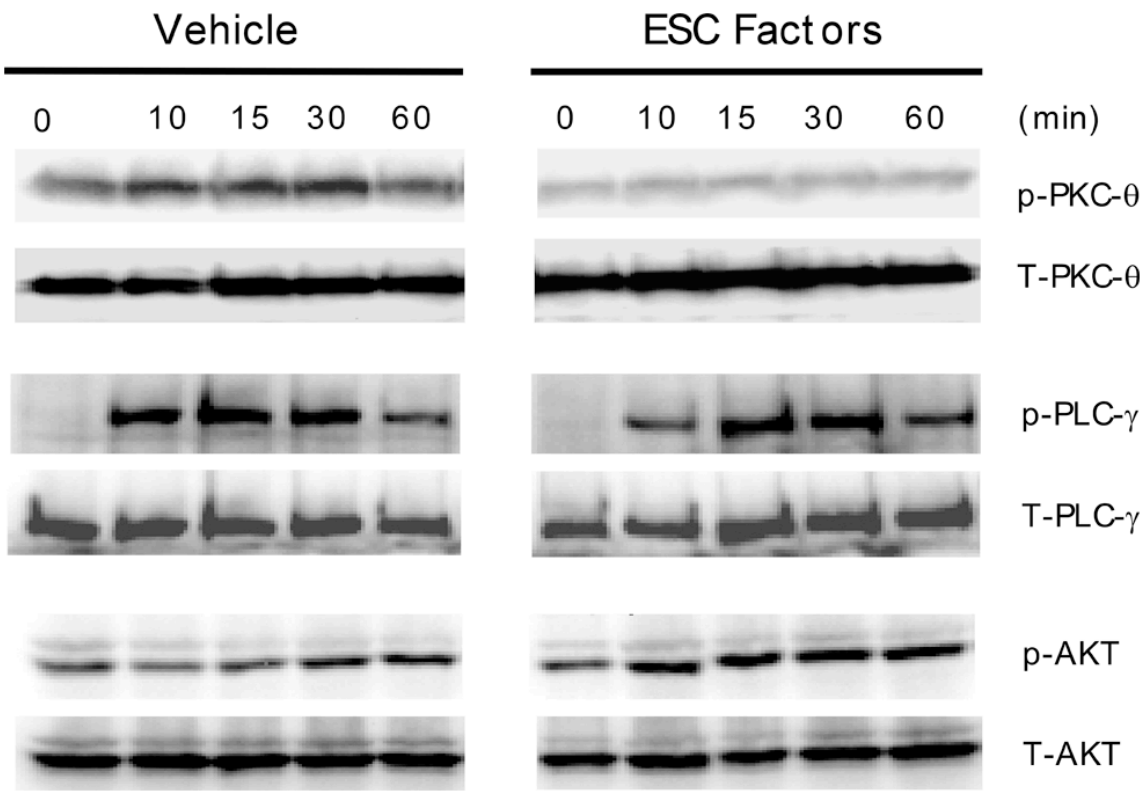
mESC extracts directly affect T cell activation and proliferation by inhibiting PKC-θ phosphorylation and subsequent activity.

mESC extracts specifically inhibit PKC-θ activation without affecting upstream T cell signaling.

Although ESC-derived factors have the capacity to inhibit PMA mediated PKC-θ activation, it is unclear whether they also affect other signaling components emanating from the TCR and CD28. Several studies have established that PKC-θ activation is mediated by signaling molecules PLC-γ and PI3K originating from the TCR and CD28, respectively [179, 184, 185]. As such, we determined whether these signaling molecules were affected by ESC-derived factors following anti-CD3 and anti-CD28 stimulation. Splenocytes were pre-treated overnight with ESC extracts and stimulated with anti-CD3 and anti-CD28 for the indicated time periods. Subsequently, phosphorylation of PKC-θ, PLC-γ and AKT (used as a surrogate marker for PI3K) were examined. Consistent with the data obtained with PMA stimulation (Figure 22), we found that splenocytes treated with ESC-extracts and activated with anti-CD3 and anti-CD28 had little or no phosphorylation of PKC-θ at all time points, whereas phosphorylation was detected in controls (Figure 23). In contrast, phosphorylation of PLC-γ and AKT could be detected in both ESC-derived factor and control treated splenocytes at all time points (Figure 23). Therefore, it can be concluded that ESC extracts specifically inhibit PKC-θ without influencing its known up-stream activators.

Figure 23. ESC extracts specifically inhibit PKC-θ activation without affecting upstream signaling molecules.

C57BL/6 splenocytes were pre-treated with ESC extracts overnight and stimulated with anti-CD3/anti-CD28 for the indicated periods of time. Subsequently, the cells were harvested and lysed. Lysates were examined by western blotting for PLC-γ, AKT and PKC-θ phosphorylation. Results are representative of 3 separate experiments.


Vehicle
ESC Factors
0
10
15
30
60
0
10
15
30
60
(min)
p-PKC-θ
T-PKC-θ
p-PLC-γ
T-PLC-γ
p-AKT
T-AKT

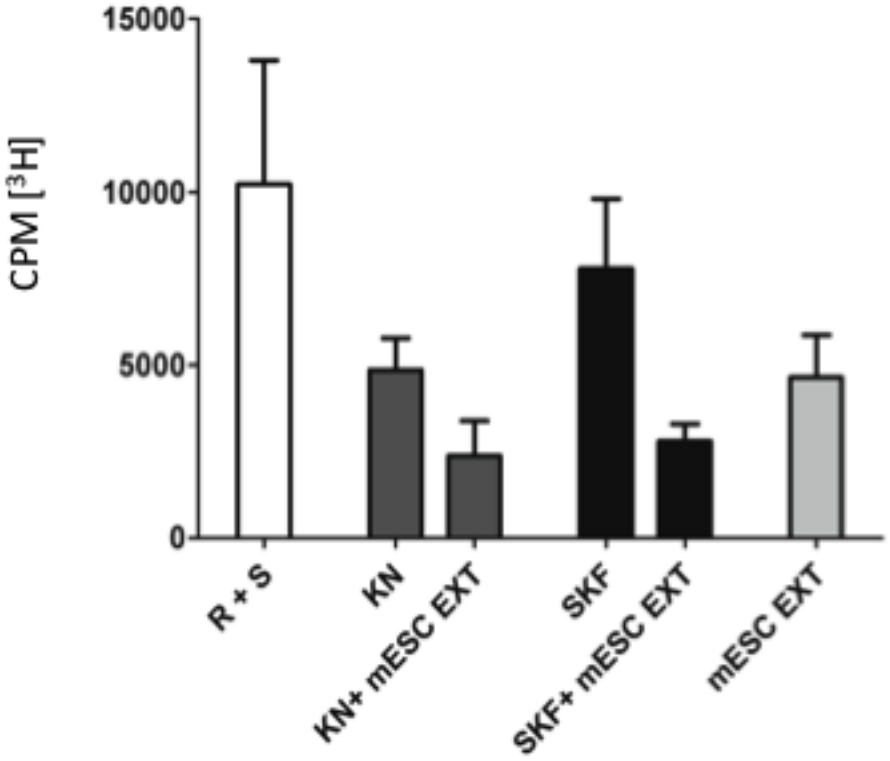
mESC extracts can be used in combination with calcium channel blockers to enhance inhibition of allogeneic immune activation.

After determining that ESC extracts specifically inhibit PKC-θ, we wanted to examine whether T cell proliferation could be further prevented in combination with inhibitors of the calcium-signaling pathway. MLR were carried out by treating the cells with SKF-96365 and KN-93 that inhibit extracellular receptor mediated calcium entry and release of intracellular stores of calcium, respectively [186, 187]. Both SKF-96365 and KN-93 were able to decrease cell proliferation (Figure 24a). Similarly, mESC extracts were able to inhibit proliferation of MLR as previously shown. Treatment of MLRs with SKF-96365 and KN-93 in combination of with mESC extracts enhanced inhibition of MLRs and had an additive effect (Figure 24a). The effect of mESC extracts was also tested in combination with cyclosporine A, a drug that specifically in inhibits calcineurin activation and is widely used in the clinic to prevent allogeneic organ rejection [188, 189]. Treatment of MLR with cyclosporine A resulted in decreased cell proliferation (Figure 24b). Once again, cyclosporine A used in combination with mESC extracts decreased MLR proliferation than either cyclosporine A or mESC extracts used alone (Figure 24b). Therefore, these data indicate that mESC in combination with inhibitors of the calcium-signaling pathway has an additive effect in preventing allo-immune activation. Moreover, it can be concluded that ESC extracts may hold the potential to supplement already clinically established drugs to promote allogeneic graft survival.

