DEPLOYING THE TOLEROGENIC EFFECTS OF IDO ENZYME AND SKIN FIBROBLASTS IN PREVENTION OF GRAFT REJECTION

by

Mohsen Khosravi Maharlooei

MD, Shiraz University of Medical Sciences, 2011

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

August 2017

Abstract

Indoleamine 2,3-dioxygenase (IDO) is an immunosuppressive enzyme with tolerogenic effects on different immune cells. Our group has previously shown that co-transplantation of IDO-expressing fibroblasts with donor tissues can delay immune rejection by inducing local immunosuppression. We first asked a question whether we can improve this effect by delivering the IDO-fibroblasts through a systemic intraperitoneal approach, instead of local co-transplantation, and secondly whether this effect is only delivered by the immunosuppressive effects of IDO or the fibroblast cells have additional immunosuppressive effects.

We employed a systemic approach to improve allograft survival without using any immunosuppressive medication. To achieve this, 10 million lentiviral transduced IDO-expressing donor derived fibroblasts were injected into the peritoneal cavity of allograft recipients. We showed that IDO-fibroblast therapy increases the survival of both islets and skin allografts and decreases the infiltration of immune cells in subcutaneous transplanted skins. Indirect pathway of allo-reactive T cell activation was suppressed more than the direct pathway. Injected IDO-fibroblasts were found in peritoneal cavity and mesenteric lymph nodes of the recipient mice. In conclusion, fibroblasts have tolerogenic effects on DCs and IDO-expressing fibroblast therapy proved to be a novel approach in improving the allogeneic graft survival.

There is controversy about the immunomodulatory effect of fibroblasts on dendritic cells (DCs). In a mouse model, we showed that intra- peritoneal injection (IP) of both syngeneic and allogeneic fibroblasts significantly increased the expression level of co-inhibitory and co-stimulatory molecules on DCs. Priming of DCs with syngeneic and allogeneic fibroblasts reduced the proliferation of CD4+ and CD8+ T cells. Even activation of fibroblast-primed DCs failed to restore their ability to induce T-cell proliferation. Likewise, priming of DCs with fibroblasts blocked the ability of ovalbumin-pulsed DCs to induce proliferation of ovalbumin-specific CD4+ T cells. Compared with non-activated DCs, fibroblast-primed DCs had significantly higher expression levels of interleukin-10 and IDO. Fibroblast-primed DCs had a significantly reduced interleukin- 12 expression level compared with that of activated DCs. After priming with fibroblasts, DCs were able to migrate to lymphatic tissues and present fibroblast-derived antigens (ovalbumin).

Lay Summary

Every year, irreversible damage to the body organs such as liver, pancreas, kidney, etc cause hundreds of thousands of deaths, unless transplantation is done to replace these organs. The immune system of recipients naturally recognizes the transplanted organs as foreigners in the body, and therefore it starts attacking and destroying the life-saving transplant, which is the main challenge of organ transplantation. We engineered skin fibroblast cells obtained from the organ donor to express an immunosuppressive molecule in order to instruct the recipient's immune system not to react to the transplanted organ. We tested two different transplantation settings in mouse models, including pancreatic islet transplantation for treatment of type 1 diabetes and skin transplantation that is used in situations of extensive skin loss such as burns. Our approach delayed graft rejection without using the immunosuppressive medications. Our approach can be combined with immunosuppressive medications to reduce the toxicity of these medications.

PREFACE

This thesis was compiled under the guidance of Dr. Aziz Ghahary who devised the original concept for the research.

Chapters 2 and 3 are based on work conducted in the BCPFF Burn and Wound Firefighters Laboratory under the supervision of Dr. A. Ghahary. I was responsible for designing the experiments, performing them, analyzing the results and writing the manuscripts.

A version of Chapter 2 is also published. M Khosravi-Maharlooei, MR Pakyari, R B. Jalili, R T. Kilani, A Ghahary (2016) Intraperitoneal injection of IDO-expressing dermal fibroblasts improves the allograft survival. Clinical Immunology. I designed and conducted the experiments, analysed the data and wrote the manuscript, which was critically reviewed by Dr. R B. Jalili and Dr. A. Ghahary. M Pakyari, R B. Jalili and R T. Kilani helped me in performing part of the experiments.

A version of Chapter 3 is published. M Khosravi-Maharlooei, MR Pakyari, R B. Jalili, S Salimi-Elizei, J C. Y. Lai, M Poormasjedi-Meibod, R T. Kilani, J Dutz and A Ghahary. (2016) Tolerogenic effect of mouse fibroblasts on dendritic cells. Immunology. I designed and conducted the experiments, analysed the data and wrote the manuscript, which was critically reviewed by Dr. R B. Jalili, Dr. J Dutz and Dr. A. Ghahary. M Pakyari, R B. Jalili, S Salimi-Elizei, J C. Y. Lai, M Poormasjedi-Meibod and R T. Kilani helped me in performing part of the experiments.

Check the first pages of these chapters to see footnotes for similar information.

The work described in this thesis has been conducted with the approval of the University of British Columbia Biohazards Committee under the certificate number B09-0298. All animal studies have been conducted under the close supervision of the University of British Columbia Animal Care Committee and under the protocol number A10-0147.

TABLE OF CONTENTS

LIST OF FIGURES

Acknowledgements

I would like to thank Dr. Aziz Ghahary for having me in his lab.

I would like to thank the members of my Masters advisory committee at the University of British Columbia; Dr. Megan Levings and Dr. Pauline Johnson for their great guidance and advice regarding my research.

I am also greatly thankful to Dr. Reza Jalili who has been a great mentor, support and colleague for me. Dr. Jalili's role for my research was critical.

Many thanks also to my laboratory colleagues Dr. Ruhangiz Kilani, Dr. MohammadReza Pakyari, Dr. Sanam Salili and Mr. Ali Farrokhi.

I am very grateful to the Transplant Training Program, CIHR Skin Research Training Centre, Friedman Foundation and UBC graduate studies for their financial assistance.

Finally, I would like to mention my family. Thank you for your love and support.

Dedication

This work is dedicated to:

My Mother, Ashraf

My father, Aziz

My sister, Elham

and

My brother, Erfan

Chapter 1

Introduction and specific aims

1.1. Overview

Organ transplantation is the standard therapeutic practice for end-stage organ failure in modern medicine. Although current immunosuppressive regimens provide short-term protection against immune rejection of transplanted organs, long-term outcomes remain unsatisfactory [1]. Moreover, the general immunosuppression state caused by these medications makes patients susceptible to increased risk of infection and tumor development [2,3]. Therefore, there is a need for novel strategies to induce long-term, drug-free, graft acceptance with normal graft function, a condition defined as "transplantation tolerance". The approaches that specifically induce tolerance against donor antigens are more preferred.

1.2. Pancreatic islet transplantation

Pancreatic islet transplantation has been introduced as an alternative approach to transplantation of the pancreas to replace the lost beta cells in type 1 diabetes. This procedure does not require major surgery and few complications arise. However, lifelong immunosuppression is still needed to preserve the transplanted islets. Although during the past two decades significant progress in islet transplantation conditions and outcomes has been achieved, challenges remain that hinder the use of this therapy as a widely available treatment for T1D. The major challenges of islet transplantation could be categorized into four distinct categories relative to the transplantation time: islet source limitation, sub-optimal engraftment of islets, lack of oxygen and blood supply, and immune rejection [4]. As shown in Fig 1.1, one of the major challenges of islet transplantation is immune rejection.

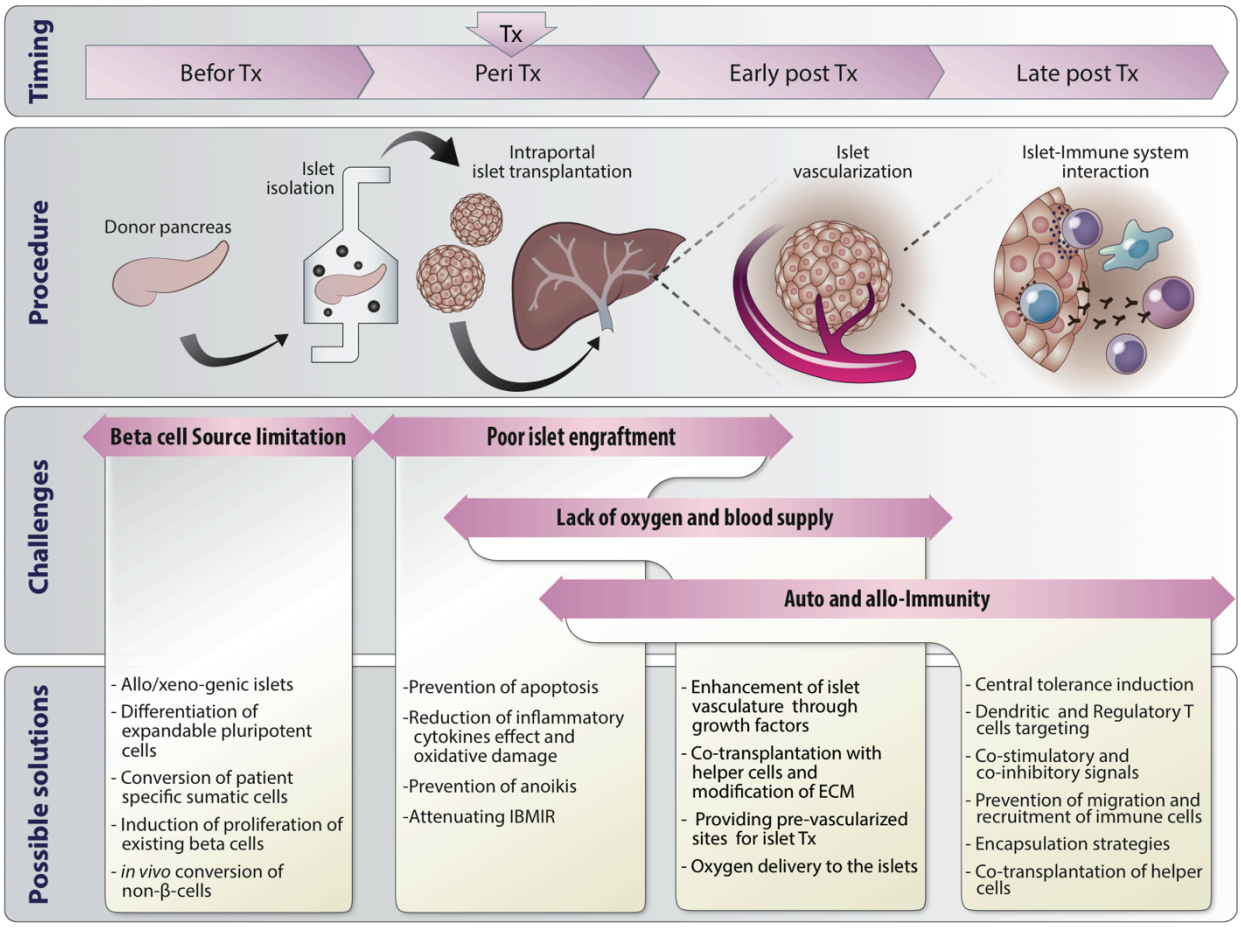

Figure 1.1. Major challenges of islet transplantation.

1.3. Skin transplantation

Skin is considered one of the most antigeneic tissues, because the dermis and epidermis are full of antigen presenting cells, including dendritic cells [5,6]. Therefore, grafted cadaveric skins activate the recipient immune system and induce rapid rejection, and thus only provide temporary wound coverage [7]. As burn patients are prone to infections[8], immunosuppressant medications cannot be used to prevent immune rejection of skin allo-grafts. Therefore, an alternative approach to induce immune tolerance to allogeneic skin grafts is very crucial.

1.4. Indoleamine 2,3 dioxygenase (IDO), an immunomodulatory enzyme

In 1998, Mellor group proved that IDO expression in maternal-fetal interface is needed to protect the fetus from maternal immune system attack [9]. Since then, IDO has been considered as a promising potent immunosuppressive molecule for induction of immune tolerance either in transplantation setting or autoimmunity or even allergies. IDO is an enzyme that is physiologically responsible for metabolizing the amino acid tryptophan. This immunosuppressive effect of IDO is attributed to both local depletion of tryptophan and generation of by-products of tryptophan metabolism [10–12]. Both local depletion of tryptophan which triggers the amino-acid sensing signal-transduction pathways and production of by-products like kynurenine, a natural ligand for the aryl hydrocarbon receptor (AhR), are considered as the ways through which IDO applies its immunoregulatory effect [13]. There is a non-enzymatic effect described for IDO as well, through which IDO contributes to TGF-β-driven tolerance in noninflammatory contexts and maintenance of a stable regulatory phenotype in plasmacytoid DCs [14].

1.5. Effect of IDO on T cells

Effect of IDO on CD8 T cells is through the molecular stress-response pathway, GCN2, which in turn leads to anergy [15,16]. Especially, it has been shown that overexpression of IDO in vivo suppresses the formation of both central memory CD8+ T cells (T(CM)) and effector memory CD8+ T cells (T(EM)) [17]. IDO also reduces the cytotoxic effects of CD8 T cells which is accompanied by defects in production of granule cytotoxic proteins, including perforin and granzyme A and B [18]. Effects on CD4 T cells include generation of regulatory T cells from uncommitted CD4 T cells [19–21] and also blockade of Th1 and Th17 differentiation [22–24].

1.6. IDO-based therapies in transplantation

Several groups have tried to increase the survival of allografts by delivery of IDO gene to the donor or recipient organs. The results have been controversial. Laurence J. et al. showed that IDO gene delivery with an adeno-associated virus to the rat donor liver does not increase the allograft survival [25]. However, it has been shown that IDO gene transfer can prolong cardiac [26,27] and pancreatic islet allograft [28,29] survival. All these experiments used adeno- or adeno associated viruses for gene delivery, which are known to be transiently present.

As an alternative approach, some groups have over-expressed IDO in DCs and have injected those cells to the recipients to increase the graft survival. Funeshima et al. showed that injection of IDO-DCs into the footpads of C57BL/6 mice caused a reduced T-cell response against allo-antigen [30]. Also, it has been shown that pre-treatment of recipient mice with donor derived DCs overexpressing IDO increases skin graft survival [31]. It has been recently shown that IDO expressing donor DCs can enhance small bowel transplant survival in mouse allogeneic setting mostly through expansion of regulatory T cells [32].

Expression of IDO in other cells rather than DCs has also been shown to block T cell proliferation. Kupffer cells pre-treated with IFN-gamma to express IDO were able to inhibit allogeneic T-cell response [33]. Likewise, it has been shown that inhibition of allogeneic T-cell responses by human bone marrow stromal cells relies on IDO-mediated tryptophan degradation [34]. With regard to tumor induced T cell tolerance, it has been shown that IDO expression by myeloid-derived suppressor cells plays an essential role [35].

1.7. Our IDO-based therapeutic approach

Our group has developed a new way of IDO delivery though overexpressing it in skin fibroblasts in order to prevent graft rejection [36,37]. As fibroblasts live longer than DCs, they might keep high level of IDO

expression for a longer duration in the body. Besides, it is easier to transduce these cells. Additionally, as fibroblasts are differentiated cells and part of their genome is inactive, there would be lower risk of insertion of virus into undesired parts and eventually lower risk of tumor formation. All together, it makes them a translationally desirable carrier cell type to deliver IDO to the recipient body.