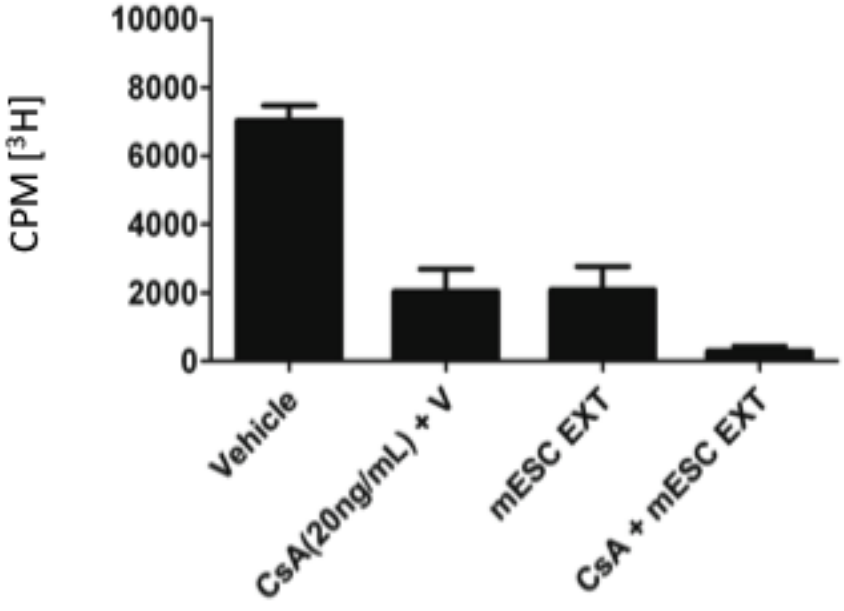
Figure 24. ESC-derived factors in combination with cyclosporin A enhance inhibition of allogeneic immune activation.

A one way mixed lymphocyte reaction was performed, C57BL/6 splenocytes were used as responders and CD1 splenocytes as stimulators in the presence of vehicle control, ESC-derived factors and CsA. Moreover, ESC-derived factors were used in combination with CsA to determine whether they can complement one another in preventing allo-immune activation. Tritiated thymidine was added on day 3 and the cells were cultured for an additional 16 to18 hours. Results are displayed as counts per minute (CPM) of triplicate wells ± SD. Results are representative of at least 3 separate experiments.

a


15000
10000
5000
0
CPM [3H]
R + S
KN
KN+ mESC EXT
SKF
SKF+ mESC EXT
mESC EXT

b


10000
8000
6000
4000
2000
0
CPM [3H]
Vehicle
CsA(20ng/mL) + V
mESC EXT
CsA + mESC EXT

Chapter 5. Discussion:

Embryonic stem cell research has the potential to unlock fundamental scientific questions in the field of developmental and cellular biology. The discovery that ESCs possess immune privileged and immune modulatory properties further enhances their significance since deciphering these properties may lead to more broad applications in autoimmune diseases, allergy and transplantation. Several groups have determined that the ESCs have powerful immune modulatory properties that not only facilitate their own survival but also reduce immune responses towards third party antigen and promote the survival of solid organ transplants [41-52, 150, 190]. However, the use of live ESCs to overcome allogeneic immune responses is associated with the adverse risk of teratoma formation [1-4, 24, 25, 27, 53-55]. These studies indicate a direct correlation between the number of ESCs injected into mice and the incidence of teratoma formation, with the most effective number of ESCs to overcome the immune system causing teratomas in 90% of the recipients [44]. Therefore, the immediate application of live ESCs for immune modulation is not feasible at the current state of stem cell research and alternate strategies are needed to harness these properties.

In order to circumvent teratoma formation we hypothesized that ESC derived cellular extracts may retain the immune modulatory properties of live cells and abrogate allo-immune responses. Indeed, our studies demonstrate that human and mouse ESC extracts were capable of inhibiting PBMC and splenocyte proliferation in response to allo-antigen in MLR assays, respectively. ESC-extracts had a dose

dependent effect on cell proliferation in these assays as increasing concentrations resulted in greater inhibition. Further experiments elucidated that ESC-extracts independently affected dendritic and T cell activation.

Initial studies involving MLR revealed that ESC-extracts may have a specific effect on DCs. We determined hESC extracts were able to inhibit maturation of human monocyte-derived DCs. DC maturation was evaluated not only on the basis of the expression of specific surface markers but also according to typical DC functions, such as phagocytic capacity, production of polarizing cytokines and the ability to trigger T-cell responses in MLR. In effect, hESC extract-treated DCs expressed decreased levels of activation markers CD80, HLA-DR, and CD83, and retained a greater ability to phagocytose dextran beads compared to controls even after treatment with TNF-α (Figure 9). In addition, ELISA assays revealed hESC extract-treated DCs produced up to 10-fold less IL-12p40 than controls. Accordingly, hESC extracts-treated DCs were found to be poor activators of purified allogeneic T cells in MLR. However, we did find an up-regulation of CD86, indicating that the phenotypes of these DCs were skewed and perhaps maturation was not completely prevented. Interestingly, MSCs modulate DC maturation by inhibiting up-regulation of CD80 and HLA-DR while CD86 is not affected [163]. Taken together, the accumulated data from the experiments described in this thesis suggests that hESC extract treatment during monocyte to DC differentiation impairs the cells from becoming fully mature. This conclusion is supported by the observation that immature hESC extract treated DCs do not have enhanced suppressive capacity compared to immature vehicle treated DCs (Figure 12). As a result, we conclude

that hESC extracts impact the transition between immature to mature DC. These studies firmly establish a direct effect of ESC extracts on DC maturation and to the best of our knowledge, provide the first evidence that human ESCs have an immune modulatory effect on DCs.

While addressing the impact of ESC extracts on DCs we observed the potential for an impact of ESC extracts on T cells. ESC extracts were found to prevent T cell proliferation in response to anti-CD3/anti-CD28 stimulation. The effect of ESC extracts also caused down regulation of T cell activation markers (CD25, CD44 and CD69), reduced production of IFN-γ both at the mRNA and protein level while causing an increase in TGF-β and Foxp3 expression. Hence, ESCs extracts have the capacity to decrease expression of important T cell markers required for proper activation and proliferation. These data also suggest that ESC extracts may favour development of Treg cells over Th1 cells. Significantly, ESC extracts were found to modulate T cell activation due to a specific effect on PKC-θ phosphorylation. PKC-θ phosphorylation at Thre-538 was inhibited (reflective of PKC-θ activity) in ESC treated T cells without an effect on upstream T cell signaling components PLC-γ and PI3K derived from both CD3 and CD28 receptors, respectively [183]. Accordingly, ESC extract mediated PKC-θ inhibition may provide an explanation for the described modulation of T cell responses described (see further discussion below). It is noteworthy to mention that this is the first study that demonstrates ESC mediated PKC-θ inhibition as mechanism of immune modulation.

The ability of ESC extract treatment to prevent DC maturation, reduce T cell proliferation and favour Treg development, suggest that ESC extracts may have potent immune modulatory properties that do not simply suppress the immune system but rather induce an altered immune response. Several pieces of evidence support this notion. To begin with, ESC extracts do not enhance cell death compared to controls either when PBMC were examined, encompassing several cell types, or T cells specifically. Further support is observed when examining the changes in cytokine secretion by both DCs and T cells. ESC extract treatment of DCs results in a decrease of IL-12p40, a cytokine that favours Th1 development, compared to controls. However, unlike IL-12p40, TGF-β levels were not down regulated in ESC extract treated DCs. The combination of unchanged TGF-β secretion along with a decrease IL-12p40 suggests that ESC extract treated DCs would most likely induce an altered T cell response. Similarly, MLRs treated with ESC extracts induced lower levels of IL-2 and IFN-γ while at the same time an increase in TGF-β expression. In line with these findings CD8 T cells were found to produce lower levels of IFN-γ. Finally, a greater number of CD4 T cells were found to express Foxp3 compared to controls. The ability of ESC extracts to affect an allo-immune response both at the level of APC and effectors cells makes it a powerful immune modulatory agent that could potentially protect allogeneic cells and solid organs from immune rejection. As such, appropriate animal transplantation models need to be examined to determine the efficacy of ESC derived extracts. If our in-vitro findings hold true in vivo and if similar findings are observed with other stem cell sources such as IPS, the use of IPS or IPS extracts may represent a more

favorable approach to treat immune based conditions. Recipient animals could receive their own strain specific IPS cell components rather than allogeneic ESCs thereby avoiding unforeseen complications such as antibody development towards allogeneic proteins.

The specific components contributing to ESC-mediated immune modulation have previously been attributed to various factors. Studies carried out with rat and mouse ESCs indicate the possible involvement of FasL and TGF-β expression in evading the immune system respectively. However, studies carried out with hESCs exclude FasL as a causative factor. Moreover, microarray studies that examined 300 immune related genes, including adhesion markers, chemokines and cytokines, did not find significant expression of these genes in hESCs. Among these genes, the study found negligible expression of IL-10 and TGF-β which was confirmed in our studies [41]. Using mouse ESCs, another independent study examined 87 immunologically relevant genes using Taqman assay and obtained similar results [48]. The study found that IL-10, TGF-β1, indoleamine 2,3-dioxygenase, arginase-1 and 2 do not contribute to immune privilege of mouse ESCs [48]. Recently, two studies carried with human ESCs have shown that arginase-1, an enzyme that depletes L-arginine in the microenvironment, and HLA-G may play a role in ESC-mediated immune modulation [50, 52]. We recently reviewed a list of highly abundant proteins found in hESCs using quantitative mass spectrometry and have not found any known candidate factors such as CTLA-4, PDL-1, PDL-2, arginase-1 and HLA-G. Moreover, in our experiments the vehicle/lysis buffer is supplemented with 50mM L-arginine as this has been found to enhance protein stability and prevent dimer

formation [191]. Since the presence of excess L-arginine does not influence the inhibitory effect of hESC-extracts on DC and T cell maturation and functions in our study, it appears that ESC-mediated immune modulation is not due to a single factor but rather a number of different factors. There also exists the possibility that different ESC lines evade the immune system using distinct mechanisms from one another and explaining the diverse results obtained.

Here, we have made the novel discovery that ESC extracts inhibit T cell proliferation by inhibiting PKC-θ activation while the calcium signaling pathway remains unhindered. We believe that ESC mediated PKC-θ inhibition may be an important mechanism used by the cells to evade the immune system and several pieces of evidence reported by other groups and elucidated in our studies support this notion. The role of PKC-θ and its contribution to T cell function and phenotype has been supported by several important findings. Zanin-Zhorov's et al. have recently elucidated that PKC-θ is recruited much less in T regulatory cells compared to effector T cells [192]. They established that PKC-θ has a negative impact on T regulatory cells rendering them less effective in suppressing effector T cell function [192]. Moreover, inhibition of PKC-θ was shown to enhance T regulatory potency in suppressing effector T cell proliferation and IFN-γ secretion [192]. This fits in very well with our results that show an increase in T regulatory cells and a decrease in IFN-γ production when T cells are activated in the presence of ESC extracts. Meanwhile, Valenzuela et al. have found that PKC-θ is required for allo-antigen responses in a graft versus host disease (GVHD) model [193]. Whereas wild type T cells induced severe GVHD resulting in a lethal outcome for all mice, PKC-θ-KO T cells induced

very mild or no GVHD leading to the survival of most transplanted mice [193]. Additionally, several studies have established that in the absence of PKC-θ activation, an anergic genetic program is initiated [194-197]. This anergic state is induced by prolonged calcium flux leading to persistent activation and translocation of NFAT to the nucleus in the absence of NFκB and AP-1, two transcription factors that coordinately bind with NFAT to induce the normal T cell program [195-197]. The binding of NFAT alone induces expression of E3 ubiquitin ligases that negatively regulate T cell signaling [194-198]. Interestingly, the expression of CD86 in DCs has been shown to be dependent on calcium signaling and may explain why ESC extracts could not prevent upregulation of this marker in DCs [199]. The inability of ESC extracts to inhibit ionomycin mediated T cell proliferation and the fact that ESC extracts can be combined with CsA, a calcium signaling pathway inhibitor, are in line with the above described findings. Our current results provide the first connection between ESC immune modulation and PKC-θ inhibition and may provide a key to identifying other factors that are expressed by ESCs.

Future directions.

There are two key aspects of ESC extract mediated immune modulation that need to be addressed in order to improve and validate its efficacy. The most immediate focus should be identifying the specific factor or factors that contribute to ESC extract mediated PKC-θ inhibition. The current protocol used to obtain ESC extracts requires further refinement to identify the contributing molecules. The combined use of proteomic analyses and biochemical approaches may provide the means of elucidating the specific factors responsible for immune modulation. The

most obvious approach to identify ESC factors may be to perform immuno-precipitation assays with purified PKC-θ. Other options include sucrose gradient centrifugation or separation through filters with specific protein size restrictions to identify a specific fraction with immune modulatory properties. Alternatively, ESC extracts could be biotinylated and incubated with T cells or DCs. Any biotinylated proteins that attached to the surface of or enter the cells could be subsequently purified with streptavidin columns and examined by mass spectrometry. The in-vivo efficacy of the ESC extracts also needs to be determined. The use of transplantation or contact hypersensitivity models may provide the means to test the ability of ESC extracts to modulate the immune system. Identifying the most appropriate model to show the efficacy of ESC extracts may depend on the severity of immune responses induced in each model. The optimal dosage of ESC-extracts may also have to be determined for in-vivo models and may not be as simple as extrapolating from in-vitro assays. It may also be beneficial to first identify the specific factors that contribute to in-vitro immune modulatory properties of ESC's prior to attempting in-vivo experiments. These future studies provide an exciting opportunity for the discovery of novel immune modulatory proteins and may take ESC-extracts one-step closer to potential clinical application ahead of the use of live cells.

Final Conclusions.

Cellular extracts from both human and mouse ESCs retain the immune modulatory properties of live ESCs. ESC extracts modulate immune responses by independently affecting DCs and T cells thereby reducing allo-immune responses in

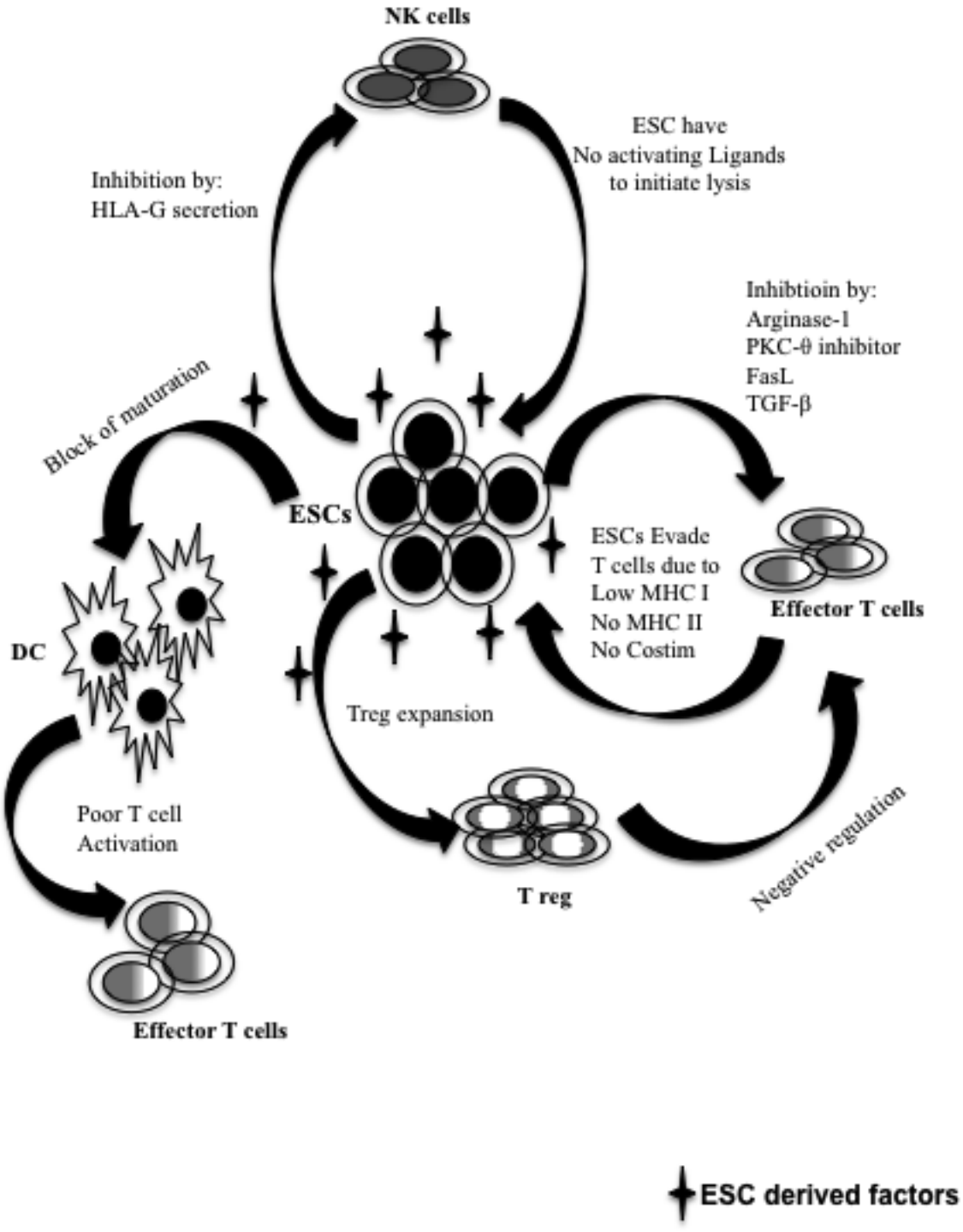
MLR. ESC extracts block DCs from becoming fully mature. Similarly, ESC extracts reduce the number of activated T cells and seem to favour T reg over Th1 development. ESC extracts may achieve modulation in T cell responses by specifically inhibiting PKC-θ phosphorylation and its subsequent activity. Therefore, ESC extracts represent a potentially safe and practical means to harness the immune modulatory properties of live ESCs to treat aberrant immune responses that comprise a large burden of current medical illness

Our accumulated data complements previous findings using live ESCs. Those studies showed that live ESC had the ability to inhibit DC and T cell activity without providing further details. Here we have been able to show the impact of ESCs on DCs and T cell in more detail by closely examining specific functions, phenotype and characteristic of the cells. Moreover, we have described a new mechanism of T cell inhibition, prevention of PKC-θ phosphorylation, which can be attributed to ESCs.

The immune modulatory properties of ESCs and their potential application in overcoming aberrant immune conditions have become convincing. It is apparent, based on our studies and those from other groups, that ESCs may use several mechanisms to evade the immune system by influencing both APC and effector cells independently. A current model of ESC mediate immune evasion and modulation may be described as follows: 1) ESCs are not readily recognized by the immune system as they express very low levels of MHCI, express no MHCII and costimulatory receptors. 2) Despite absence of MHC expression they evade NK cell mediated lysis due to a lack of ligands for NK activating receptors. 3) ESCs actively

inhibit the maturation of DCs that may be able to take up antigen and present it to effector cells that may mediate their rejection. 4) ESCs actively inhibit T cell activation. 5) ESCs promote an increase in T regulatory cells that may negatively regulate effector T cells (Figure 25). This may explain the ability of ESCs to survive across allogeneic barrier for prolonged periods of time without immune suppression. Therefore the use of ESC-extracts could harness most of these properties and improve the outcome of autoimmune disease, transplantation and chronic inflammation.

Figure 25. A model of ESC and ESC-extracts mediated immune modulation.