Our group has previously shown that co-transplantation of IDO expressing fibroblasts with the allografts can increase the survival of skin [38] and islet [39,40] tissues. In the first study, IDO was overexpressed in syngeneic fibroblasts using an adenoviral vector, which were co-transplanted with allogeneic islets in a three-dimensional composite under the kidney capsule of recipient mice. It was shown that as long as IDO is expressed locally, T cells cannot enter the graft and blood glucose is normal. However, as adenovirus mediated expression of IDO is transient, we tested a lentiviral vector. We have shown that lentivirus transduced fibroblasts stably express IDO even at very high passages in vitro. However, although local co-transplantation of these cells with allogeneic islets under kidney capsule of recipient mice prolonged graft survival, this effect was still transient (51.0 ± 2.9 days vs 11.6 ± 1.5 days in control group)[40]. That is why we thought of a systemic approach to affect the immune system in a way that favours the protection of allografts. In this study, we investigated the effect of intraperitoneal (IP) injection of IDO fibroblasts as the systemic method of IDO delivery to the allograft recipients in two models of skin and pancreatic islet transplantation. Also, we tried to find out the underlying mechanisms involved in protection of allografts. Besides, we investigated the pure effect of fibroblasts on the recipient immune system.

1.8. Effect of fibroblasts on recipient immune system antigen presenting cells

DCs are the most important antigen presenting cells in the body and are responsible for activation of T cells against foreign antigens as well as regulating the immune response against self-molecules. It is well known that cytokines secreted from other types of cells affect the fate of DCs and ultimately, the subtypes

of induced T cells[41]. Skin is a barrier to protect the body from foreign antigens, but they can enter when this barrier is breached. Langerhans cells and other DCs in the skin engulf these antigens and present them to the naïve T cells located within the skin draining lymph nodes[42]. On the other hand, dermal fibroblasts are the major cells in proximity of DCs during their migration toward lymph nodes. There is a controversy in the literature about the effect of fibroblasts on DCs. Some studies are in favor of stimulatory effect of fibroblasts on DCs. A series of studies by Simon's group shows that fibroblasts can induce maturation of DCs[43], promote migration of DCs[44] and support expansion of IL-17 producing T cells[45]. On the other hand, there are some clues in favor of immunoregulatory effects of fibroblasts on DCs. For instance, it has been shown that fibroblasts can reduce the production of IL12-p70 upon activation of DCs[46]. Also, it has been shown that cell-cell contact with fibroblasts is required for the suppression of MHC class II and CD40 on DCs, while soluble prostaglandin E2 (PGE2) released by fibroblasts is responsible for suppression of production of IL-12 and TNF-α by DCs[47].

So far, we know that the effect of other types of stromal cells on DCs is to some extent tolerogenic. For instance, mesenchymal stem cells (MSCs) are known for their tolerogenic effects on DCs. It has been shown that MSCs can impair the ability of ovalbumin-pulsed DCs *in vivo* to induce ovalbumin-specific T cell proliferation. However, this effect was mostly attributed to decreased migration of ovalbumin-pulsed DCs injected subcutaneously to the mouse recipients of MSCs[48]. Also, it has been shown that hematopoietic stem cell derived DCs cultured *in vitro* with MSCs are defective in stimulating allo-reactive T cells and this effect is being attributed to activation of Notch pathway in DCs[49]. MSC-treated mature DCs gain immature phenotype and it cannot be reversed by LPS activation[50]. MSCs from other sources rather than bone marrow, including umbilical cord blood can have a similar impact on DCs[51]. Further, renal fibroblasts are shown to induce increased expression of co-inhibitory molecules, B7H1 and B7DC on DCs, in addition to decreased expression of IL-12. Also, renal fibroblast conditioned DCs are less potent in stimulating T cell proliferation[52].

As reviewed above, there is no consensus about the effect of fibroblasts, which reside in almost all tissues, on DCs. Understanding more about this interaction might shed light on the physiology of cell-cell interactions in the skin. Besides, it can help us design cell therapy approaches to treat skin related diseases.

1.9. Hypothesis

In the current study, we aimed to develop a strategy for induction of a systemic and alloantigen-specific tolerance via intraperitoneal (IP) injection of IDO-expressing primary dermal fibroblasts at the time of transplantation. We hypothesized that IP injection of IDO-fibroblasts will prevent skin and islet allograft rejection and induces a donor-specific immune tolerance through different mechanisms including: 1) Suppression of alloreactive T cell clones, 2) Generation of donor-specific Tregs and 3) Induction of a tollerogenic phenotype in antigen presenting cells without compromising the general immune system.

Besides, we hypothesized that conditioning of DCs with dermal fibroblasts can convert the immature DCs to tolerogenic ones. Tolerogenic DCs are a subset of DCs that are responsible for regulating the immune responses[53]. The main features of these cells include high expression level of co-inhibitory molecules, IL-10 and indoleamine 2, 3 dioxygenase (IDO) and low expression of IL-12, the ability to induce anergy in effector T cells, expansion of regulatory T cells, and migratory capacity to T-cell areas in secondary lymphoid tissues[54]. In this study, we tried to test these features in DCs upon encountering the fibroblasts.

1.10. Aims

Aim 1: Evaluating skin and islet allograft survival after IP injection of stable IDO-expressing primary dermal fibroblasts in recipient mice.

Aim 2: Evaluating the effect of fibroblasts on recipient DCs.

Chapter 2

Intraperitoneal injection of IDO-expressing dermal fibroblasts improves the allograft survival

Indoleamine 2,3-dioxygenase (IDO) is an immunosuppressive enzyme with tolerogenic effects on different immune cells. Our group has previously shown that co-transplantation of IDO-expressing fibroblasts with donor tissues can delay immune rejection by inducing local immunosuppression. In this study, we have employed a systemic approach to improve allograft survival without using any immunosuppressive medication. To achieve this, 10 million lentiviral transduced IDO-expressing donor derived fibroblasts were injected into the peritoneal cavity of allograft recipients. We showed that IDO-fibroblast therapy increases the survival of both islets and skin allografts and decreases the infiltration of immune cells in subcutaneous transplanted skins. Indirect pathway of allo-reactive T cell activation was suppressed more than the direct pathway. Injected IDO-fibroblasts were found in peritoneal cavity and mesenteric lymph nodes of the recipient mice. In conclusion, IDO-expressing fibroblast therapy proved to be a novel approach in improving the allogeneic graft survival.

2.1. Methods

2.2. Mice

8-week-old female C3H/Hej (C3H), male C57BL/6 (B6) and Balb/c mice were purchased from Jackson Laboratories, Bar Harbor, ME and kept in Blusson Spinal Cord Injury Center Animal Care Facility in an environmentally controlled room (temperature, 24±2°C; humidity, 55±5%, 12-h light/dark cycle) with access to food and water. The University of British Columbia Animal Care Committee (UBC-ACC) has approved this study. All animals were maintained and undergone procedures in accordance with the principles of laboratory animal care and the guidelines of the UBC-ACC.

2.3. Mouse dermal fibroblast culture and viral transduction

As we described before [40], a 2x2cm piece of full thickness skin from back of a sacrificed B6 mouse (after hair removal with hair clipper and sterilization with povidone iodine) was used to culture fibroblasts. Washed several times in PBS containing 2% penicillin/streptomycin, the hypodermis fat tissue was removed with a sterile blade in sterile condition. The skin was cut into small pieces and each piece was put on the 150x25mm culture dish (Corning, NY) with the dermis facing down. Small drops of FBS, was put onto each piece of skin and the dish was kept in 37^{o}C incubator supplemented with 5% CO2 for 4 hours. Afterwards, DMEM media (Hyclone, Utah) containing 10% FBS and 1% penicillin/streptomycin was added very slowly to the dish. Detachment of the pieces from dish surface should be avoided. After one to two weeks, the fibroblasts migrate out of the skin and adhere to the dish surface. Then, the cells were subcultured.

Fibroblasts were transduced with a lentiviral vector that contained IDO and mCherry under the control of EF1-alpha promoter described before [55]. Transduced cells were isolated by fluorescence-activated cell sorting (FACS). For functional quality control, prior to the cell transplantation, the kynurenine level (byproduct of IDO enzyme activity in tryptophan degradation pathway) was measured in fibroblast-conditioned medium collected 72 hours after viral transduction by a procedure described before [55]. Briefly, proteins in the conditioned medium were precipitated by trichloroacetic acid (TCA). After centrifugation, 0.5 ml of supernatant was incubated with an equal volume of Ehrlich's reagent at room temperature for 10 min. The reaction mixture was measured spectrophotometrically at 490 nm. The concentration of kynurenine in the conditioned medium was calculated according to a standard curve of defined kynurenine concentration. Only IDO-fibroblasts with kynurenine level of above 13μg/ml were used for transplantation studies.

2.4. Mouse islet isolation

Pancreatic islets were obtained from 6- to 8-week-old male C57BL/6 (B6) mice (The Jackson Laboratories, Bar Harbor, ME) as previously reported [40]. Briefly, immediately after euthanizing the mice, 3 ml of Hanks' balanced salt solution (HBSS; Life Technologies, Gaithersburg, MD) containing 1 mg/ml of collagenase (Type V; Sigma Chemical Co., St. Louis, MO) were injected through the pancreatic duct into the pancreases. Then, after removal of the pancreases with scissors, they were kept on ice to prevent collagenase activity. While all the pancreases were removed, they were digested at 37°C for 10 min in a 50 ml tube containing collagenase. After shaking the tube for 30 seconds, the digested pancreatic tissue was washed with HBSS containing %0.25 BSA and passed through a metal mesh to remove the fat and fibrotic tissues. Afterwards, the suspension was passed through 100 μm cell strainer (BD Biosciences, Bedford, MA) to isolate the big islets that were trapped on the strainer. In the next step, the flow-through was passed through a 70 μm cell strainer to isolate the remaining small islets. Finally, to increase the purity of isolated islets, they were handpicked, counted and cultured in HAM's F10 medium (Sigma) supplemented with 2 mmol/L L-glutamine, 10% heat-inactivated fetal calf serum, penicillin, and streptomycin in 95% air, 5% CO2 at 37° C. Islets were counted the next day and transplanted to the recipient mice.

2.5. Transplantation of islets in fibroblast IP injected diabetic mice and blood glucose monitoring

First, recipient Balb/c mice became diabetic by IP injection of 200mg/kg streptozotocin (Sigma). Diabetes was defined as a minimum of two consecutive blood glucose measurements of above 15mmol/l. After proof of diabetes induction, recipient mice received IP injection of either 10^7 B6 fibroblasts, B6 IDO-fibroblasts or vehicle medium (500μl of DMEM medium containing 10% FBS). Two to three days later, the recipient mice were anesthetized with isoflurane and 500 B6 pancreatic islets were transplanted under the left kidney capsule. Blood glucose level was checked every other day after transplantation using an

Accu-Chek Compact Plus blood glucose monitoring system. Graft rejection was defined as two consecutive blood glucose measurement of above 10mmol/l [39].

2.6. Skin transplantation on the back site

Three days before skin transplantation, recipient mice received IP injection of either 10^7 B6 fibroblasts, B6 IDO-fibroblasts or vehicle medium. At the day of transplantation, donor B6 mice were sacrificed and the tails were cut to harvest the tail skin. The tail was prepped with povidone-iodine and washed with PBS. Then, the skin was harvested from tail by making a longitudinal incision on the tail using a sterile blade. The donor skin was cut into 6mm round pieces by punch biopsy tool. The skin was placed into petri dish containing saline afterward and kept on ice.

Recipient C3H mice were anesthetized using isoflurane. The back area was shaved and swabbed with povidone-iodine. Meloxicam was injected SQ as the painkiller. Graft bed was prepared by removing a circular 6mm diameter full thickness skin from the back of the recipient mice using curved scissors. The grafts were placed on the graft bed and sutured to the surrounding skin by 7.0 prolene sutures in 4 points and the gaps between these were sutured by 4 to 8 stiches of 8.0 nylon. Opsite was sprayed on top of the grafts and the wounds then were covered by Tegaderm film and coflex band-aid. Mice were checked until full recovery. The next doses of painkiller were given every 12h until two days after surgery. The bandages were removed at day 7. Images from the site of transplantation were taken every four days afterwards. To prevent drying the transplanted skin, the graft site was covered with a small gauze, which in turn was attached to the surrounding skin with a tape. At each time point to take pictures, the bandade was removed and changed.

2.7. Skin transplantation into subcutaneous space

As an alternative method, we developed a method to transplant the donor skin in sub-cutaneous space of recipient mice. Similarly, three days before skin transplantation, recipient mice received IP injection of either 10^7 B6 fibroblasts, B6 IDO-fibroblasts or vehicle medium. As the day of transplantation, donor skin was prepared as the previous method from B6 mouse tail. Recipient C3H back area was also prepped as the previous method. However, instead of a circular cut, using scissors only a 5mm linear incision was made on the back of the mice. Surrounding subcutaneous connective tissues were separated with the tip of tweezer to make more space for the donor skin tissue. The 6mm diameter donor skin tissue was put in the created space and the incision site was sutured with 6.0 prolene stiches. Ten days after transplantation, the recipient mice in different groups were sacrificed and the graft was checked for infiltration of immune cells.

2.8. H&E and Immunofluorescent staining

Subcutaneous skin grafts and the recipient skin covering above that area were excised ten days after transplantation and were fixed in %4 formaldehyde over night. After embedding in paraffin, 5µm-thick sections were cut from the center of the graft and placed on superfrost plus glass slides (Fisher Scientific). Slides were stained with hematoxylin and eosin dyes to detect the amount of immune cell infiltration. Slides were visualized by Nicon Eclipse 80i (Japan) microscope. Photos were taken through NIS-Elements D version 4.00.03 software by Nicon DS Ri1 camera.

To detect T cell infiltration, slides were stained with CD3 antibody. Briefly, after deparaffinization and rehydration, heat induced antigen retrieval was done using sodium citrate buffer in a microwave. Afterwards, blocking was done with 10% goat serum in TBS for 2 hours at room temperature. Primary rabbit anti mouse CD3 antibody (Abcam, ab828) was added to the slides and incubated overnight at 2-8°C. After washing, Rhodamine goat anti rabbit IgG secondary antibody (R-6394, Life Technologies)

was added and kept for 1h at room temperature in a dark place. Finally, after washing the secondary antibody, DAPI mounting solution (Vector Laboratories) was added and the tissue was covered with a cover slip. The slides were visualized by Zeiss fluorescent microscope. Pictures were taken through AxioVision 4 software by AxioCam camera (Carl Zeiss).