NK cells
ESC have
No activating Ligands
to initiate lysis
Inhibition by:
HLA-G secretion
Inhibtioin by:
Arginase-1
PKC-θ inhibitor
FasL
TGF-β
Block of maturation
ESCs
ESCs Evade
T cells due to
Low MHC I
No MHC II
No Costim
Effector T cells
DC
Treg expansion
Poor T cell
Activation
T reg
Negative regulation
Effector T cells
ESC derived factors

References:

1. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature 1981;292(5819):154-6.
2. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proceedings of the National Academy of Sciences of the United States of America 1981;78(12):7634-8.
3. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282(5391):1145-7.
4. Thomson JA, Kalishman J, Golos TG, et al. Isolation of a primate embryonic stem cell line. Proceedings of the National Academy of Sciences of the United States of America 1995;92(17):7844-8.
5. Itskovitz-Eldor J, Schuldiner M, Karsenti D, et al. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Molecular medicine (Cambridge, Mass 2000;6(2):88-95.
6. Schuldiner M, Yanuka O, Itskovitz-Eldor J, Melton DA, Benvenisty N. Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America 2000;97(21):11307-12.
7. Schuldiner M, Eiges R, Eden A, et al. Induced neuronal differentiation of human embryonic stem cells. Brain research 2001;913(2):201-5.
8. Reubinoff BE, Itsykson P, Turetsky T, et al. Neural progenitors from human embryonic stem cells. Nat Biotechnol 2001;19(12):1134-40.
9. Martin CH, Woll PS, Ni Z, Zuniga-Pflucker JC, Kaufman DS. Differences in lymphocyte developmental potential between human embryonic stem cell and umbilical cord blood-derived hematopoietic progenitor cells. Blood 2008;112(7):2730-7.
10. Slukvin, II, Vodyanik MA, Thomson JA, Gumenyuk ME, Choi KD. Directed differentiation of human embryonic stem cells into functional dendritic cells through the myeloid pathway. J Immunol 2006;176(5):2924-32.
11. Koller BH, Hagemann LJ, Doetschman T, et al. Germ-line transmission of a planned alteration made in a hypoxanthine phosphoribosyltransferase gene by homologous recombination in embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America 1989;86(22):8927-31.
12. Nagy A, Gocza E, Diaz EM, et al. Embryonic stem cells alone are able to support fetal development in the mouse. Development (Cambridge, England) 1990;110(3):815-21.
13. Assady S, Maor G, Amit M, Itskovitz-Eldor J, Skorecki KL, Tzukerman M. Insulin production by human embryonic stem cells. Diabetes 2001;50(8):1691-7.
14. Kaufman DS, Hanson ET, Lewis RL, Auerbach R, Thomson JA. Hematopoietic colony-forming cells derived from human embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America 2001;98(19):10716-21.
15. Lu SJ, Feng Q, Caballero S, et al. Generation of functional hemangioblasts from human embryonic stem cells. Nature methods 2007;4(6):501-9.

16. Rambhatla L, Chiu CP, Kundu P, Peng Y, Carpenter MK. Generation of hepatocyte-like cells from human embryonic stem cells. Cell transplantation 2003;12(1):1-11.
17. Amariglio N, Hirshberg A, Scheithauer BW, et al. Donor-derived brain tumor following neural stem cell transplantation in an ataxia telangiectasia patient. PLoS medicine 2009;6(2):e1000029.
18. Goldman S. Stem and progenitor cell-based therapy of the human central nervous system. Nat Biotechnol 2005;23(7):862-71.
19. Hanna J, Wernig M, Markoulaki S, et al. Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. Science 2007;318(5858):1920-3.
20. Kehat I, Kenyagin-Karsenti D, Snir M, et al. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. The Journal of clinical investigation 2001;108(3):407-14.
21. Carpenter MK, Inokuma MS, Denham J, Mujtaba T, Chiu CP, Rao MS. Enrichment of neurons and neural precursors from human embryonic stem cells. Experimental neurology 2001;172(2):383-97.
22. Zhan X, Dravid G, Ye Z, et al. Functional antigen-presenting leucocytes derived from human embryonic stem cells in vitro. Lancet 2004;364(9429):163-71.
23. Taylor CJ, Bolton EM, Pocock S, Sharples LD, Pedersen RA, Bradley JA. Banking on human embryonic stem cells: estimating the number of donor cell lines needed for HLA matching. Lancet 2005;366(9502):2019-25.
24. Nakagawa M, Koyanagi M, Tanabe K, et al. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol 2008;26(1):101-6.
25. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131(5):861-72.
26. Campbell KH, McWhir J, Ritchie WA, Wilmut I. Sheep cloned by nuclear transfer from a cultured cell line. Nature 1996;380(6569):64-6.
27. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126(4):663-76.
28. Yu J, Vodyanik MA, Smuga-Otto K, et al. Induced pluripotent stem cell lines derived from human somatic cells. Science 2007;318(5858):1917-20.
29. Zaltzman JS, Pei Y, Maurer J, Patterson A, Cattran DC. Cyclosporine nephrotoxicity in lung transplant recipients. Transplantation 1992;54(5):875-8.
30. Carstens J, Andersen HK, Spencer E, Madsen M. Cytomegalovirus infection in renal transplant recipients. Transpl Infect Dis 2006;8(4):203-12.
31. Penn I. Malignant melanoma in organ allograft recipients. Transplantation 1996;61(2):274-8.
32. Penn I. Why do immunosuppressed patients develop cancer? Critical reviews in oncogenesis 1989;1(1):27-52.
33. Goulmy E, Termijtelen A, Bradley BA, van Rood JJ. Y-antigen killing by T cells of women is restricted by HLA. Nature 1977;266(5602):544-5.
34. Mach B. Genetics of histocompatibility. Current opinion in hematology 1994;1(1):4-11.

35. Krishnan NS, Higgins RM, Lam FT, et al. HA-1 mismatch has significant effect in chronic allograft nephropathy in clinical renal transplantation. Transplantation proceedings 2007;39(5):1439-45.
36. Gerrits JH, van de Wetering J, Postma S, et al. Stable T-cell reactivity after successful tapering of azathioprine in HLA-identical living-related kidney transplant recipients despite minor histocompatibility antigen mismatches. Nephrol Dial Transplant 2007;22(2):353-61.
37. Clausen J. Stem cells, nuclear transfer and respect for embryos. Human reproduction and genetic ethics;16(1):48-59.
38. Gore A, Li Z, Fung HL, et al. Somatic coding mutations in human induced pluripotent stem cells. Nature;471(7336):63-7.
39. Hussein SM, Batada NN, Vuoristo S, et al. Copy number variation and selection during reprogramming to pluripotency. Nature;471(7336):58-62.
40. Bonde S, Zavazava N. Immunogenicity and engraftment of mouse embryonic stem cells in allogeneic recipients. Stem cells (Dayton, Ohio) 2006;24(10):2192-201.
41. Drukker M, Katchman H, Katz G, et al. Human embryonic stem cells and their differentiated derivatives are less susceptible to immune rejection than adult cells. Stem cells (Dayton, Ohio) 2006;24(2):221-9.
42. Drukker M, Katz G, Urbach A, et al. Characterization of the expression of MHC proteins in human embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America 2002;99(15):9864-9.
43. Fandrich F, Lin X, Chai GX, et al. Preimplantation-stage stem cells induce long-term allogeneic graft acceptance without supplementary host conditioning. Nature medicine 2002;8(2):171-8.
44. Koch CA, Geraldes P, Platt JL. Immunosuppression by embryonic stem cells. Stem cells (Dayton, Ohio) 2008;26(1):89-98.
45. Li L, Baroja ML, Majumdar A, et al. Human embryonic stem cells possess immune-privileged properties. Stem cells (Dayton, Ohio) 2004;22(4):448-56.
46. Magliocca JF, Held IK, Odorico JS. Undifferentiated murine embryonic stem cells cannot induce portal tolerance but may possess immune privilege secondary to reduced major histocompatibility complex antigen expression. Stem cells and development 2006;15(5):707-17.
47. Menard C, Hagege AA, Agbulut O, et al. Transplantation of cardiac-committed mouse embryonic stem cells to infarcted sheep myocardium: a preclinical study. Lancet 2005;366(9490):1005-12.
48. Robertson NJ, Brook FA, Gardner RL, Cobbold SP, Waldmann H, Fairchild PJ. Embryonic stem cell-derived tissues are immunogenic but their inherent immune privilege promotes the induction of tolerance. Proceedings of the National Academy of Sciences of the United States of America 2007;104(52):20920-5.
49. Swijnenburg RJ, Tanaka M, Vogel H, et al. Embryonic stem cell immunogenicity increases upon differentiation after transplantation into ischemic myocardium. Circulation 2005;112(9 Suppl):I166-72.
50. Verloes A, Van de Velde H, LeMaoult J, et al. HLA-G expression in human embryonic stem cells and preimplantation embryos. J Immunol 2011;186(4):2663-71.