2.9. T cell proliferation assay

C3H recipient mice received sub-cutaneous B6-skin grafts three days after being IP injected with either 10^7 B6 fibroblasts, B6 IDO-fibroblasts or vehicle medium (3 mice per group). Ten days after skin transplantation, recipient mice were sacrificed and pooled cells of spleen and mesenteric and skin draining lymph nodes were used as responder cells in MLR. Dendritic cells (DCs) from B6 and C3H mice were used as stimulator cells. DCs were differentiated in a 10 day differentiation protocol as previously published by Lutz et al [56]. At day 10, C3H DCs were loaded with B6 antigens (for 12 h) to enable them to present B6-derived antigens to B6-presensitized C3H T cells. To prepare the cell lysate, a pellet of 10^7 B6 bone marrow cells resuspended in 100µl PBS was frozen in -80 °C and thawed for four times, followed by passing through a 70µm cell strainer (Fisher Scientific) to remove the big chunks. From this solution, 10µl was added to each dish of C3H DCs [57]. Both B6 and C3H DCs were activated with 200ng/ml of the TLR9 agonist CpG oligodeoxynucleotide (ODN) 1826 (5'-TCCATGACGTTCCTGACGTT-3') (custom synthesized by Sigma-Aldrich (Oakville ON)) and 200ng/ml of LPS (Sigma). At day 11, DCs were washed and seeded in round-bottom 96-well plates at a concentration of $5x10^4$ cell/well. Pooled donor cells were stained with 2.5µg/ml CFSE for 10 minutes and added to DCs at a concentration of $4x10^5$ cells per well. After 4 days, cells were harvested, stained with anti-mouse CD3 APC (1:200, ebioscience) and CD4 Percp (1:200, ebioscience) and read with flowcytometer (BD Accuri C6) to detect T cell proliferation.

2.10. Evaluating the regulatory T cells (Tregs)

Spleen and MLN cells from the same recipients as MLR experiment were used to evaluate the Tregs. First, cells were stained with anti-mouse CD4 FITC (1:200, ebioscience) and anti-mouse CD25 APC (1:200, ebioscience). After 30 min incubation at 2-8°C, cells were washed and fixed and permeabilized (as the eBioscience protocol for intracellular staining) and then stained with anti-mouse FoxP3 PE (1:200, ebioscience). After another 30 min incubation and final washing steps, cells were read with flowcytometer (BD Accuri C6). 123count eBeads (eBioscience) were also added to the samples to count the total number of Tregs. For analysis, BD Accuri C6 software was used. Cells were first gated on CD4+ cells and double positive cells for CD25 and FoxP3 were considered as Tregs.

2.11. Tracking of IP injected fibroblasts

To track the injected cells in MLNs, recipient mice were sacrificed at weeks 2, 4 and 6 after IP injection of mCherry-IDO-fibroblasts. Peritoneal cavity was washed with 4ml of PBS. Peritoneal lavage cells were stained with anti mouse MHC-I (H-2Kb) APC (1:200, eBioscience) and read with flowcytometer to detect the mCherry positive cells. Also, mesenteric lymph nodes were frozen in cryomatrix (ThermoScientific, CA). Using the cryostat (ThermoScientific), 5 μm sections were obtained. The slides were fixed for 15 min in 1:1 acetone/ethanol followed by 15 min in PBS. The slides were visualized by Zeiss fluorescent microscope. Pictures were taken through AxioVision 4 software by AxioCam camera (Carl Zeiss).

2.12. Results

2.13. Intra peritoneal injection of IDO-expressing fibroblasts increases allograft skin survival

The skin transplant sites were checked every other day for signs of rejection, including the changes in colour, texture and size of the grafts. Photos were taken every 4 days. The rejection process was associated with scaling of epidermis followed by shrinkage of the graft site (Figure 2.1A). As shown in figure 2.1B, IDO-fib group significantly increased the skin graft survival as compared to both control ($p < 0.005$, $n = 5$) and B6-fibroblast ($p < 0.005$, $n = 4$). The mean (+/-SD) survival for control, B6-fibroblast and IDO-fibroblast groups were 19.6 (+/-2.3), 19.5 (+/-3) and 25 (+/-4.3), respectively. However, eventually the grafted skins were rejected in all groups. The log-rank test was used as the statistical test.

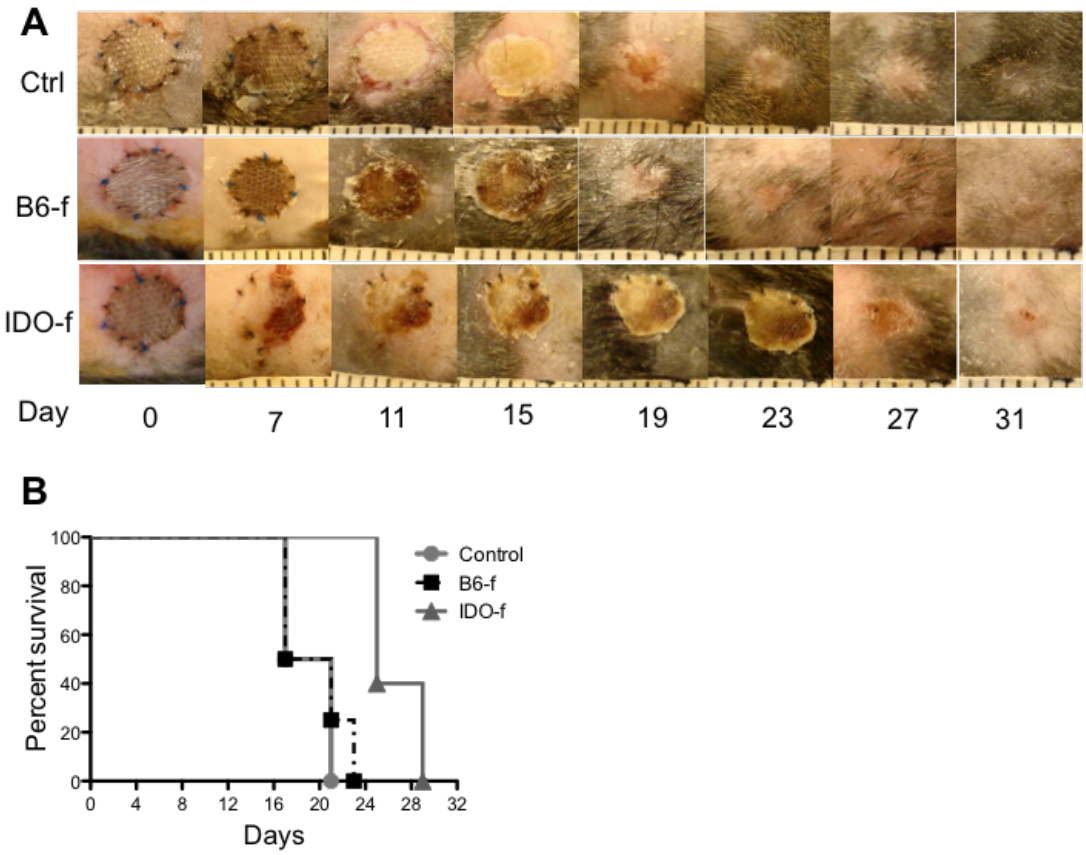

Figure 2.1. Skin graft survival. Skin grafts from B6 donor mice were transplanted on the back of recipient C3H mice. Four days before skin transplantation, recipient mice receive IP injection of either

IDO-expressing B6 fibroblasts (IDO-f), non-IDO expressing B6 fibroblasts (B6-f) or vehicle medium (Ctrl). (A) Shows the representative pictures of transplanted skin tissues during the course of follow up until rejection. (B) Shows the survival graph of all mice in three treatment groups. IDO-fib group significantly increased the graft survival compared to other two groups ($p=0.004$ comparing IDO-fib and control and $p=0.003$ comparing IDO-fib and B6-fib, $n=5$ in IDO-fib group and $n=4$ in other two groups).

2.14. Intraperitoneal injection of IDO-expressing fibroblasts decreases immune cells infiltration into the allo-skin tissue

Ten days after transplantation, the recipient mice were sacrificed; the transplanted skin was cut and processed for immunohistological staining. As shown in figure 2.2A, the thickness and cellularity of the transplanted skin is much lower in IDO-fibroblast treated group as compared to other groups. Also, the number of infiltrated T cells into the grafted skin was much reduced in IDO-fibroblast treated group as compared to B6-fibroblast and control groups (figure 2.2B).

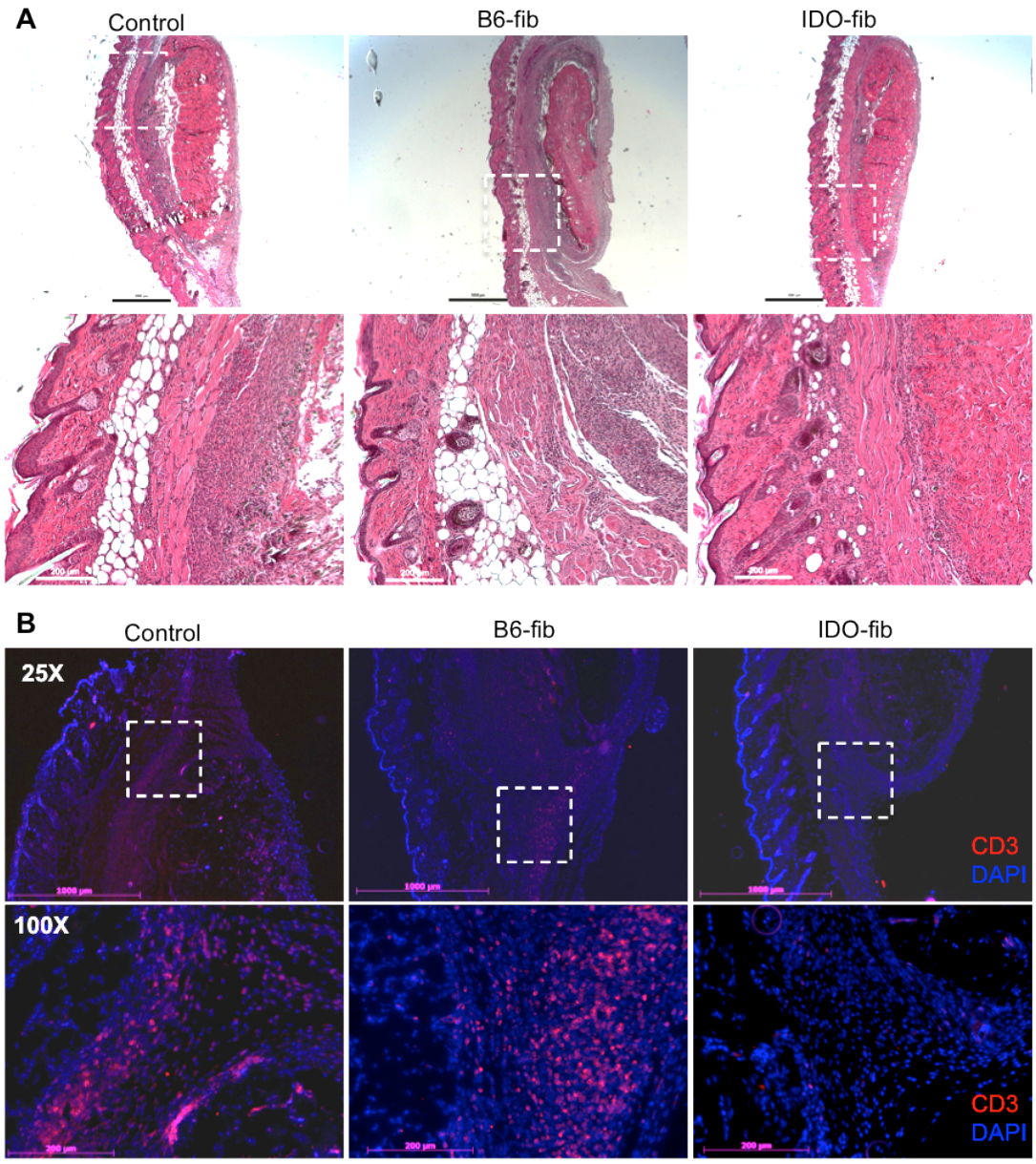

Figure 2.2. Infiltration of immune cells into sub-cutaneously transplanted skin grafts. Four days after IP injection of either IDO expressing B6 fibroblasts (IDO-f), regular B6 fibroblasts (B6-f) or vehicle medium (Control)**,** B6 donor tail skin pieces were transplanted in subcutaneous space of recipient C3H mice. Two weeks after transplant, mice were sacrifices and the grafts were fixed and slides were prepared from paraffin embedded blocks. (A) Shows the hematoxylin and eosin (H&E) staining and (B) shows the immune fluorescent staining with anti mouse CD3 antibody. CD3+ T cells are shown in red and nuclei staining with DAPI is shown in blue.

2.15. Intra peritoneal injection of IDO-expressing fibroblasts increases allograft pancreatic islet survival

Recipient mice that kept normoglycemia for more than 100 days were considered immune tolerant. As shown in figure 2.3, IDO-fibroblast treatment significantly increased the graft survival as compared to that of control group (p=0.026, n=5). The mean (+/-SD) survival for control, B6-fibroblast and IDO-fibroblast groups were 23.4 (+/-5.4), 39.6 (+/-15.5) and 61.4 (+/-16.3), respectively. Two mice in IDO-fib group reached immune tolerance. Although one of the mice in B6-fibroblast group reached tolerance, there was no significant difference between this group and control group.

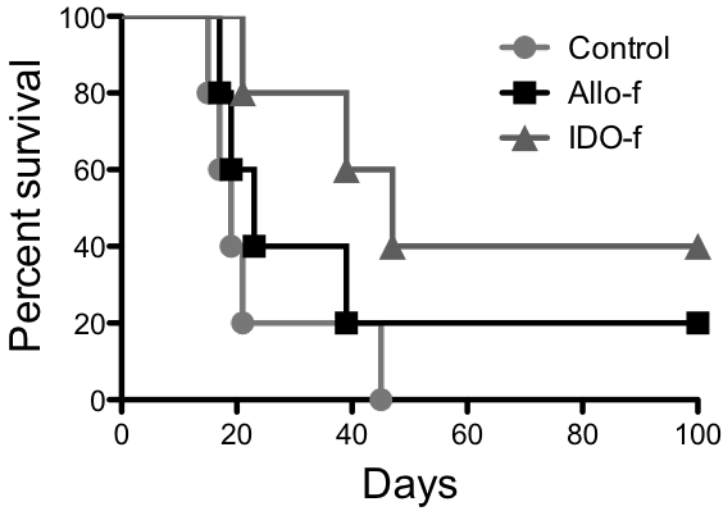

Figure 2.3. Pancreatic islet graft survival. Pancreatic islet transplantation was done from B6 to balb/c mice. Four days before islet transplantation, recipient mice in treatment groups were injected with ten million B6-fibroblasts or B6-IDO-fibroblasts dissolved in 500µl of DMEM containing 10% FBS in peritoneal cavity. In control group, the vehicle medium was injected without cells. IDO-fibroblast (IDO-f) treatment group significantly increased the graft survival compared to control groups (p=0.026, n=5).

2.16. Intra peritoneal injection of IDO-fibroblasts and non-IDO expressing B6-fibroblasts suppress indirectly-activated allo-reactive CD4 T cells

To test the mechanisms involved in prolongation of graft survival, we set MLR assays. Ten days after subcutaneous transplantation of B6-skin in different groups, splenocytes and LN cells from skin draining and mesenteric lymph nodes of recipient mice were isolated and pooled as responder cells. Proliferation of recipient T cells in response to donor and recipient dendritic cells (in vitro) was tested as a measure of recipient T cell allo-reactivity in direct and indirect pathways, respectively. As shown in figure 2.4, both B6-fibroblast and IDO-fibroblast treatments decreased the proliferation of recipient T cells in response to B6-loaded C3H DCs (indirect activation). However, only IDO-fibroblast treatment could significantly decrease the proliferation of recipient CD4 T cells in response to B6 DCs (direct activation) ($p < 0.05$, $n = 3$). Indeed, B6-fibroblast treatment even enhanced direct activation of recipient CD4 T cells (Figure 2.4).

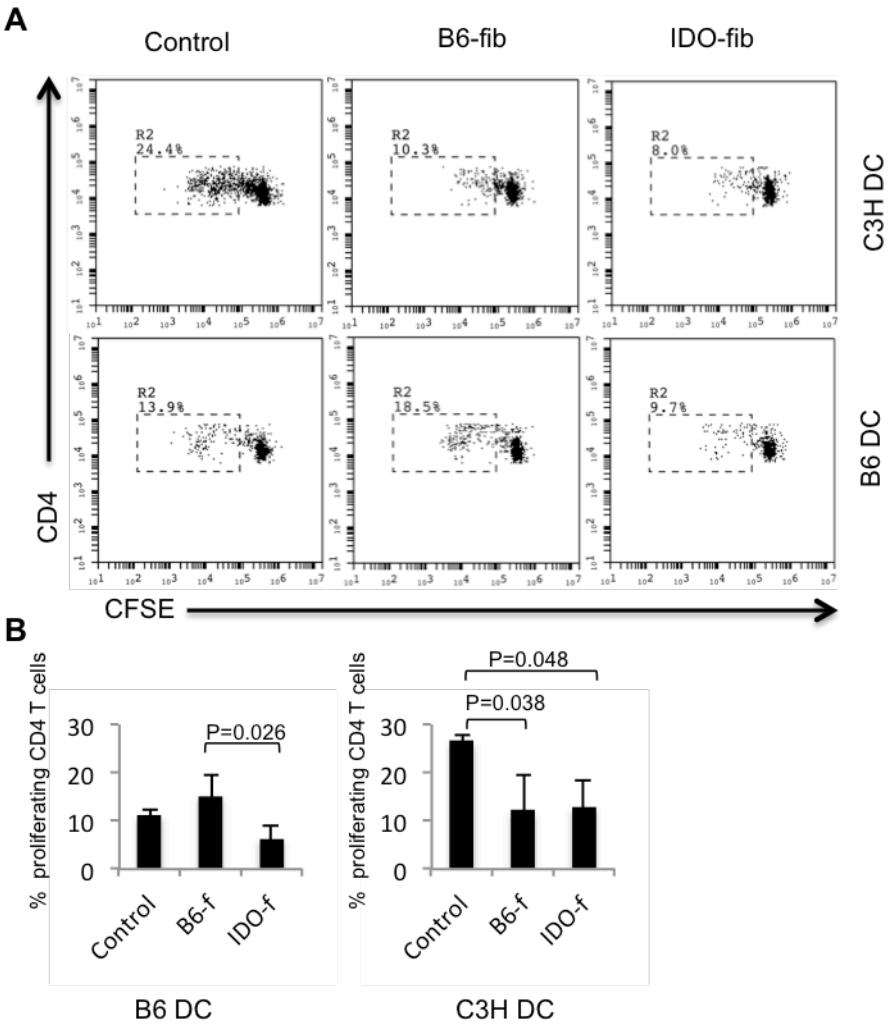

Figure 2.4. Allo-reactive T cell proliferation. Four days after IP injection of either IDO expressing B6 fibroblasts (IDO-f), non-IDO expressing B6 fibroblasts (B6-f) or vehicle medium (Control)**,** B6 donor tail skin pieces were transplanted in subcutaneous space of recipient C3H mice. After 10 days, the mice were sacrificed and pooled splenocytes and lymphocytes from spleen, skin draining and mesenteric lymph nodes were stained with CFSE and used as responder cells. Bone marrow derived DCs from B6 (donor) and C3H (recipient) mice were treated with mitomycin C and used as stimulator cells. (A) shows representative plots of CD4 proliferation in different mentioned groups. (B) shows accumulated data from three independent experiments. Data are presented as the Mean +/- SD. P values are shown above the bars that are significantly different. P value < 0.05 was considered to be significant.

2.17. Expansion of Tregs in lymphatic tissues after IP injection of both IDO-fibroblasts and non-IDO expressing B6-fibroblasts

In mesenteric lymph nodes (MLNs) of recipient mice, total number of Tregs in both B6-fibroblast and IDO-fibroblast treated groups was significantly increased ($p < 0.05$, $n = 3$). Total number of Tregs in spleen of IDO-fibroblast treated group was also significantly increased compared to those of control group ($p < 0.05$, $n = 3$). The percentage of Tregs among CD4+ T cells didn't change significantly (Figure 2.5).

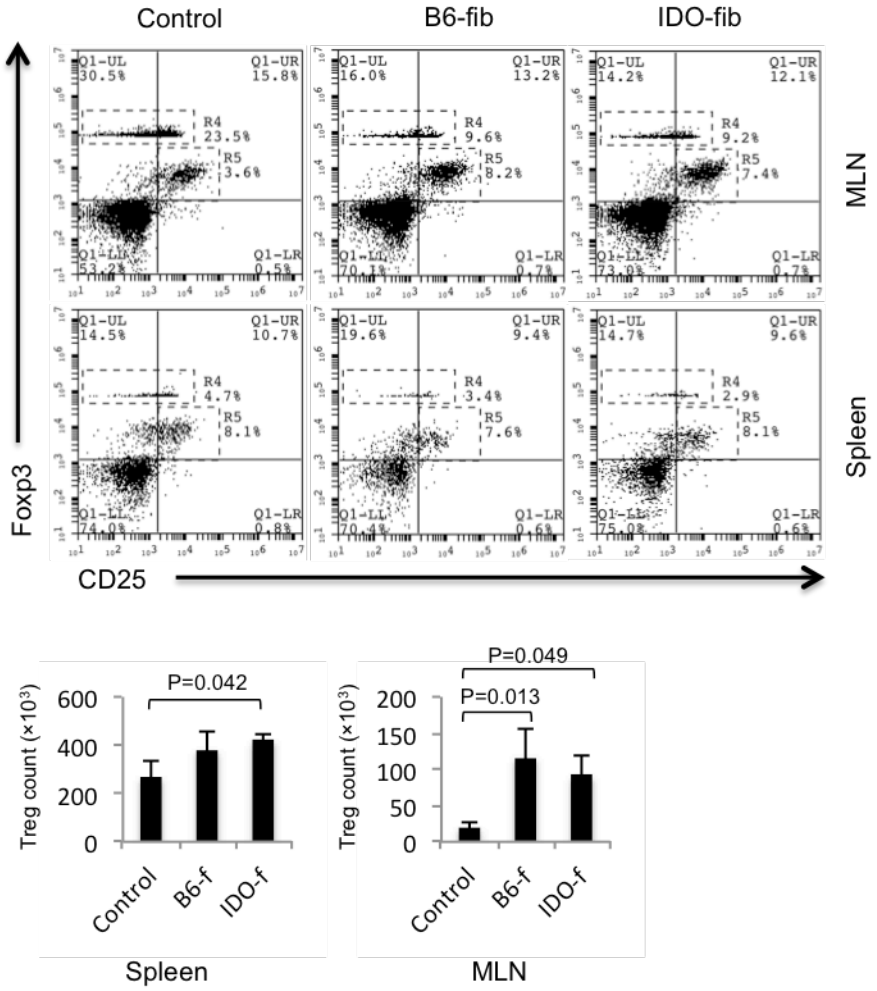

Figure 2.5. Tregs in lymphatic tissues. Four days after IP injection of either IDO expressing B6 fibroblasts (IDO-f), non-IDO expressing B6 fibroblasts (B6-f) or vehicle medium (Control)**,** B6 donor tail

skin pieces were transplanted in sub-cutaneous space of recipient C3H mice. After 10 days, the mice were sacrificed and regulatory T cells were checked in spleen and mesenteric lymph nodes (MLNs) of recipient mice by flowcytometer. First, cells were gated on CD4 cells and then double positive CD25 and FoxP3 cells were considered as regulatory T cells. Counting beads were used to measure the total number of Tregs. (A) shows the representative plots of T regs in spleen and MLNs of mentioned groups. (B) shows accumulated data of total number Tregs from three independent experiments. Data are presented as the Mean +/- SD. P values are shown above the bars that are significantly different. P value < 0.05 was considered to be significant.

2.18. Tracking of IP injected fibroblasts

To find out the fate of injected cells, cherry red IDO-fibroblasts were injected into the peritoneal cavity of recipient C3H mice. Two, four and six weeks later, MLNs and peritoneal lavage of recipient mice were checked to track the injected cells. Injected cells were present in MLNs even six weeks after injection. Also, they were present in peritoneal lavage after two weeks. However, at weeks four and six they were not detectable in peritoneal lavage (Figure 2.6).

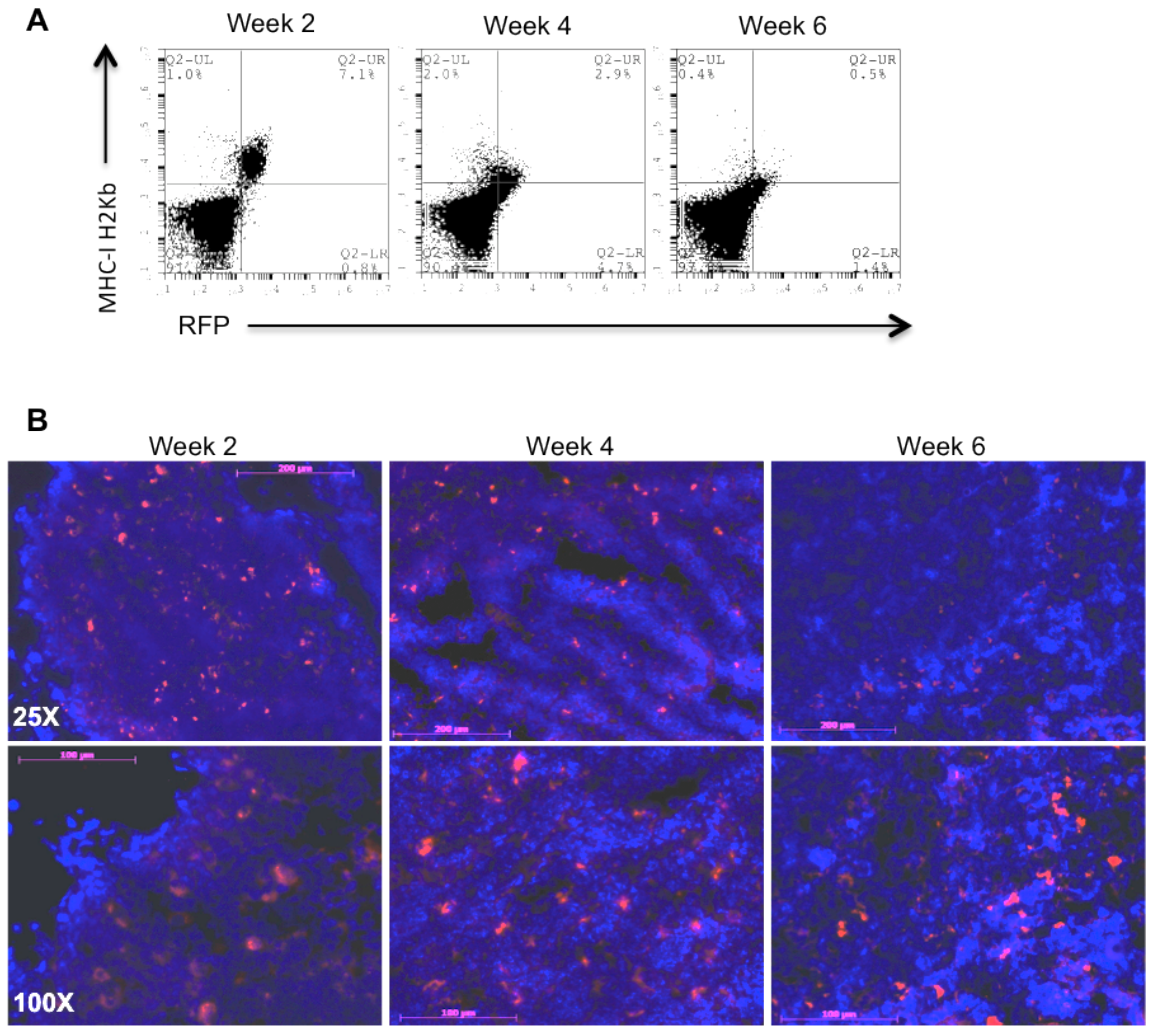

Figure 2.6. Tracking of IP injected mCherry-IDO-fibroblasts. Ten million mCherry-IDO-fibroblasts from B6 mouse were transplanted into the peritoneal cavity of C3H mice. After 2, 4 and 6 weeks, recipient mice were sacrificed and injected cells were tracked in peritoneal cavity and mesenteric lymph nodes (MLNs). (A) To track the cells in peritoneal cavity, the cavity was washed with 4 ml of PBS and then the peritoneal lavage cells were stained with anti mouse MHC-I (H-2Kb) APC (1:200, eBioscience) and read with flowcytometer to detect the mCherry positive cells. (B) To detect the IDO-fibroblasts in MLNs, frozen sections were visualized by Zeiss fluorescent microscope. 25X (above row) and 100X (lower row) magnifications are shown.

2.19. Conclusion

In summary, we showed that injection of 10^7 donor-derived fibroblasts transduced with lentiviral vector containing IDO into the peritoneal cavity of recipient mice could significantly increase the survival of allografts. This effect was more tangible for pancreatic islet transplantation compared to skin transplantation. Besides, with a novel model of subcutaneous skin transplantation, we showed that infiltration of immune cells especially T cells was much less in IDO-fibroblast treated group. We showed that, recipient CD4+ T cells in IDO-fibroblast treated group proliferated less than control group in response to recipient DCs presenting donor antigens. However, fibroblast treated group showed a similar effect as well. It means that both IDO-fibroblast and regular fibroblast treatments suppressed allo-reactive T cells in indirect and semi-direct pathways. While the percentage of Tregs was not changed in IDO-treated group, it is not clear whether an increase seen in total number of Tregs has any effect in prevention of graft rejection. We showed that two weeks after IP injection of IDO-fibroblasts, they could be tracked in peritoneal cavity of the recipient mice, while their number is reduced at weeks four and six. In contrast, they could be tracked in mesenteric lymph nodes at all three time points. It means that these cells can migrate to the draining lymph nodes and probably employ part of their effect through contacting the immune cells there. In summary, intraperitoneal injection of IDO expressing fibroblast is new approach that can increase the survival of allografts. While the systemic effect of IP IDO-fibroblast therapy shows superiority to the local co-transplantation approach, there might be synergistic tolerogenic effects when both approaches are combined. Although IDO-therapy might have limitations to induce long-term immunosuppressant free tolerance state, it can at least reduce the amount of immunosuppressive medications needed to protect the graft. Large animal studies are needed to verify the translational potential of this approach.