51. Wu DC, Boyd AS, Wood KJ. Embryonic stem cells and their differentiated derivatives have a fragile immune privilege but still represent novel targets of immune attack. Stem cells (Dayton, Ohio) 2008;26(8):1939-50.
52. Yachimovich-Cohen N, Even-Ram S, Shufaro Y, Rachmilewitz J, Reubinoff B. Human Embryonic Stem Cells Suppress T Cell Responses via Arginase I-Dependent Mechanism. J Immunol 2009(184):1300-8.
53. Kleinsmith LJ, Pierce GB, Jr. Multipotentiality of Single Embryonal Carcinoma Cells. Cancer research 1964;24:1544-51.
54. Solter D, Skreb N, Damjanov I. Extrauterine growth of mouse egg-cylinders results in malignant teratoma. Nature 1970;227(5257):503-4.
55. Stevens LC. The development of transplantable teratocarcinomas from intratesticular grafts of pre- and postimplantation mouse embryos. Developmental biology 1970;21(3):364-82.
56. Motzer RJ, Amsterdam A, Prieto V, et al. Teratoma with malignant transformation: diverse malignant histologies arising in men with germ cell tumors. The Journal of urology 1998;159(1):133-8.
57. Logothetis CJ, Samuels ML, Trindade A, Johnson DE. The growing teratoma syndrome. Cancer 1982;50(8):1629-35.
58. International, Society, For, Stem, Cell, Research. Guidelines for the Clinical Translation of Stem Cells: ISSCR Publications; 2008.
59. International, Society, For, Stem, Cell, Research. Guidelines for the Conduct of Human Embryonic Stem Cell Research: ISSCR Publications; 2006.
60. Nichols J, Zevnik B, Anastassiadis K, et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 1998;95(3):379-91.
61. Chambers I, Colby D, Robertson M, et al. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 2003;113(5):643-55.
62. Mitsui K, Tokuzawa Y, Itoh H, et al. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 2003;113(5):631-42.
63. Solter D, Knowles BB. Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). Proceedings of the National Academy of Sciences of the United States of America 1978;75(11):5565-9.
64. Berstine EG, Hooper ML, Grandchamp S, Ephrussi B. Alkaline phosphatase activity in mouse teratoma. Proceedings of the National Academy of Sciences of the United States of America 1973;70(12):3899-903.
65. Loh YH, Wu Q, Chew JL, et al. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nature genetics 2006;38(4):431-40.
66. Hough SR, Clements I, Welch PJ, Wiederholt KA. Differentiation of mouse embryonic stem cells after RNA interference-mediated silencing of OCT4 and Nanog. Stem cells (Dayton, Ohio) 2006;24(6):1467-75.
67. Hyslop L, Stojkovic M, Armstrong L, et al. Downregulation of NANOG induces differentiation of human embryonic stem cells to extraembryonic lineages. Stem cells (Dayton, Ohio) 2005;23(8):1035-43.

68. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. Experimental cell research 1961;25:585-621.
69. Shahdadfar A, Fronsdal K, Haug T, Reinholt FP, Brinchmann JE. In vitro expansion of human mesenchymal stem cells: choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptome stability. Stem cells (Dayton, Ohio) 2005;23(9):1357-66.
70. Becker KA, Ghule PN, Therrien JA, et al. Self-renewal of human embryonic stem cells is supported by a shortened G1 cell cycle phase. Journal of cellular physiology 2006;209(3):883-93.
71. Suda Y, Suzuki M, Ikawa Y, Aizawa S. Mouse embryonic stem cells exhibit indefinite proliferative potential. Journal of cellular physiology 1987;133(1):197-201.
72. Zwaka TP, Thomson JA. Differentiation of human embryonic stem cells occurs through symmetric cell division. Stem cells (Dayton, Ohio) 2005;23(2):146-9.
73. Nichols J, Chambers I, Taga T, Smith A. Physiological rationale for responsiveness of mouse embryonic stem cells to gp130 cytokines. Development (Cambridge, England) 2001;128(12):2333-9.
74. Niwa H, Burdon T, Chambers I, Smith A. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes & development 1998;12(13):2048-60.
75. Ying QL, Nichols J, Chambers I, Smith A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell 2003;115(3):281-92.
76. Xu RH, Peck RM, Li DS, Feng X, Ludwig T, Thomson JA. Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nature methods 2005;2(3):185-90.
77. Vallier L, Alexander M, Pedersen RA. Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. Journal of cell science 2005;118(Pt 19):4495-509.
78. Levenstein ME, Ludwig TE, Xu RH, et al. Basic fibroblast growth factor support of human embryonic stem cell self-renewal. Stem cells (Dayton, Ohio) 2006;24(3):568-74.
79. Yoshida K, Chambers I, Nichols J, et al. Maintenance of the pluripotential phenotype of embryonic stem cells through direct activation of gp130 signalling pathways. Mechanisms of development 1994;45(2):163-71.
80. Wang G, Zhang H, Zhao Y, et al. Noggin and bFGF cooperate to maintain the pluripotency of human embryonic stem cells in the absence of feeder layers. Biochemical and biophysical research communications 2005;330(3):934-42.
81. Bendall SC, Hughes C, Campbell JL, et al. An enhanced mass spectrometry approach reveals human embryonic stem cell growth factors in culture. Mol Cell Proteomics 2009;8(3):421-32.
82. Wang D, Matsumoto R, You Y, et al. CD3/CD28 costimulation-induced NF-kappaB activation is mediated by recruitment of protein kinase C-theta, Bcl10, and IkappaB kinase beta to the immunological synapse through CARMA1. Molecular and cellular biology 2004;24(1):164-71.

83. Wu AM, Till JE, Siminovitch L, McCulloch EA. A cytological study of the capacity for differentiation of normal hemopoietic colony-forming cells. Journal of cellular physiology 1967;69(2):177-84.
84. Becker AJ, Mc CE, Till JE. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature 1963;197:452-4.
85. Morrison SJ, Wandycz AM, Hemmati HD, Wright DE, Weissman IL. Identification of a lineage of multipotent hematopoietic progenitors. Development (Cambridge, England) 1997;124(10):1929-39.
86. Martin GR, Evans MJ. The morphology and growth of a pluripotent teratocarcinoma cell line and its derivatives in tissue culture. Cell 1974;2(3):163-72.
87. Jami J, Ritz E. Multipotentiality of single cells of transplantable teratocarcinomas derived from mouse embryo grafts. Journal of the National Cancer Institute 1974;52(5):1547-52.
88. McBurney MW. Clonal lines of teratocarcinoma cells in vitro: differentiation and cytogenetic characteristics. Journal of cellular physiology 1976;89(3):441-55.
89. Brinster RL. The effect of cells transferred into the mouse blastocyst on subsequent development. The Journal of experimental medicine 1974;140(4):1049-56.
90. Bradley A, Evans M, Kaufman MH, Robertson E. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature 1984;309(5965):255-6.
91. Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America 1993;90(18):8424-8.
92. Beddington RS, Robertson EJ. An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. Development (Cambridge, England) 1989;105(4):733-7.
93. Schwartzberg PL, Goff SP, Robertson EJ. Germ-line transmission of a c-abl mutation produced by targeted gene disruption in ES cells. Science 1989;246(4931):799-803.
94. Doetschman TC, Eistetter H, Katz M, Schmidt W, Kemler R. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. Journal of embryology and experimental morphology 1985;87:27-45.
95. Jensen J, Hyllner J, Bjorquist P. Human embryonic stem cell technologies and drug discovery. Journal of cellular physiology 2009;219(3):513-9.
96. Owen R. Immunogetic consequences of vascular anastomoses between bovine twins. . Science 1945;102:400-1.
97. Anderson D, Billingham RE, Medawar PB. The use of skin grafting to distinguish between monozygotic and dizygotic twins in cattle. . Heredity 1951;6:379-97.
98. Billingham RE, Brent L, Medawar PB. Actively acquired tolerance of foreign cells. Nature 1953;172(4379):603-6.
99. Ostensen M, Villiger PM. Immunology of pregnancy-pregnancy as a remission inducing agent in rheumatoid arthritis. Transplant immunology 2002;9(2-4):155-60.

100. Ishitani A, Sageshima N, Lee N, et al. Protein expression and peptide binding suggest unique and interacting functional roles for HLA-E, F, and G in maternal-placental immune recognition. J Immunol 2003;171(3):1376-84.
101. Munn DH, Zhou M, Attwood JT, et al. Prevention of allogeneic fetal rejection by tryptophan catabolism. Science 1998;281(5380):1191-3.
102. Hunt JS, Vassmer D, Ferguson TA, Miller L. Fas ligand is positioned in mouse uterus and placenta to prevent trafficking of activated leukocytes between the mother and the conceptus. J Immunol 1997;158(9):4122-8.
103. Aluvihare VR, Kallikourdis M, Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. Nature immunology 2004;5(3):266-71.
104. Lin H, Mosmann TR, Guilbert L, Tuntipopipat S, Wegmann TG. Synthesis of T helper 2-type cytokines at the maternal-fetal interface. J Immunol 1993;151(9):4562-73.
105. Cadet P, Rady PL, Tyring SK, Yandell RB, Hughes TK. Interleukin-10 messenger ribonucleic acid in human placenta: implications of a role for interleukin-10 in fetal allograft protection. American journal of obstetrics and gynecology 1995;173(1):25-9.
106. Cheng HL, Schneider SL, Kane CM, et al. TGF-beta 2 gene and protein expression in maternal and fetal tissues at various stages of murine development. Journal of reproductive immunology 1993;25(2):133-48.
107. Haupl T, Ostensen M, Grutzkau A, Radbruch A, Burmester GR, Villiger PM. Reactivation of rheumatoid arthritis after pregnancy: increased phagocyte and recurring lymphocyte gene activity. Arthritis and rheumatism 2008;58(10):2981-92.
108. Zhao T, Zhang ZN, Rong Z, Xu Y. Immunogenicity of induced pluripotent stem cells. Nature;474(7350):212-5.
109. Lindahl KF, Wilson DB. Histocompatibility antigen-activated cytotoxic T lymphocytes. II. Estimates of the frequency and specificity of precursors. The Journal of experimental medicine 1977;145(3):508-22.
110. Marrack P, Kappler J. T cells can distinguish between allogeneic major histocompatibility complex products on different cell types. Nature 1988;332(6167):840-3.
111. Jones G. The number of reactive cells in mouse lymphocyte cultures stimulated by phytohemagglutinin, concanavalin A or histocompatibility antigen. J Immunol 1973;111(3):914-20.
112. Sheldon S, Poulton K. HLA typing and its influence on organ transplantation. Methods in molecular biology (Clifton, NJ 2006;333:157-74.
113. Akatsuka Y, Nishida T, Kondo E, et al. Identification of a polymorphic gene, BCL2A1, encoding two novel hematopoietic lineage-specific minor histocompatibility antigens. The Journal of experimental medicine 2003;197(11):1489-500.
114. Terakura S, Murata M, Warren EH, et al. A single minor histocompatibility antigen encoded by UGT2B17 and presented by human leukocyte antigen-A*2902 and -B*4403. Transplantation 2007;83(9):1242-8.
115. Shimmura H, Tanabe K, Tokumoto T, et al. Impact of HLA-identity on results of ABO-incompatible living kidney transplantation. Transplantation proceedings 2004;36(7):2172-4.