Chapter 3

Tolerogenic effect of mouse fibroblasts on dendritic cells

In the previous chapter, we used IDO-expressing fibroblasts in order to delay the immune rejection of allogeneic islets and skin. The immunoregulatory effects of IDO enzyme are well-described. However, we were wondering if fibroblasts per se have any immunomodulatory effect. We focused on the effect of fibroblasts on DCs, the main regulators of the immune system. There is controversy about the immune-modulatory effect of fibroblasts on dendritic cells. To clarify this issue, in this study, we have evaluated different features of fibroblast-primed DCs including their ability to express co-inhibitory and co-stimulatory molecules, pro- and anti-inflammatory cytokines and their ability to induce T cell proliferation. We also examined migratory capacity of DCs to lymphatic tissues and present fibroblast-derived antigens after encountering fibroblasts. The results of our *in vitro* study showed that both co-inhibitory (PD-L1, PD-L2 and B7H4) and co-stimulatory (CD86) molecules were up-regulated when DCs were co-cultured with fibroblasts. In mouse model we showed that intra- peritoneal injection (IP) of both syngeneic and allogeneic fibroblasts significantly increased both total DC count and expression level of co-inhibitory and co-stimulatory molecules on DCs. Priming of DCs with syngeneic and allogeneic fibroblasts reduced the proliferation of CD4+ and CD8+ T cells. Even activation of fibroblast- primed DCs failed to restore their ability to induce T cell proliferation. Likewise, priming of DCs with fibroblasts blocked the ability of ovalbumin-pulsed DCs to induce proliferation of ovalbumin-specific CD4+ T cells. Compared to non-activated DCs, fibroblast-primed DCs had significantly higher expression levels of IL-10 and IDO. Fibroblast-primed DCs had significantly reduced IL-12 expression level as compared to that of activated DCs. After priming with fibroblasts, DCs were able to migrate to lymphatic tissues and present fibroblast-derived antigens (ovalbumin). In conclusion, after priming with fibroblasts, DCs gain tolerogenic features. This finding suggests the potential role of fibroblasts in maintenance of immune-tolerance.

3.1. Methods

3.2. Mice

8-week-old female C3H/Hej (C3H) and C57bl/6 (B6) mice were purchased from Jackson Laboratories, Bar Harbor, ME and kept in Blusson Spinal Cord Injury Center Animal Care Facility. OT-II mice and Thy1.1 congenic C57bl/6 mice purchased from Jackson laboratories (Sacramento, CA) were bred and maintained as OT-II x Thy1.1 homozygous mice at the Child & Family Research Institute Animal Care Facility. The OT-II mice have CD4 T cells that harbor a T cell receptor that recognizes OVA. Mice expressing a membrane bound chicken ovalbumin OVA gene under the direction of the chicken beta actin promoter (Act-mOVA) were purchased from Jackson laboratories. Care and maintenance of all animals were in accordance with the principals of laboratory animal care and the guidelines of the institutional Animal Policy and Welfare Committee.

3.3. Isolation and culture of dendritic cells and fibroblasts

To culture DCs, we followed the method established by Lutz et al[58]. After removing the surrounding tissues from femur bones in sterile condition, both ends of the bones were cut and the marrow was flushed out. Two million cells were plated in non-adherent 60 x 15 mm dishes (VWR, Canada) in RPMI medium containing 10% FBS (Hyclone, Utah), 1% penicillin/streptomycin (Invitrogen) and GM-CSF (eBioscience, CA). At days 3, 6 and 8, half of the medium in each dish was taken and centrifuged at 500g for 5min. The supernatant was discarded and the pellet was suspended in fresh medium containing GM-CSF and added to the same dishes. At day 10, naïve DCs are ready for characterization or co-culturing studies. DCs characterized by expression of both CD11c and MHC-II molecules consist more than 80% of cells in suspension

To culture fibroblasts, after removing the hairs with hair clipper and sterilization of back skin with povidone iodine, a 2x2cm piece of full thickness skin was taken from the sacrificed mouse. Washed several times in PBS containing 2% penicillin/streptomycin, the hypodermis fat tissue was removed with a sterile blade in sterile condition. The skin was cut into small pieces and each piece was put on the 150x25mm culture dish (Corning, NY) with the dermis facing down. Small drops of FBS, was put onto each piece of skin and the dish was kept in 37^{o}C incubator supplemented with 5% CO2 for 4 hours. Afterwards, DMEM media (Hyclone, Utah) containing 10% FBS and 1% penicillin/streptomycin was added very slowly to the dish. Care should be taken to avoid detachment of the pieces from dish surface. After one to two weeks, the fibroblasts migrate out of the skin and adhere to the dish surface. The cells can be subcultured. At passages 5-9, the fibroblasts were used for co-culturing studies[59].

3.4. Conditioning of dendritic cells

After 10 days of culturing the DCs from bone marrow cells with the mentioned protocol, the non-adherent cells in the dishes were collected for further conditioning in different groups. For conditioning, 2x10^{6} DCs were cultured in 6ml of RPMI medium containing 10% FBS, 1% penicillin/streptomycin and GM-CSF in non-adherent dishes for another 48 hours. To activate the DCs, 200ng/ml of the TLR9 agonist CpG oligodeoxynucleotide (ODN) 1826 (5'-TCCATGACGTTCCTGACGTT-3') (custom synthesized by Sigma-Aldrich (Oakville ON)), was added to the culture medium[60]. For co-culturing with fibroblasts, 2x10^{5} fibroblasts were seeded in non-adherent 6cm dishes 4 days before adding DCs.

B6 cell lysate was added to C3H DCs to enable them to present B6-derived antigens to B6-presensitized C3H T cells[61]. To prepare the cell lysate, a pellet of 10^{7} B6 bone marrow cells resuspended in 100µl PBS was frozen in -80 °C and thawed for four times, followed by passing through a 70µm cell strainer (Fisher Scientific) to remove the big chunks. From this solution, 10µl was added to each dish of C3H DCs. In

experiments that presentation of chicken albumin on B6 DCs was needed, 1mg/ml of ovalbumin (OVA) protein (Sigma) was added to each dish of B6 DCs for 24h and then washed out[62].

3.5. Co-culturing of DCs/lymphocytes and T cell proliferation assay

Non-adherent cells from each dish of conditioned DCs were collected and treated with 50µg/ml of mitomycin-C (Sigma) for half an hour. Then, $5x10^4$ DCs suspended in 100µl media were seeded in round bottom 96-well plates (Corning, NY).

Skin draining lymph node cells from B6-presensitized C3H and OT-II mice were used as responder cells. Twenty million lymphocytes were stained with 2.5µg/ml of CFSE for 10 minutes. After washing the cells, $2x10^5$ lymphocytes in 100µl of media were added to DCs. RPMI medium containing 10% FBS, 1% penicillin/streptomycin and 30U/ml of IL-2 (Roche, Germany) was used for co-culturing of DCs and lymphocytes and was changed every other day[63]. After 6 days, the cells were stained with CD4 and CD8 antibodies and 7AAD and read with FACS to assess T cell proliferation.

3.6. Flow cytometry and kynurenine assay

All flow cytometry analyses were performed with a BD Accuri C6 Flow Cytometer (BD Bioscience). All the antibodies were bought from eBioscience, CA. For characterization of C3H DCs, they were stained with Anti-Mouse CD11c FITC (1:200) and Anti-Mouse MHCII (I-AK) PerCP-eFluor 710 (1:200). B6 DCs were stained with Anti-Mouse CD11c FITC (1:200) and Anti-Mouse MHCII (I-Ab) APC (1:200). Co-stimulatory and co-inhibitory antibodies that were used to characterize the DCs include Anti-Mouse CD86 PE (1:200), Anti-Mouse CD274 (PD-L1 or B7H1) PE (1:200), Anti-Mouse CD273 (PD-L2 or B7DC) PE (1:200) and Anti-Mouse B7-H4 PE (1:200).

For T cell proliferation assays, 6 days after co-culturing with DCs, lymphocytes were stained with Anti-Mouse CD4 PE (1:1000), Anti-Mouse CD8 APC (1:300), 7AAD (1:75) and read with FACS.

To track the GFP+ DCs, 3 days after injection of 10 million C3H fibroblasts into the peritoneal cavity of B6-GFP^{+} mice, the peritoneal lavage cells were re-injected into the peritoneal cavity of B6 non-GFP mice. Three and 10 days after injection, the recipient mice were sacrificed, their peritoneal lavage and mesenteric LN cells were stained with Anti Mouse CD11c APC (1:200) and read with FACS to track the GFP^{+} DCs.

To check the presentation of ovalbumin on B6-DCs after in vitro co-culturing with B6 and Act-mOVA fibroblast or after IP injection of B6 and Act-mOVA to B6 recipients, co-cultured DCs and peritoneal lavage and MLN cells of the recipient mice were stained with Anti Mouse CD11c APC (1:200) and MHC-I/OVA (H-2Kb / SIINFEKL) PE (1:200) and read with FACS.

After treating B6 and C3H DCs in different groups for 48 hours, the supernatants were collected and then the concentration of mouse IL-12 p70 and IL-10 was determined by BD Cytometric Bead Array kit (CBA)(BD Biosciences, San Jose, CA) according to the manufacturer's instructions using C6 Accuri flowcytometer (BD Biosciences). Also, kynurenine (by-product of IDO enzyme activity) level was measured in the supernatans as previously described [55]. Briefly, proteins in the conditioned medium were precipitated by trichloroacetic acid (TCA). After centrifugation, 0.5 ml of supernatant was incubated with an equal volume of Ehrlich's reagent at room temperature for 10 min. The reaction mixture was measured spectrophotometrically at 490 nm. The concentration of kynurenine in the conditioned medium was calculated according to a standard curve of defined kynurenine concentration.

3.7. Quantitative PCR

After treating B6 and C3H DCs in different groups for 48h, total RNA was isolated and purified using RNeasy kit (Qiagen, Valencia, CA) through following the manufacturer's instructions. The concentration and purity of the extracted RNA were checked by Nano Drop 2000 Spectrophotometer (ThermoScientific). The absorbance ratio at 260/280 nm was checked as a measure of purity of RNA. After DNase I treatment (Invitrogen, CA), cDNA was synthesized from 1mg of total RNA using a Superscript II First Strand cDNA Synthesis kit (Invitrogen, CA). Q-PCR was performed on Applied Biosystems 7500 PCR machine using the following PCR cycling conditions: 95°C for 5 min, 40 cycles at 95°C for 15 seconds and 60°C for 1 min. U6 snRNA expression was measured as the house keeping gene (F: 5'-ctcgcttcggcagcaca-3', R: 5'-aacgcttcacgaatttgcgt-3'). The following primers were used for Q-PCR reactions: mouse IL-10 (F: 5'-cagccgggaagacaataacg-3', R: 5'-ccgcagctctaggagcatg-3'), mouse IL-12p35 (F: 5'-gtgattctgaagtgctgcgt-3', R: 5'-ctttgatgatgaccctgtgc-3'), Mouse IDO (F: 5'-aagggcttcttcctcgtctc-3', R: 5'-aaaaacgtgtctgggtccac-3') (Invitrogen). CYBR Green dye (Roche, Germany) was used for detection of PCR products and relative quantity (RQ) of expression of each gene was measured by Delta Delta CT method. Non-activated B6 DC sample was assigned as the reference sample and fold change of all the samples was compared to this one.

3.8. Fluorescent microscopy

To confirm the existence of GFP^+ DCs in MLNs of recipient non-GFP B6 mice, the lymph nodes were frozen in cryomatrix (ThermoScientific, CA). Using the cryostat (ThermoScientific), 5 μm sections were obtained. The slides were fixed for 15 min in 1:1 acetone/ethanol followed by 15 min in PBS. The slides were visualized by Zeiss fluorescent microscope. Pictures were taken through AxioVision 4 software by AxioCam camera (Carl Zeiss).

3.9. Statistical analysis

Graphs are generated using GraphPad Prism software version 5.04. Data are shown as mean+/- SD of three or more observations. Statistical analysis was done using SPSS Statistics software version 22. To perform analysis where two groups were compared, T test was applied. To compare more than two groups, One-way ANOVA with post-hoc evaluation of Tukey was used. P value<0.05 was considered statistically significant.

3.10. Results

3.11. Co-inhibitory and co-stimulatory molecules are increased on DCs upon co-culturing with dermal primary fibroblasts

Bone marrow-derived DC (BM-DCs) from B6 and C3H mice were cultured for 48h with or without fibroblasts from B6 mice. Afterwards, non-adherent cells were collected and DCs were checked for expression of co-inhibitory and co-stimulatory molecules. B6 DCs, which were cultured with syngeneic fibroblasts, had a significantly higher expression of co-inhibitory molecules, PD-L1 and PD-L2. Also, C3H DCs that were cultured with allogeneic fibroblasts had elevated expression of co-inhibitory molecule, PD-L1. In both groups, co-stimulatory molecule, CD86, was also significantly increased (figure 3.1).

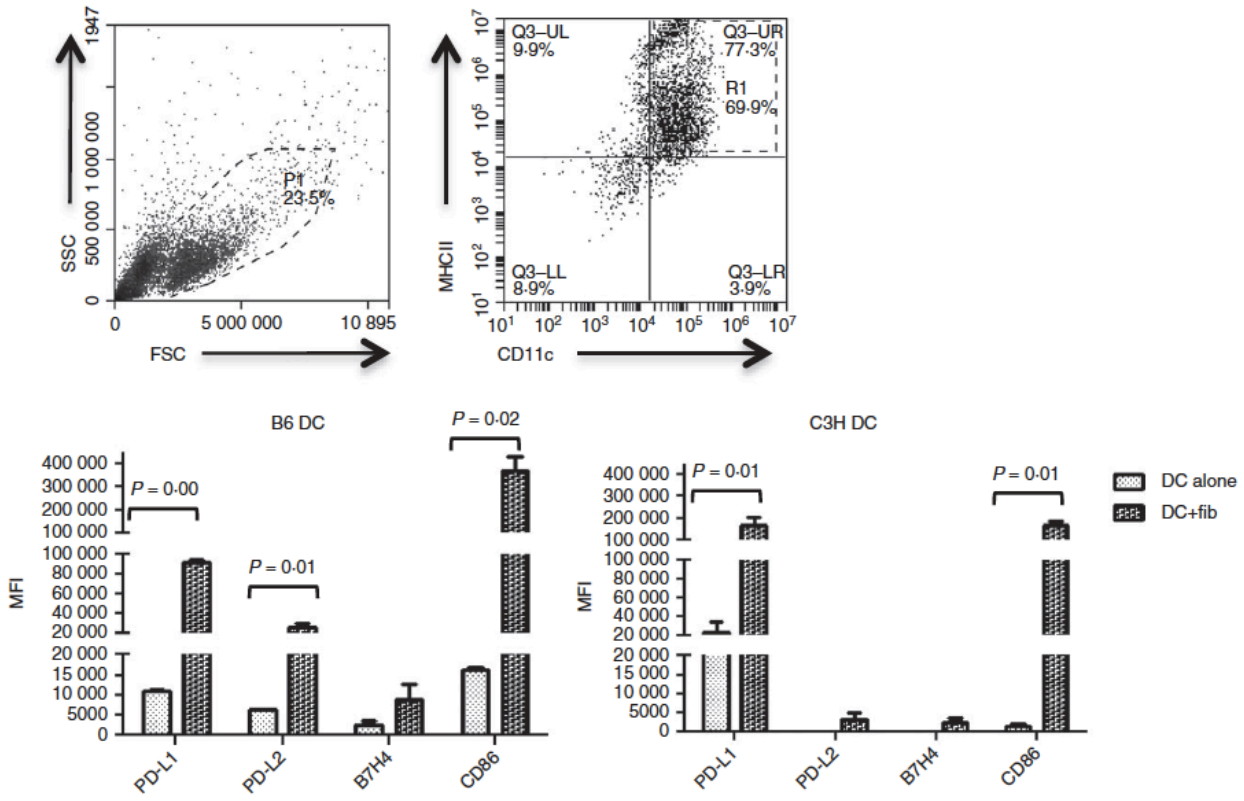

Figure 3.1. Evaluation of co-inhibitory and co-stimulatory molecules on DCs upon co-culturing with primary dermal fibroblasts. B6 and C3H BM-DCs were cultured in the absence (control) or presence of B6 fibroblasts (B6-fib) for 48 hours. Representative flow cytometry plots are shown on top row of this figure. After gating the cells based on the size (FSC) and granularity (SSC), cells having CD11c and MHCII were identified as DCs and the MFI of co-inhibitory and co-stimulatory molecules were checked on DCs. Lower row graphs summarize the MFI of co-inhibitory molecules, PD-L1, PD-L2 and B7H4, and also the co-stimulatory molecule, CD86, on B6 (left) and C3H DCs (right). Data were presented as the Mean +/- SD of at least 3 separate experiments. P value < 0.05 was considered to be significant.

To validate this finding *in vivo,* we injected 10^6 B6 fibroblasts in DMEM medium plus 10% FBS as the vehicle (Control) into the peritoneal cavity of B6 and C3H mice. After 10 days, the peritoneal lavage cells were collected and checked for the expression of co-inhibitory and co-stimulatory molecules on DCs. DCs were characterized as $CD11c^+$ $MHCII^+$ cells. The result showed that both syngeneic and allogeneic

fibroblasts caused a significant increase in total DC count within the peritoneal cavity as compared to that of control. The mean florescence intensity (MFI) of co-inhibitory molecule, PD-L1 significantly increased on DCs in both syngeneic and allogeneic fibroblast groups. However, the levels of PD-L2 and B7H4 were only increased in allogeneic fibroblasts treated group. Although the MFI of CD80 in allogeneic fibroblast group and CD40 and CD86 in both allogeneic and syngeneic fibroblast groups are higher than control group, this difference is not statistically significant. Only the MFI of CD40 is significantly increased on DCs in syngeneic fibroblast treated group (figure 3.2).

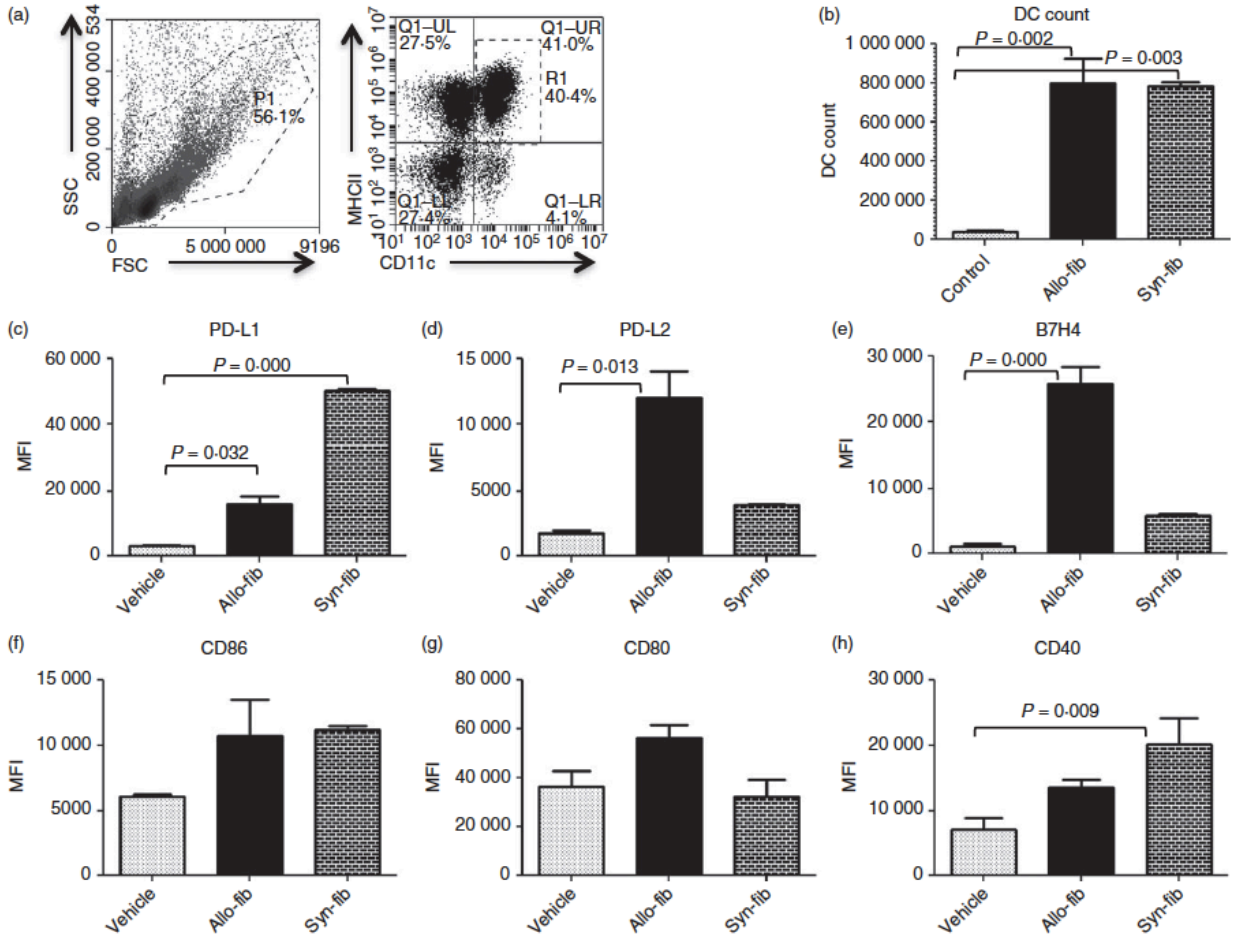

Figure 3.2. Detection of co-inhibitory and co-stimulatory molecules on DCs isolated from peritoneal cavity of the mice that received intra-peritoneal (IP) injection of fibroblasts. Ten days after IP injection of B6 fibroblasts or control vehicle to C3H and B6 mice, the peritoneal lavage cells were analyzed by flow cytometry. (A) After gating for size (FSC) and granularity (SSC), $CD11c^{+}$ $MHCII^{+}$ cells

were gated and defined as dendritic cells. The MFI of co-inhibitory and co-stimulatory molecules was measured on DCs. Next panel's show the results of statistical analysis on data collected from all repeats of the experiments. (B) Total PL DCs were counted in different groups using counting beads. (C-H) The MFI of co-inhibitory molecules, PD-L1, PD-L2 and B7H4, and also the co-stimulatory molecules, CD80, CD86 and CD40 on PL DCs are shown, respectively. Data were presented as the Mean +/- SD of at least 3 separate experiments. P value < 0.05 was considered to be significant.

3.12. Direct and indirect pathways of antigen presentation in DCs were impaired upon priming with fibroblasts

To investigate whether contact with fibroblasts changes the ability of DCs to induce T cell proliferation, a series of experiments were conducted. After *in vitro* culturing of B6 and C3H BM-DCs, they were divided into four groups. B6 DCs were cultured either alone, treated with CpG, co-cultured with B6-fibroblasts, or co-cultured with fibroblast and CpG. C3H DCs were also treated in the same manner. Moreover, B6 bone marrow cell lysate was also added to all groups of C3H DCs to enable them to present B6-derived antigens. After 48 hours of incubation, DCs were further treated with mitomycin-C and used as stimulator cells. Lymphocytes of skin draining lymph nodes of B6-pre-sensitized C3H mice (received B6 skin transplant one month before) were stained with CFSE and used as responder cells. After 6 days of mixed co-culture, the cells were analyzed by flow cytometry to check their proliferative status. As shown in figure 3.3A, only CpG activated DCs were able to induce T cell proliferation in CD4 and CD8 T cells. Priming of DCs from both B6 and C3H mice with B6 fibroblasts resulted in reduced proliferation of CD4 and CD8 T cells. Even simultaneous activation with CpG couldn't restore the ability of DCs to induce T cell proliferation. As B6 DCs can directly activate allogeneic C3H lymphocytes, we considered it as direct pathway of antigen presentation. However, C3H DCs present B6-derived antigen to syngeneic B6-pre-sensitized C3H lymphocytes in indirect and/or semi-direct pathway. This finding shows that both direct and indirect pathways of T cell activation are impaired upon priming of DCs with

fibroblasts. However, it cannot be excluded that presentation of B6-derived antigens on C3H DCs has happened through semi-direct pathway rather than indirect one.

3.13. Ovalbumin-pulsed activated DCs fail to induce proliferation of OTII T cells upon fibroblast priming

To further confirm the previous finding, we examined whether fibroblast priming of DCs can arrest the ability of DCs to induce proliferation of antigen-specific T cells. Similar to the previous experiment, B6 DCs were cultured *in vitro* and treated in five different groups: 1) non pulsed non-activated, 2) ovalbumin pulsed non-activated, 3) ovalbumin pulsed CpG-activated, 4) ovalbumin pulsed and B6-fibroblast primed, and 5) ovalbumin pulsed, B6-fibroblast primed, and CpG activated. Lymphocytes of OTII mice, which contain ovalbumin specific CD4 T cells, were used as responder cells. After CFSE staining and co-culturing with DCs from above-mentioned groups, proliferation of responder cells was checked using flow cytometry. Similar to the previous experiment, priming with fibroblasts inhibited the ability of ovalbumin pulsed DCs to induce proliferation of ovalbumin-specific CD4 T cells (figure 3.3B). While the DCs were pulsed with ovalbumin, they could induce proliferation of OTII cells both in the presence or absence of CpG activation. However, after being co-cultured with fibroblasts, they lost their ability to induce OTII cell proliferation. Similarly, DCs ability to induce cell proliferation was not restored even with CpG activation.

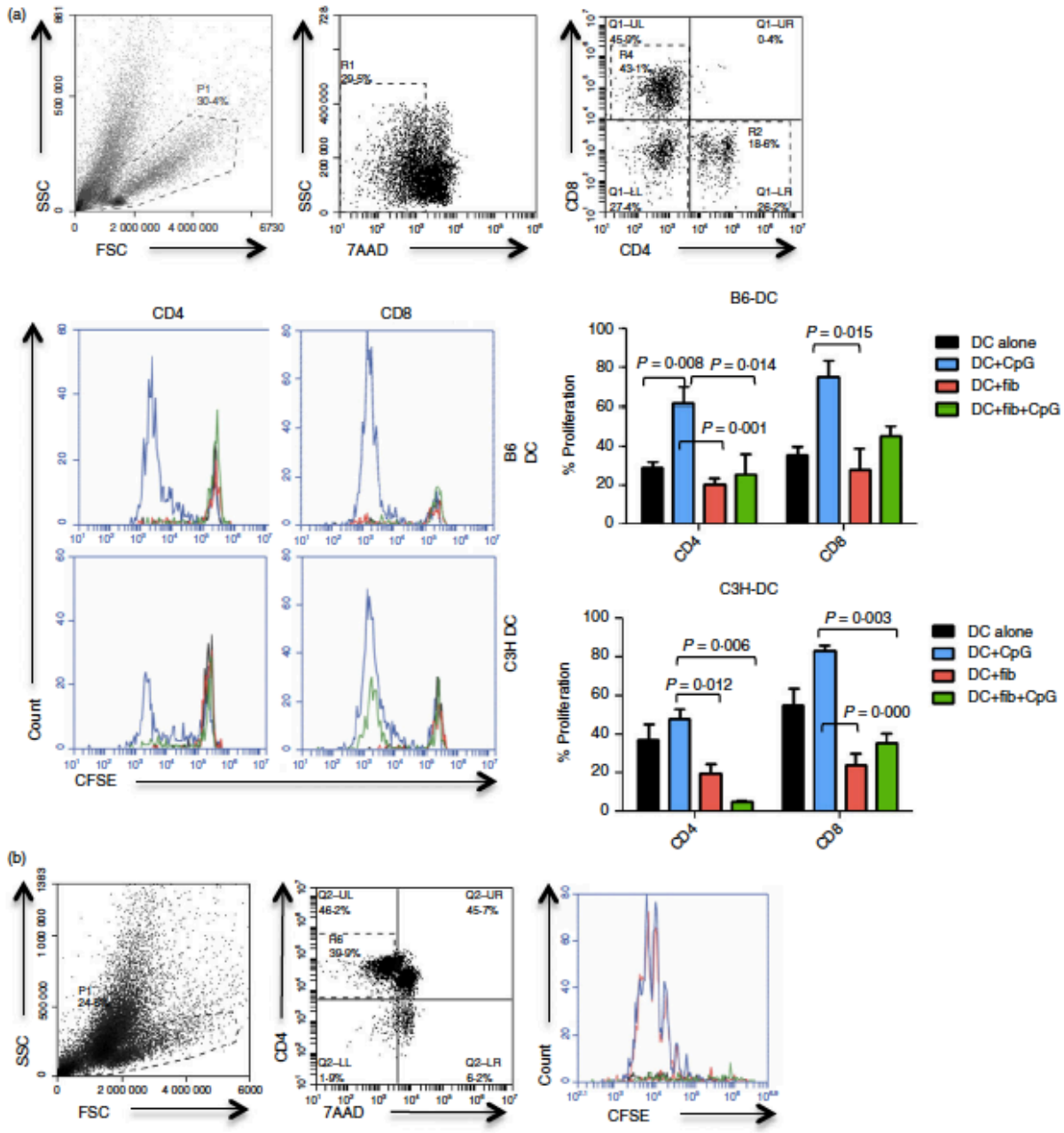

Figure 3.3. T cell proliferation in response to fibroblast-conditioned DCs. (A) Lymphocytes from B6-presensitized C3H lymph nodes were stained with CFSE as responders and cultured for 6 days with mitomycin-C treated B6 and C3H BM-DCs as stimulators. T cell proliferation was measured in the following DC treatment groups: 1) non-activated DCs (DC alone, black lines in histograms), 2) CpG-activated DCs (DC+CpG, blue lines), 3) B6-fibroblast primed DCs (DC+fib, red lines), and 4) CpG activated and B6-fibroblast primed DCs (DC+fib+CpG, green lines). After gating for FSC-SSC (not shown) and live cells ($7AAD^-$ population), CD4 and CD8 cells were identified and proliferation of these

T cell subsets was compared in the four above-mentioned groups of B6 and C3H DCs. Graphs summarize the results of T cell proliferation assay from 3 independent experiments for both B6 and C3H stimulators. Proliferation of CD4 and CD8 T cells are shown separately. (B) Ovalbumin specific lymphocytes from OT-II mouse were stained with CFSE and cultured for 6 days with mitomycin-C treated B6 BM-DCs from 5 different pre-conditioned sets: 1) non-pulsed non-activated DCs (black), 2) ovalbumin pulsed non-activated DCs (brown), 3) ovalbumin pulsed CpG-activated DCs (blue), 4) ovalbumin pulsed B6-fibroblast primed DCs (red), and 5) ovalbumin pulsed, B6-fibroblast primed and CpG activated DCs (green). After gating the live CD4 T cells ($CD4^+$ $7AAD^-$), CFSE dilution was checked as a measure of T cell proliferation. T cells in both ovalbumin pulsed DC groups either with (blue) or without (brown) CpG activation proliferated extensively.