116. Jeon BJ, Kim IG, Seong YK, Han BH. Analysis of the Results of ABO-Incompatible Kidney Transplantation: In Comparison with ABO-Compatible Kidney Transplantation. Korean journal of urology 2010;51(12):863-9.
117. Sumitran-Holgersson S. Beyond ABO and human histocompatibility antigen: other histocompatibility antigens with a role in transplantation. Current opinion in organ transplantation 2008;13(4):425-9.
118. Lechler RI, Batchelor JR. Restoration of immunogenicity to passenger cell-depleted kidney allografts by the addition of donor strain dendritic cells. The Journal of experimental medicine 1982;155(1):31-41.
119. Reed AJ, Noorchashm H, Rostami SY, et al. Alloreactive CD4 T cell activation in vivo: an autonomous function of the indirect pathway of alloantigen presentation. J Immunol 2003;171(12):6502-9.
120. Hornick PI, Mason PD, Yacoub MH, Rose ML, Batchelor R, Lechler RI. Assessment of the contribution that direct allorecognition makes to the progression of chronic cardiac transplant rejection in humans. Circulation 1998;97(13):1257-63.
121. Huang JF, Yang Y, Sepulveda H, et al. TCR-Mediated internalization of peptide-MHC complexes acquired by T cells. Science 1999;286(5441):952-4.
122. Tsang JY, Chai JG, Lechler R. Antigen presentation by mouse CD4+ T cells involving acquired MHC class II:peptide complexes: another mechanism to limit clonal expansion? Blood 2003;101(7):2704-10.
123. Larsen CP, Steinman RM, Witmer-Pack M, Hankins DF, Morris PJ, Austyn JM. Migration and maturation of Langerhans cells in skin transplants and explants. The Journal of experimental medicine 1990;172(5):1483-93.
124. Zhou P, Hwang KW, Palucki D, et al. Secondary lymphoid organs are important but not absolutely required for allograft responses. Am J Transplant 2003;3(3):259-66.
125. Pietra BA, Wiseman A, Bolwerk A, Rizeq M, Gill RG. CD4 T cell-mediated cardiac allograft rejection requires donor but not host MHC class II. The Journal of clinical investigation 2000;106(8):1003-10.
126. Reiser JB, Darnault C, Guimezanes A, et al. Crystal structure of a T cell receptor bound to an allogeneic MHC molecule. Nature immunology 2000;1(4):291-7.
127. Kreisel D, Krupnick AS, Gelman AE, et al. Non-hematopoietic allograft cells directly activate CD8+ T cells and trigger acute rejection: an alternative mechanism of allorecognition. Nature medicine 2002;8(3):233-9.
128. Hancock WW, Sayegh MH, Zheng XG, Peach R, Linsley PS, Turka LA. Costimulatory function and expression of CD40 ligand, CD80, and CD86 in vascularized murine cardiac allograft rejection. Proceedings of the National Academy of Sciences of the United States of America 1996;93(24):13967-72.
129. Yamada And A, Sayegh MH. The CD154-CD40 costimulatory pathway in transplantation. Transplantation 2002;73(1 Suppl):S36-9.
130. Blazar BR, Sharpe AH, Chen AI, et al. Ligation of OX40 (CD134) regulates graft-versus-host disease (GVHD) and graft rejection in allogeneic bone marrow transplant recipients. Blood 2003;101(9):3741-8.
131. Krummel MF, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. The Journal of experimental medicine 1995;182(2):459-65.

132. Judge TA, Wu Z, Zheng XG, Sharpe AH, Sayegh MH, Turka LA. The role of CD80, CD86, and CTLA4 in alloimmune responses and the induction of long-term allograft survival. J Immunol 1999;162(4):1947-51.
133. Zheng XX, Sayegh MH, Zheng XG, et al. The role of donor and recipient B7-1 (CD80) in allograft rejection. J Immunol 1997;159(3):1169-73.
134. Kim KS, Denton MD, Chandraker A, et al. CD28-B7-mediated T cell costimulation in chronic cardiac allograft rejection: differential role of B7-1 in initiation versus progression of graft arteriosclerosis. The American journal of pathology 2001;158(3):977-86.
135. Gramaglia I, Weinberg AD, Lemon M, Croft M. Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. J Immunol 1998;161(12):6510-7.
136. Chen AI, McAdam AJ, Buhlmann JE, et al. Ox40-ligand has a critical costimulatory role in dendritic cell:T cell interactions. Immunity 1999;11(6):689-98.
137. Yuan X, Salama AD, Dong V, et al. The role of the CD134-CD134 ligand costimulatory pathway in alloimmune responses in vivo. J Immunol 2003;170(6):2949-55.
138. Cella M, Engering A, Pinet V, Pieters J, Lanzavecchia A. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. Nature 1997;388(6644):782-7.
139. Sallusto F, Cella M, Danieli C, Lanzavecchia A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. The Journal of experimental medicine 1995;182(2):389-400.
140. Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. The Journal of experimental medicine 2001;193(2):233-8.
141. Hawiger D, Inaba K, Dorsett Y, et al. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. The Journal of experimental medicine 2001;194(6):769-79.
142. Sparwasser T, Koch ES, Vabulas RM, et al. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. Eur J Immunol 1998;28(6):2045-54.
143. Freeman GJ, Long AJ, Iwai Y, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. The Journal of experimental medicine 2000;192(7):1027-34.
144. Latchman Y, Wood CR, Chernova T, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nature immunology 2001;2(3):261-8.
145. Carter L, Fouser LA, Jussif J, et al. PD-1:PD-L inhibitory pathway affects both CD4(+) and CD8(+) T cells and is overcome by IL-2. Eur J Immunol 2002;32(3):634-43.
146. Pearson TC, Alexander DZ, Winn KJ, Linsley PS, Lowry RP, Larsen CP. Transplantation tolerance induced by CTLA4-Ig. Transplantation 1994;57(12):1701-6.
147. Lin H, Bolling SF, Linsley PS, et al. Long-term acceptance of major histocompatibility complex mismatched cardiac allografts induced by CTLA4Ig plus donor-specific transfusion. The Journal of experimental medicine 1993;178(5):1801-6.

148. Li Y, Li XC, Zheng XX, Wells AD, Turka LA, Strom TB. Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance. Nature medicine 1999;5(11):1298-302.
149. Taylor PA, Lees CJ, Waldmann H, Noelle RJ, Blazar BR. Requirements for the promotion of allogeneic engraftment by anti-CD154 (anti-CD40L) monoclonal antibody under nonmyeloablative conditions. Blood 2001;98(2):467-74.
150. Nussbaum J, Minami E, Laflamme MA, et al. Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. Faseb J 2007;21(7):1345-57.
151. Swijnenburg RJ, Schrepfer S, Govaert JA, et al. Immunosuppressive therapy mitigates immunological rejection of human embryonic stem cell xenografts. Proceedings of the National Academy of Sciences of the United States of America 2008;105(35):12991-6.
152. Jash A, Kwon HK, Sahoo A, et al. Topical application of porcine placenta extract inhibits the progression of experimental contact hypersensitivity. Journal of ethnopharmacology;133(2):654-62.
153. He H, Li W, Chen SY, et al. Suppression of activation and induction of apoptosis in RAW264.7 cells by amniotic membrane extract. Investigative ophthalmology & visual science 2008;49(10):4468-75.
154. Lutz MB, Schuler G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? Trends in immunology 2002;23(9):445-9.
155. Lee MG, Borkowski TA, Udey MC. Regulation of expression of B7 by murine Langerhans cells: a direct relationship between B7 mRNA levels and the level of surface expression of B7 by Langerhans cells. The Journal of investigative dermatology 1993;101(6):883-6.
156. Harding FA, McArthur JG, Gross JA, Raulet DH, Allison JP. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. Nature 1992;356(6370):607-9.
157. Linsley PS, Brady W, Grosmaire L, Aruffo A, Damle NK, Ledbetter JA. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. The Journal of experimental medicine 1991;173(3):721-30.
158. Lanier LL, O'Fallon S, Somoza C, et al. CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL. J Immunol 1995;154(1):97-105.
159. Freeman GJ, Gribben JG, Boussiotis VA, et al. Cloning of B7-2: a CTLA-4 counter-receptor that costimulates human T cell proliferation. Science 1993;262(5135):909-11.
160. Zhou LJ, Tedder TF. Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. J Immunol 1995;154(8):3821-35.
161. Reid CD, Stackpoole A, Meager A, Tikerpae J. Interactions of tumor necrosis factor with granulocyte-macrophage colony-stimulating factor and other cytokines in the regulation of dendritic cell growth in vitro from early bipotent CD34+ progenitors in human bone marrow. J Immunol 1992;149(8):2681-8.
162. Peters JH, Xu H, Ruppert J, Ostermeier D, Friedrichs D, Gieseler RK. Signals required for differentiating dendritic cells from human monocytes in vitro. Advances in experimental medicine and biology 1993;329:275-80.

163. Beyth S, Borovsky Z, Mevorach D, et al. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. Blood 2005;105(5):2214-9.
164. Macatonia SE, Hosken NA, Litton M, et al. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. J Immunol 1995;154(10):5071-9.
165. Rissoan MC, Soumelis V, Kadowaki N, et al. Reciprocal control of T helper cell and dendritic cell differentiation. Science 1999;283(5405):1183-6.
166. van Beelen AJ, Zelinkova Z, Taanman-Kueter EW, et al. Stimulation of the intracellular bacterial sensor NOD2 programs dendritic cells to promote interleukin-17 production in human memory T cells. Immunity 2007;27(4):660-9.
167. Chen W, Jin W, Hardegen N, et al. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. The Journal of experimental medicine 2003;198(12):1875-86.
168. Oppmann B, Lesley R, Blom B, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity 2000;13(5):715-25.
169. Linke A, Kuhn R, Muller W, Honarvar N, Li C, Langhorne J. Plasmodium chabaudi chabaudi: differential susceptibility of gene-targeted mice deficient in IL-10 to an erythrocytic-stage infection. Experimental parasitology 1996;84(2):253-63.
170. Kehrl JH, Wakefield LM, Roberts AB, et al. Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. The Journal of experimental medicine 1986;163(5):1037-50.
171. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986;136(7):2348-57.
172. Langrish CL, Chen Y, Blumenschein WM, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. The Journal of experimental medicine 2005;201(2):233-40.
173. McGeachy MJ, Bak-Jensen KS, Chen Y, et al. TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. Nature immunology 2007;8(12):1390-7.
174. Takahashi T, Kuniyasu Y, Toda M, et al. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. International immunology 1998;10(12):1969-80.
175. Bonnard GD, Yasaka K, Jacobson D. Ligand-activated T cell growth factor-induced proliferation: absorption of T cell growth factor by activated T cells. J Immunol 1979;123(6):2704-8.
176. Budd RC, Cerottini JC, Horvath C, et al. Distinction of virgin and memory T lymphocytes. Stable acquisition of the Pgp-1 glycoprotein concomitant with antigenic stimulation. J Immunol 1987;138(10):3120-9.
177. Moretta A, Poggi A, Pende D, et al. CD69-mediated pathway of lymphocyte activation: anti-CD69 monoclonal antibodies trigger the cytolytic activity of different lymphoid effector cells with the exception of cytolytic T lymphocytes expressing T cell receptor alpha/beta. The Journal of experimental medicine 1991;174(6):1393-8.

178. Testi R, Phillips JH, Lanier LL. T cell activation via Leu-23 (CD69). J Immunol 1989;143(4):1123-8.
179. Samelson LE. Signal transduction mediated by the T cell antigen receptor: the role of adapter proteins. Annual review of immunology 2002;20:371-94.
180. Isakov N, Altman A. Human T lymphocyte activation by tumor promoters: role of protein kinase C. J Immunol 1987;138(10):3100-7.
181. Touraine JL, Hadden JW, Touraine F, Hadden EM, Estensen R, Good RA. Phorbol myristate acetate: a mitogen selective for a T-lymphocyte subpopulation. The Journal of experimental medicine 1977;145(2):460-5.
182. McCrady CW, Ely CM, Westin E, Carchman RA. Coordination and reversibility of signals for proliferative activation and interleukin-2 mRNA production in resting human T lymphocytes by phorbol ester and calcium ionophore. The Journal of biological chemistry 1988;263(34):18537-44.
183. Passalacqua M, Pedrazzi M, Sparatore B, Patrone M, Pontremoli S, Melloni E. Functional role of the charge at the T538 residue in the control of protein kinase Ctheta. Archives of biochemistry and biophysics 2009;481(2):202-9.
184. Sanchez-Lockhart M, Marin E, Graf B, et al. Cutting edge: CD28-mediated transcriptional and posttranscriptional regulation of IL-2 expression are controlled through different signaling pathways. J Immunol 2004;173(12):7120-4.
185. Weiss A, Irving BA, Tan LK, Koretzky GA. Signal transduction by the T cell antigen receptor. Semin Immunol 1991;3(5):313-24.
186. Tessier S, Karczewski P, Krause EG, et al. Regulation of the transient outward K(+) current by Ca(2+)/calmodulin-dependent protein kinases II in human atrial myocytes. Circulation research 1999;85(9):810-9.
187. Merritt JE, Armstrong WP, Benham CD, et al. SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. The Biochemical journal 1990;271(2):515-22.
188. Borel JF, Feurer C, Gubler HU, Stahelin H. Biological effects of cyclosporin A: a new antilymphocytic agent. Agents and actions 1976;6(4):468-75.
189. Borel JF, Meszaros J. Skin transplantation in mice and dogs. Effect of cyclosporin A and dihydrocyclosporin C. Transplantation 1980;29(2):161-2.
190. Drukker M. Recent advancements towards the derivation of immune-compatible patient-specific human embryonic stem cell lines. Semin Immunol 2008;20(2):123-9.
191. Reddy KR, Lilie H, Rudolph R, Lange C. L-Arginine increases the solubility of unfolded species of hen egg white lysozyme. Protein Sci 2005;14(4):929-35.
192. Zanin-Zhorov A, Ding Y, Kumari S, et al. Protein kinase C-theta mediates negative feedback on regulatory T cell function. Science;328(5976):372-6.
193. Valenzuela JO, Iclozan C, Hossain MS, et al. PKCtheta is required for alloreactivity and GVHD but not for immune responses toward leukemia and infection in mice. The Journal of clinical investigation 2009;119(12):3774-86.
194. Jeon MS, Atfield A, Venuprasad K, et al. Essential role of the E3 ubiquitin ligase Cbl-b in T cell anergy induction. Immunity 2004;21(2):167-77.
195. Soto-Nieves N, Puga I, Abe BT, et al. Transcriptional complexes formed by NFAT dimers regulate the induction of T cell tolerance. The Journal of experimental medicine 2009;206(4):867-76.

196. Macian F, Garcia-Cozar F, Im SH, Horton HF, Byrne MC, Rao A. Transcriptional mechanisms underlying lymphocyte tolerance. Cell 2002;109(6):719-31.
197. Macian F, Garcia-Rodriguez C, Rao A. Gene expression elicited by NFAT in the presence or absence of cooperative recruitment of Fos and Jun. The EMBO journal 2000;19(17):4783-95.
198. Kriegel MA, Rathinam C, Flavell RA. E3 ubiquitin ligase GRAIL controls primary T cell activation and oral tolerance. Proceedings of the National Academy of Sciences of the United States of America 2009;106(39):16770-5.
199. Kandilci A, Grosveld GC. SET-induced calcium signaling and MAPK/ERK pathway activation mediate dendritic cell-like differentiation of U937 cells. Leukemia 2005;19(8):1439-45.

Appendix:

Curriculum Vitae

Education

2011 **University of Ottawa,** Ottawa Ontario Canada

Ph.D. candidate: *Department of Biochemistry, Microbiology and Immunology*

Thesis: “Stem cell derived extracts possess novel immune suppressive properties and may have *in-vivo* therapeutic potential.”

Supervisor: Dr. Lisheng Wang

2007 **University of Western Ontario,** London Ontario Canada

M.Sc.: *Department of Microbiology and Immunology*

Thesis: “Indoleamine-2,3-dioxygenase induces renal tubular epithelial cell injury following ischemia reperfusion.”

Supervisor: Dr. Anthony M. Jevnikar

2004 **University of Waterloo,** Waterloo Ontario Canada

B.Sc. Honours Biology

Honours thesis: “CK-1: A putative rainbow trout chemokine.”

Supervisor: Dr. Brian Dixon

Publications

Peer reviewed Manuscripts

Mohib K, Al-Khamees B, Allan DS, Wang L. Embryonic stem cell-derived factors inhibit T effector activation and induce T regulatory cells by suppressing PKC-θ activation. *PloS One.* 2012 Mar; 7(3): e32420.

Mohib K, Allan DS, Wang L. Human embryonic stem cell-extracts inhibit the differentiation and function of monocyte-derived dendritic cells**.** *Stem Cell Rev*. 2010 Dec; 6(4): 611-621**.**

Li L, Wang BH, Wang S, Moalim-Nour L, **Mohib K**, Lohnes D, Wang L. Individual cell movement, asymmetric colony expansion, rho-associated kinase, and e-cadherin impact the clonogenicity of human embryonic stem cells. *Biophys J.* 2010 Jun 2;98(11):2442-51.

Li L, Wang S, Jezierski A, Moalim-Nour L, **Mohib K**, Parks RJ, Retta SF, Wang L. A unique interplay between Rap1 and E-cadherin in the endocytic pathway regulates self-renewal of human embryonic stem cells. *Stem Cells*. 2010 Feb;28(2):247-57.