3.14. Elevated expression of anti-inflammatory cytokines and reduced expression of pro-inflammatory cytokine in fibroblast-primed DCs

To further characterize fibroblast-conditioned DCs, their expressions of pro- and anti-inflammatory cytokines were checked using Real Time qPCR. B6 and C3H BM-DCs were divided into four groups, including non-activated, CpG activated, B6-fibroblast primed, and CpG activated plus B6-fibroblast primed. As shown in figure 3.4, compared to non-activated DCs, both B6 and C3H fibroblast-primed DCs had significantly higher mRNA and secreted protein levels of IL-10. IDO mRNA level was only significantly elevated in fibroblast-primed B6 DC., while kynurenine (by-product of IDO enzyme activity) level was significantly increased in fibroblast-primed C3H DC group. Upon activation, both B6 and C3H DCs had significantly higher amounts of pro-inflammatory cytokine IL-12. However, priming with fibroblasts, significantly reduced the expression of IL-12 even upon CpG activation (figure 3.4C).

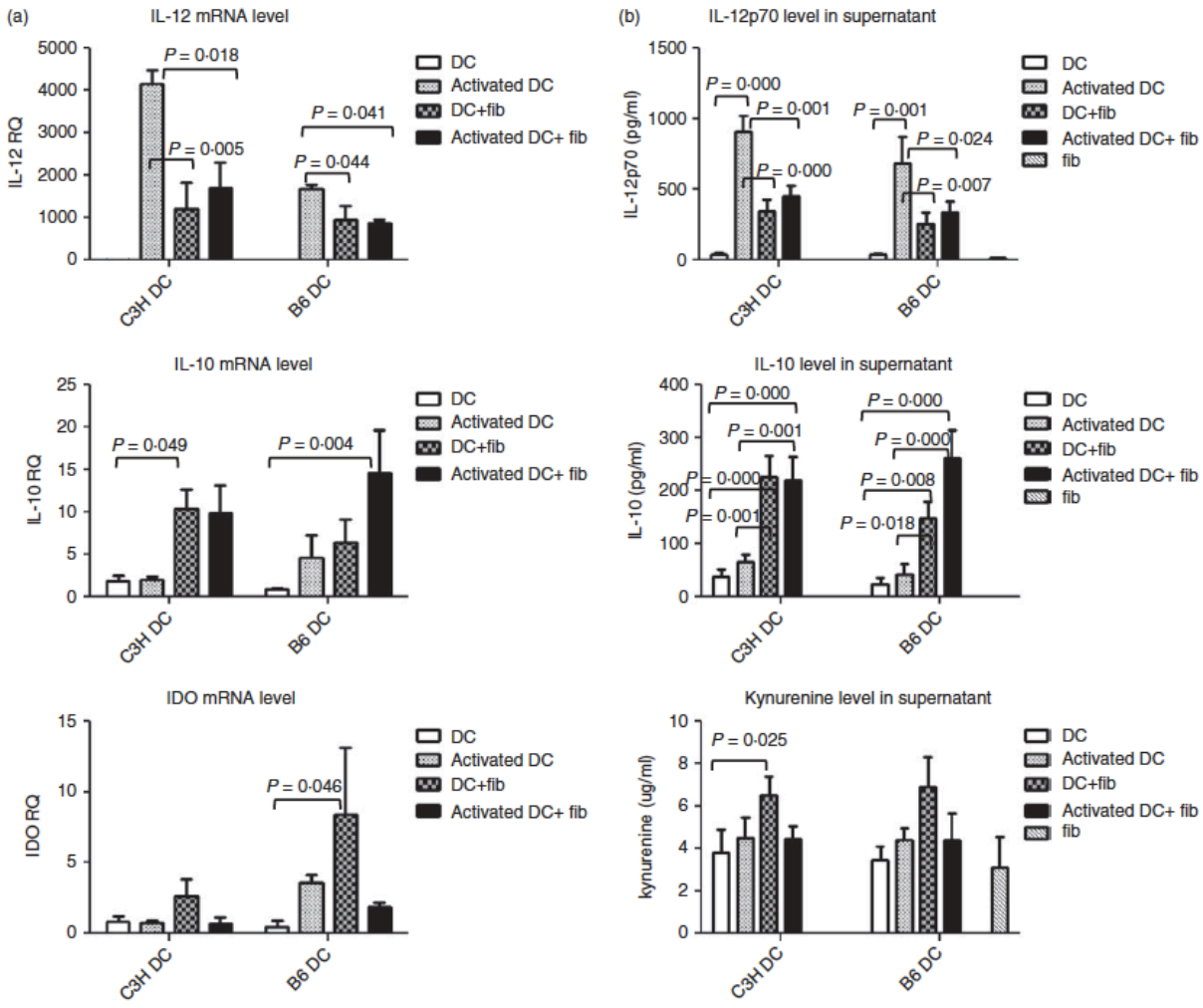

Figure 3.4. Cytokine expression in DCs cultured in the presence or absence of fibroblasts. B6 and C3H BM-DCs were cultured in four different groups for 48 hours: 1) non-activated DCs (DC), 2) CpG-activated DCs (activated DC), 3) B6-fibroblast treated DCs (DC+fib), and 4) CpG activated and B6-fibroblast primed DCs (activated DC+ fib). (A) The mRNA expression levels of IL-12, IL-10 and IDO in different groups of treated DCs are shown in three graphs from top to bottom (N=3). The relative quantity (RQ) of each cytokine is calculated by normalizing to the amount of B6 non-activated DC group using Delta Delta C(T) method. U6 was used as the internal control. (B) Supernatants of DC culture dishes in different groups were collected after 48h of conditioning and secreted levels of IL-12P70 and IL-10 were measured by BD Cytometric Bead Array kit. The level of kynurenine (by-product of IDO enzyme activity) was measured by spectrophotometric Kynurenine assay. Graphs are shown respectively from top to bottom. Data were presented as the Mean +/- SD of at least 3 separate experiments. P value < 0.05 was considered to be significant.

3.15. DCs are able to migrate to lymphatic tissues upon priming with fibroblasts

To test the ability of DCs to migrate to the lymphatic tissues after being primed with fibroblasts, we designed the following experiment. Ten million C3H fibroblasts were injected into the peritoneal cavity of GFP B6 mice. After two days, the peritoneal cavity was washed and the peritoneal lavage cells were injected into peritoneal cavity of non-GFP B6 mice. After 3 and 10 days, peritoneal lavage and mesenteric LN cells of these mice were checked by flow cytometric analysis to track the GFP^+ DCs. Further, fluorescence microscopy was used to locate GFP^+ cells within the frozen sections of the recipients' mesenteric lymph nodes. As presented in figure 3.5, GFP^+ $CD11c^+$ DCs were present in peritoneal lavage and also mesenteric lymph nodes of recipient mouse 3 days after injection, but not after 10 days. This finding confirms that after priming with fibroblasts, DCs can migrate to lymphatic tissues.

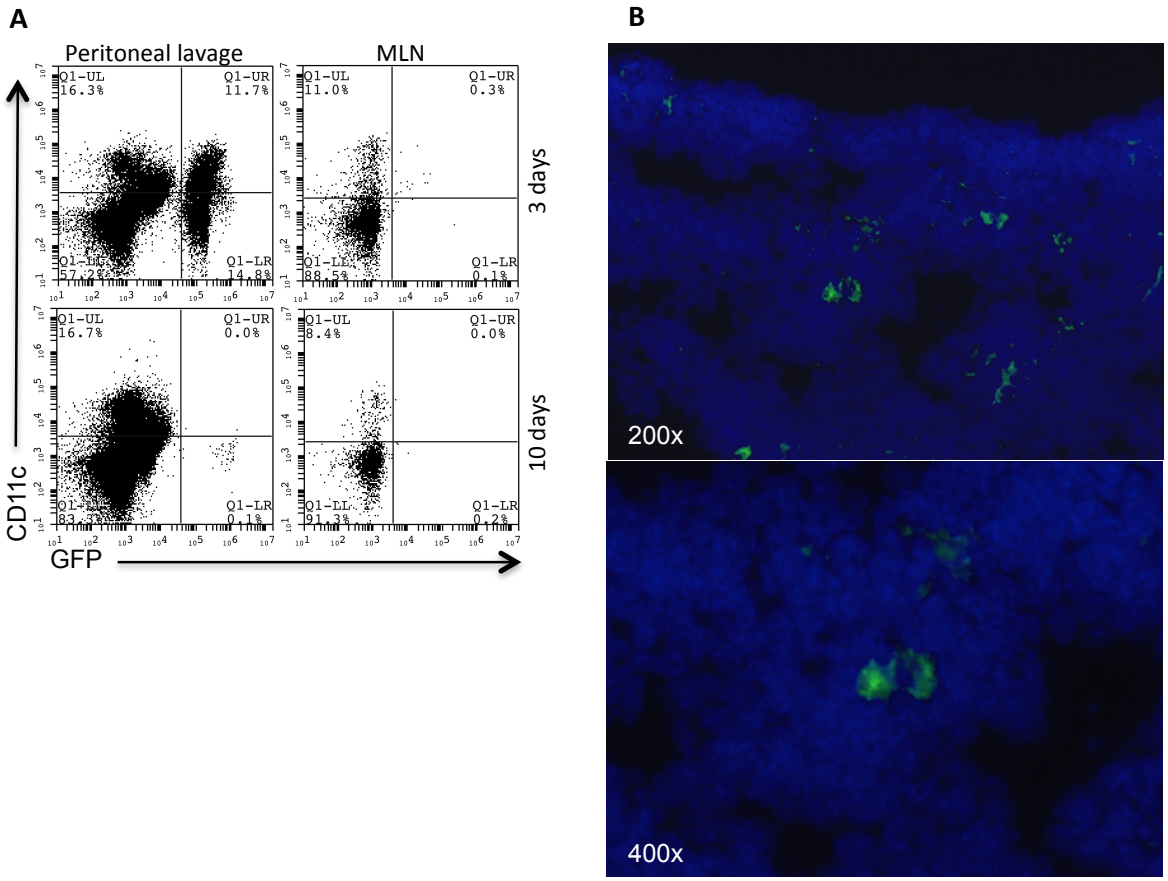

Figure 3.5. Evaluating the ability of dendritic cells to migrate to the lymph nodes after priming with fibroblasts. To test the migratory capacity of DCs after priming with fibroblasts, C3H fibroblasts were injected into the peritoneal cavity of the B6-GFP mouse. Two days later, the peritoneal lavage cells were injected into the peritoneal cavity of non-GFP B6 mice. Three and ten days later, the GFP^+ cells were tracked in peritoneal cavity and mesenteric lymph nodes (MLNs) of recipient mice. (A) Three (upper plots) and ten days (lower plots) after IP injection of GFP^+ cells, peritoneal lavage (left plots) and MLN cells (right panels) were stained for CD11c and tested with flow cytometry. (B) The migration of GFP^+ cells was further confirmed by detecting them in MLNs using fluorescence microscopy.

3.16. DCs are able to present fibroblast-derived antigens

To investigate the ability of DCs to present fibroblast-derived antigens, B6 or Act-mOVA fibroblasts were co-cultured in vitro with B6 DCs. After 48 hours of co-culturing, DCs were checked for presentation of ovalbumin on their MHC-I. A percentage of Act-mOVA fibroblast-primed DCs stained positive with the antibody that detects MHC-I/ovalbumin (figure 3.6A). Likewise, to see whether DCs can present fibroblast-derived antigens in draining LNs *in vivo*, 10^6 B6 or Act-mOVA fibroblasts were injected into the peritoneal cavity of B6 mice. After 3 days, mesenteric LN cells were checked with flow cytometry to detect $CD11c^+$ DCs that present MHC-I/ovalbumin. There were a percentage of positive DCs in Act-Ovalbumin fibroblasts treated group but not present in the B6-fibroblast treated group (figure 3.6B).

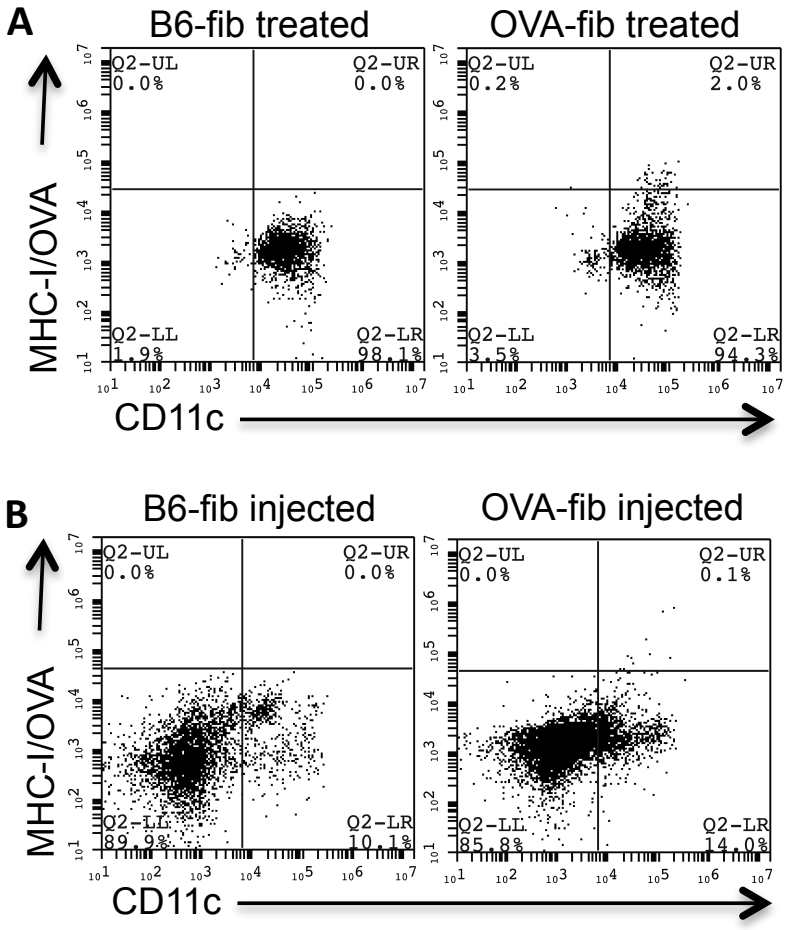

Figure 3.6. Evaluating the capacity of DCs to present fibroblast-derived antigens in the context of MHC-I. (A) B6 DCs were cultured in the presence of B6 fibroblasts (left plot) or Act-OVA fibroblasts (right plot). $CD11c^+$ DCs that present ovalbumin on MHC-I were detected with flow cytometry. (B) Similarly, after injection of B6-fibroblasts (left plot) or Act-OVA fibroblasts (right plot) into the peritoneal cavity of B6 mice, $CD11c^+$ DCs that present ovalbumin on MHC-I were tracked in mesenteric lymph nodes using flow cytometry.