Conley AJ, **Mohib K**, Menassa R, Jevnikar AM, Brandle JE. Recombinant erythropoietin produced in transgenic tobacco attenuates tissue injury in a kidney cell model. *Plant Biotechnol J.* 2009 Feb;7(2):183-99. Epub 2008 Nov 26.

Mohib K, Wang S, Guan Q, Mellor AL, Sun H, Du C, Jevnikar AM. Indoleamine 2,3-dioxygenase expression promotes renal ischemia-reperfusion injury*. Am J Physiol Renal Physiol.* 2008 Jul; 295(1): F226-34.

Mohib K, Guan Q, Dao H, Du C, Jevnikar AM. Pro-apoptotic activity of indoleamine 2,3-dioxygenase expressed in renal tubular epithelial cells. *Am J Physiol Renal Physiol.* 2007 Sep; 292(3): F801-12.

Book Chapters

Mohib K and Wang L. Differentiation and characterization of dendritic cells from human embryonic stem cells**.** *Current Protocols in Immunology***. Book Chapter submitted.**

Published Abstracts

Mohib K, Al-Khamees B, Allan DS, Wang L. Embryonic stem cell derived factors inhibit T effector cell activation and induce T regulatory cells by modulating PKC-θ activation. *J. Immunol.* 182: 63.29.

Wang S, Jiang J, **Mohib K**, Sun T, Garcia B, Wang H, Zhang Z, Mellor AL, Du C, Jevnikar AM. Deficiency in donor kidney Indoleamine 2,3-dioxygenase improves ischemia reperfusion injury (IRI) but accelerates allograft loss in murine model. *Am J Trans.* 2008 April; Suppl. 8(2): 129.

Khan KA, Wang S, Zhang Z, Huang Z, **Mohib K**, Yin Z, Qin HY, Singh B, Jevnikar AM. Murine serpin inhibitor protease 6 (PI6) expression by tubular epithelial cells (TEC) may provide endogenous resistance to endogenous granzyme B injury*. Am J Trans*. 2008 April; Suppl. 8(2): 129.

Mohib K, Guan Q, Dao H, Du C, Jevnikar AM. Renal indoleamine 2,3- dioxygenase (IDO) metabolites promotes Fas/FasL dependent tubular epithelial cell (TEC) self injury during inflammation*. Am J Trans***.** 2007 May;Suppl. 2(7): 388.

Mohib K, Wang S, Guan Q, Zhong R, Du C, Jevnikar AM. Indoleamine 2,3-dioxygenase (IDO) expression in renal tubular epithelial cells contributes to renal ischemia reperfusion injury*. Am J Trans*. 2006 Aug; Suppl. 6(2): 200-201.

Presentations

June 2011 **The International Society for Stem cell Research 9th Annual Meeting.** *Poster Presentation.* Kanishka Mohib, Al-Khamees B, David S. Allan, Lisheng Wang. "*Embryonic stem cell derived factors inhibit T effector cell activation and induce T regulatory cells by inhibiting PKC-θ activation"*

May 2011 **The American Association of Immunologists 98th Annual Meeting.** *Poster Presentation.* Kanishka Mohib, Al-Khamees B, David S. Allan, Lisheng Wang. "*Embryonic stem cell derived factors inhibit T effector*

cell activation and induce T regulatory cells by inhibiting PKC-θ activation"

Oct. 2010 **Yale University School of Medicine, Department of Immunobiology.** *Oral presentation.* Kanishka Mohib, David S. Allan and Lisheng Wang. "*Human embryonic stem cell-extracts inhibit the differentiation and function of monocyte-derived dendritic cells.*"

May 2010 **University of Ottawa, Department of Biochemistry, Microbiology and Immunology Graduate Seminar Symposium.** *Poster Presentation*
Kanishka Mohib, David S. Allan and Lisheng Wang. "*Human embryonic stem cell-extracts inhibit the differentiation and function of monocyte-derived dendritic cells.*"

Feb. 2009 **University of Ottawa, Department of Biochemistry, Microbiology and Immunology Graduate Seminar Symposium.** *Oral Presentation*. Kanishka Mohib, David S. Allan and Lisheng Wang. "*Stem cell derived extracts possess novel immune suppressive properties and may have in-vivo therapeutic potential."*

May 2007 **American Transplant Congress:** *Poster Presentation*.
Kanishka Mohib, Qiunong Guan , Hong Dao, Caigan Du, Anthony M. Jevnikar. "*Renal indoleamine 2,3- dioxygenase (IDO) metabolites promotes Fas/FasL dependent tubular epithelial cell (TEC) self injury during inflammation."*

March 2007 **Canadian Society of Transplantation**: *Oral Presentation.*
Kanishka Mohib, Shuang Wang, Qiunong Guan, Caigan Du, Anthony M. Jevnikar: "*Indoleamine 2,3-dioxygenase enhances renal tubular epithelial apoptosis and ischemia reperfusion injury through the build up of kynurenine enzymatic pathway catabolites*"

July 2006 **World Transplant Congress:** *Oral Presentation*
Kanishka Mohib, Shuang Wang, Qiunong Guan, Robert Zhong, Caigan Du, Anthony M. Jevnikar: *"Indoleamine 2,3-dioxygenase (IDO) expression in renal tubular epithelial cells contributes to renal ischemia reperfusion injury."*

May 2006 **University of Western Ontario Department of Medicine Research Day:** *Poster Presentation***.** Kanishka Mohib, Shuang Wang, Qiunong Guan, Robert Zhong, Caigan Du and Anthony M. Jevnikar: "*Indoleamine-2,3-dioxygenase (IDO) activity induces renal tubular epithelial cell death and renal ischemia reperfusion injury."*

March 2006 **Canadian Society of Transplantation:** *Oral Presentation.*
Kanishka Mohib, Shuang Wang, Qiunong Guan, Robert Zhong, Caigan Du and Anthony M. Jevnikar: *"Inhibition of indoleamine-2,3-dioxygenase (IDO) activity prevents renal ischemia reperfusion injury."*

Nov. 2005 Robarts Research Institute: *Poster Presentation.*

Kanishka Mohib, Shuang Wang, Qiunong Guan, Robert Zhong, Caigan Du and Anthony M. Jevnikar: *"Indoleamine-2,3-dioxygenase (IDO) activity may contribute renal tubular epithelial cell injury as a result of ischemia reperfusion."*

Feb. 2005 **Lawson Health Research Institute Sister Mary Doyle Research Day:** *Poster Presentation.*
Kanishka Mohib, Shuang Wang, Qiunong Guan, Robert Zhong, Caigan Du and Anthony M. Jevnikar: *"Indoleamine-2,3-dioxygenase (IDO) influences renal tubular epithelial cell death under inflammatory conditions."*

Patents

2009 Wang L, **Mohib K**, Li L, Allan D. Stem cell extracts and uses thereof for immune modulation. United States of America Patent. 61/258,300.

Awards And Honours

2011-12 Canadian Blood Services Graduate Fellowship Award: *Research Award ($42,000)*

2010-12 University of Ottawa Excellence Award: *Distinction ($12,000)*

2008-10 Canadian Blood Services Graduate Fellowship Award: *Research Award ($42,000)*

2008-10 University of Ottawa Excellence Award: *Distinction ($12,000)*

2007-08 University of Ottawa Admission Award: *Distinction ($6,000)*

2007 American Transplant Congress: *Poster of Distinction Award*

2006 World Transplant Congress: *Young Investigator Award ($1,000)*

2004-06	University of Western Ontario: *Faculty of Graduate Studies Scholarship* *($9,000)*
2004	Canadian Millennium Scholarship *($2,500)*
2003	University of Waterloo Faculty of Science: *Dean's Honour Roll*
2004	University of Waterloo Faculty of Science: *Dean's Honour Roll*
2002	Canada Millennium Scholarship *($2,500)*
2000	Province of Ontario Scholar

Professional Activity and University Service

2010	Clinical Observer ship - Dr. David Allan (Hematologist), Bone Marrow Transplant Clinic, Ottawa General Hospital
2010	Host-Moderator - University of Ottawa, Microbiology and Immunology Mini Seminar Symposium
2009-11	Student Representative - University of Ottawa, Department of Microbiology and Immunology Graduate Committee Member
2009-10	Faculty of Medicine Evacuation Team Member
2007-08	Volunteer - Department of Biochemistry, Microbiology and Immunology Recruitment Day
2006	Surgical Observer ship - Dr. Chris Nguan (Urologist), Kidney Transplant Clinic, University Hospital, London Health Sciences Center
2005-06	Society of Graduate Studies Representative for the Department of Microbiology and Immunology, University of Western Ontario
2005-06	Director of Hospitality University of Western Ontario, Department of Microbiology and Immunology 3M Distinguished Lectureship Committee
2005-06	Robarts Research Institute "Ice Breaker" Organizing Committee
2005-07	Representative for Department of Microbiology & Immunology University of Western Ontario Open Day

Teaching Experience

2011	Mentor for Postdoctoral Fellow - Dr. Wang, Stem Cell Research Laboratory
2009-11	Mentor for M.Sc. student - Dr. Wang, Stem Cell Research Laboratory
2007	Instructor/Volunteer - YMCA After School Science Program
2005-07	Mentor for visiting/summer students - Dr. Jevnikar, Basic Transplant Immunology Laboratory
2006	Graduate Teaching Assistant - University of Western Ontario, Department of Microbiology and Immunology

Membership in Professional Societies

2011-12	The International Society for Stem Cell Research
2006-08	Canadian Society of Transplantation

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