3.17. Conclusion

In conclusion, our results show that conditioning of DCs with fibroblasts triggers them to express higher levels of co-inhibitory molecules and anti-inflammatory cytokines and lower levels of pro-inflammatory cytokines. Fibroblast conditioning arrests the ability of DCs to induce T cell proliferation both in direct and indirect pathways. These DCs can migrate to the regional lymph nodes and present fibroblast-derived antigens. Together, these data are suggestive of tolerogenic nature of DCs after being conditioned with fibroblasts. This finding sheds light on the role of skin fibroblasts in maintenance of self-tolerance and regulation of immune responses in the skin. Moreover, it might be used to design cell therapy approaches to control the immune responses in dermal autoimmune diseases or even in the setting of transplantation.

Chapter 4

Discussion

In the second chapter, we showed that injection of 10^7 donor-derived fibroblasts transduced with lentiviral vector containing IDO into the peritoneal cavity of recipient mice could significantly increase the survival of allografts. This effect was more tangible for pancreatic islet transplantation compared to skin transplantation. It can be attributed to higher immunogenicity of skin tissue compared to pancreatic islets. However, we showed that this approach is effective in skin transplantation as well. Besides, with a novel model of subcutaneous skin transplantation, we showed that infiltration of immune cells especially T cells was much less in IDO-fibroblast treated group. Some of our mice that received islet transplantation and IDO-fibroblasts kept normoglycemic levels even up to 150 days examined, which by definition shows immune tolerance. It shows the superiority of this systemic approach to the local co-transplantation approach. We tried to find out the reasons that can describe this effect.

The first thing that we looked at is allo-reactive T cell proliferation. As shown before, IDO can suppress allo-reactive T cells through different mechanisms including tryptophan depletion and the suppressive effect of by-products like kynurenine and 3-hydroxykynurenine [13,64,65]. Here we showed that, recipient CD4+ T cells in IDO-fibroblast treated group proliferated less than control group in response to recipient DCs presenting donor antigens. However, fibroblast-treated group showed a similar effect as well. It probably means that both IDO-fibroblast and regular fibroblast treatments suppressed allo-reactive T cells in indirect and semi-direct pathways. There are two possibilities to describe this effect. First, we have recently shown that fibroblasts have a tolerogenic effect on dendritic cells both *in vivo* and *in vitro* [66]. It might be possible that allogeneic fibroblasts injected into peritoneal cavity have a tolerogenic effect on recipient DCs presenting donor derived antigens and hence, suppressing allo-reactive T cells in indirect or semi-direct pathways. Second, we have also shown that after IP injection of allogeneic fibroblasts into the peritoneal cavity, a huge influx of recipient immune cells reaches that area. It is possible that IFN-gamma produced by these immune cells induces the expression of IDO in fibroblasts, which in turn suppresses allo-reactive T cells in indirect and semi-direct pathways. Regarding allo-reactive T cells in direct pathway, we showed that IDO-fibroblast treatment decreases proliferation of allo-reactive T cells

activated in direct pathway compared to fibroblast treated group. Although the proliferation is less than control group, but is not statistically significant. It probably shows that IDO is not significantly involved in prevention of acute rejection, which is in line with some published papers [25], while opposing others [67]. If it proves to be true, IDO therapy should be combined with some short-term immunosuppressive regimens to prevent acute rejection in a translational setting.

Next, we checked the effect of our therapies on regulatory T cells. We showed that total number of regulatory T cells in mesenteric lymph nodes is significantly increased in mesenteric lymph nodes of both IDO-fibroblast and regular fibroblast treated mice. Considering the conserved percentage of Tregs among total CD4+ T cells of all groups, it might simply show a general expansion of total T cells in MLNs of both IDO-fibroblast and regular fibroblast treated mice. The same pattern was seen in spleens of both groups; however, only an increase in total number of Tregs in IDO-fibroblast treated group was statistically significant. While the percentage of Tregs was not changed in IDO-treated group, it is not clear whether an increase seen in total number of Tregs has any effect in prevention of graft rejection. Although, the number and percentage of Tregs is important, their antigen specificity for donor antigens is even more important factor. Our group has previously shown that IDO-fibroblasts can induce antigen specific Tregs that can suppress alloreactions toward fibroblast donor antigens much better than a third party [21]. More studies are needed to find out if IDO-therapy has any effect on expansion of donor specific Tregs in vivo which might simply explain the observed tolerogenic effects.

A major difference between local co-transplantation method and systemic IDO-fibroblast therapy is the number of transplanted cells. The limited space under the kidney capsule does not allow transplanting more than 2-5x10^5 IDO-fibroblasts, while with the IP method, we could transplant 10^7 IDO fibroblasts. Our group has shown IP injection of 10^7 IDO-fibroblasts can increase the mouse serum level of kynurenine [68]. Here, we showed that two weeks after IP injection of IDO-fibroblasts, they could be tracked in peritoneal cavity of the recipient mice, while; their number is reduced at weeks four and six. In contrast, they could be tracked in mesenteric lymph nodes at all three time points. It means that these cells

can migrate to the draining lymph nodes and probably employ part of their effect through contacting the immune cells there.

In summary, intraperitoneal injection of IDO expressing fibroblast is new approach that can increase the survival of allografts. While the systemic effect of IP IDO-fibroblast therapy shows superiority to the local co-transplantation approach, there might be synergistic tolerogenic effects when both approaches are combined. Although IDO-therapy might have limitations to induce long-term immunosuppressant free tolerance state, it can at least reduce the amount of immunosuppressive medications needed to protect the graft. Large animal studies are needed to verify the translational potential of this approach.

In the second chapter, we used IDO-expressing fibroblasts in order to delay the immune rejection of allogeneic islets and skin. The immunoregulatory effects of IDO enzyme are well-described. However, we were wondering if fibroblasts per se have any immunomodulatory effect. We focused on the effect of fibroblasts on DCs, the main regulators of the immune system. In the third chapter, we addressed the potential role of fibroblasts on tolerogenicity of DCs. We hypothesized that priming with fibroblasts has a tolerogenic impact on DCs. To test this hypothesis, we tested different characteristics of tolerogenic DCs in BM-DCs after being primed with fibroblasts. These features include the expression of co-inhibitory molecules and anti-inflammatory cytokines, the ability to migrate to lymphatic organs and present antigens, and induction of anergy in T cells.

The major signals needed for activation of T cells include (but are not limited to) the interaction between TCR and MHC/peptide complex and the interaction of co-stimulatory molecules on APCs with their receptors on T cells[69]. While co-stimulation helps activation of DCs upon introduction of any danger signal, co-inhibitory molecules help maintaining tolerance to self-antigens[70]. PD-L1 and PD-L2 are among the most important co-inhibitory molecules that both bind to PD-1 on T cells, which in turn starts an inhibitory signaling pathway in T cells. Expression of PD-L2 is restricted to DCs, macrophages and B1 cells[71], while PD-L1 is expressed on a wider variety of cells[70]. B7H4 is another co-inhibitory molecule of

the B7 family that is expressed on APCs and its receptor is still unknown[72]. Our results showed that these co-inhibitory molecules as well as co-stimulatory molecule, CD86, are up-regulated on DCs co-cultured with fibroblasts. We also confirmed it in an *in vivo* system by injecting the fibroblasts into the peritoneal cavity of recipient mice and checking these molecules on retrieved peritoneal lavage DCs. We found an influx of CD11c+ MHCII+ DCs into the peritoneal cavity upon injection of either syngeneic or allogeneic fibroblasts. Both syngeneic and allogeneic fibroblast groups up-regulated co-inhibitory and co-stimulatory molecules on DCs. Although co-stimulatory molecules were up regulated on DCs upon priming with fibroblasts, we believe the ratio of co-inhibitory to co-stimulatory molecules was in favor of immunomodulatory state for DCs. Indeed, tolerogenic DCs comprises a spectrum of DCs with different maturation status which includes immature DCs and other type of DCs with higher levels of co-stimulation[73]. Therefore, the expression of co-stimulatory molecule on DCs does not per se prove their stimulatory nature.

As one of the main functions of DCs is to stimulate the proliferation of naïve T cells, we have asked whether conditioning of DCs with fibroblasts could attenuate this function of DCs. Our results showed that upon priming with either syngeneic or allogeneic fibroblasts, DCs loose their capacity to stimulate the proliferation of CD4 and CD8 T cells both in direct and indirect (or semi-direct) pathways. Even activation of DCs with a TLR ligand, CpG, couldn't retrieve this ability of DCs. This finding is consistent with those previously reported for stromal cells like mesenchymal stem cells[49] and renal fibroblasts[52]. One possible explanation for reduced stimulatory ability of fibroblast-primed DCs can be the increased expression of anti-inflammatory cytokines and reduced expression of pro-inflammatory cytokines. We showed that the expression of IL-10 and IDO was increased in fibroblast-primed DCs as compared to that shown in immature DCs. In general, IL-10 suppresses T cell responses especially CD4 effector and memory cells[74]. IDO, a tryptophan degrading enzyme, also inhibits T cell proliferation[75,76] through local depletion of tryptophan[77] and production of T cell cytotoxic byproducts[78]. Moreover, IL-12p70 expression was significantly lower in fibroblast-primed DCs compared to activated DCs.

Another feature of functional DCs including tolerogenic ones, is their ability to migrate to lymphatic organs and present antigens. We used GFP^+ DCs to show the ability of fibroblast-primed DCs to migrate from peritoneal cavity to mesenteric lymph nodes. The fact that GFP^+ DCs were present on day 3 but not on day 10 post-injection in the peritoneal cavity suggests that either all of them have migrated to other places before day 10 or they have undergone apoptosis and cleared. We also showed that upon priming with fibroblasts, host DCs migrated from peritoneal cavity to mesenteric lymph nodes and presented fibroblast derived antigens. We used Act-mOVA mouse fibroblasts to prime peritoneal cavity DCs. These transgenic mice express chicken ovalbumin under the control of β-actin promoter on the surface of all body cells[79]. As this antigen does not exist in B6 recipient mice, its presentation on DCs proves that they have been able to receive the fibroblast antigen and migrate to mesenteric lymph nodes to present it to naive T cells. As there is no commercially available antibody to detect ovalbumin on MHCII of B6 mice, we were only able to evaluate its presentation on MHCI. Presentation of ovalbumin on MHCI can either show that fibroblast cell membrane is being fused to DC membrane or DCs have engulfed the fibroblast-derived antigens and crosspresented them on their MHCI.

In conclusion, our results show that conditioning of DCs with fibroblasts triggers them to express higher levels of co-inhibitory molecules and anti-inflammatory cytokines and lower levels of pro-inflammatory cytokines. Fibroblast conditioning arrests the ability of DCs to induce T cell proliferation both in direct and indirect pathways. These DCs can migrate to the regional lymph nodes and present fibroblast-derived antigens. Together, these data are suggestive of tolerogenic nature of DCs after being conditioned with fibroblasts. This finding sheds light on the role of skin fibroblasts in maintenance of self-tolerance and regulation of immune responses in the skin. Moreover, it might be used to design cell therapy approaches to control the immune responses in dermal autoimmune diseases or even in the setting of transplantation.

References

1. Meier-Kriesche, H. U., Schold, J. D., Srinivas, T. R. & Kaplan, B. Lack of Improvement in Renal Allograft Survival Despite a Marked Decrease in Acute Rejection Rates over the Most Recent Era. *Am. J. Transplant.* **4,** 378–383 (2004).

2. Sun, H. Y. & Singh, N. Opportunistic infection-associated immune reconstitution syndrome in transplant recipients. *Clinical Infectious Diseases* **53,** 168–176 (2011).

3. Wu, C. & Shapiro, R. Post-transplant malignancy: reducing the risk in kidney transplant recipients. *Expert Opin. Pharmacother.* **12,** 1719–29 (2011).

4. Khosravi-Maharlooei, M. *et al.* THERAPY OF ENDOCRINE DISEASE: Islet transplantation for type 1 diabetes: so close and yet so far away. *Eur. J. Endocrinol.* **173,** R165--R183 (2015).

5. Lee, W. P. *et al.* Relative antigenicity of components of a vascularized limb allograft. *Plastic and reconstructive surgery* **87,** 401–11 (1991).

6. Baumeister, S. *et al.* Risks of allogeneic hand transplantation. *Microsurgery* **24,** 98–103 (2004).

7. Tobin, G. R. *et al.* The History of Human Composite Tissue Allotransplantation. *Transplant. Proc.* **41,** 466–471 (2009).

8. Rafla, K. & Tredget, E. E. Infection control in the burn unit. *Burns* **37,** 5–15 (2011).

9. Munn, D. H. *et al.* Prevention of allogeneic fetal rejection by tryptophan catabolism. *Science* **281,** 1191–1193 (1998).

10. Mellor, A. L. & Munn, D. H. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol* **4,** 762–774 (2004).

11. Moffett, J. R. & Namboodiri, M. A. Tryptophan and the immune response. *Immunology and Cell*

Biology **81,** 247–265 (2003).

12. Fallarino, F. *et al.* The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor zeta-chain and induce a regulatory phenotype in naive T cells. *J. Immunol.* **176,** 6752–61 (2006).

13. Munn, D. H. & Mellor, A. L. Indoleamine 2,3 dioxygenase and metabolic control of immune responses. *Trends in Immunology* **34,** 137–143 (2013).

14. Pallotta, M. T. *et al.* Indoleamine 2,3-dioxygenase is a signaling protein in long-term tolerance by dendritic cells. *Nat. Immunol.* **12,** 870–878 (2011).

15. Forouzandeh, F., Jalili, R. B., Germain, M., Duronio, V. & Ghahary, a. Differential immunosuppressive effect of indoleamine 2,3-dioxygenase (IDO) on primary human CD4+ and CD8+ T cells. *Mol. Cell. Biochem.* **309,** 1–7 (2008).

16. Munn, D. H. *et al.* GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. *Immunity* **22,** 633–642 (2005).

17. Liu, Z. *et al.* Suppression of memory CD8 T cell generation and function by tryptophan catabolism. *J. Immunol.* **178,** 4260–4266 (2007).

18. Liu, H. *et al.* Reduced cytotoxic function of effector CD8+ T cells is responsible for indoleamine 2,3-dioxygenase-dependent immune suppression. *J. Immunol.* **183,** 1022–1031 (2009).

19. Fallarino, F. *et al.* The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor zeta-chain and induce a regulatory phenotype in naive T cells. *J. Immunol.* **176,** 6752–6761 (2006).

20. Chung, D. J. *et al.* Indoleamine 2,3-dioxygenase-expressing mature human monocyte-derived dendritic cells expand potent autologous regulatory T cells. *Blood* **114,** 555–563 (2009).

21. Curran, T. A., Jalili, R. B., Farrokhi, A. & Ghahary, A. IDO expressing fibroblasts promote the expansion of antigen specific regulatory T cells. *Immunobiology* **219,** 17–24 (2014).

22. Sharma, M. D. *et al.* Indoleamine 2,3-dioxygenase controls conversion of Foxp3+ Tregs to TH17-like cells in tumor-draining lymph nodes. *Blood* **113,** 6102–6111 (2009).

23. Baban, B. *et al.* IDO activates regulatory T cells and blocks their conversion into Th17-like T cells. *J. Immunol.* **183,** 2475–2483 (2009).

24. Yan, Y. *et al.* IDO upregulates regulatory T cells via tryptophan catabolite and suppresses encephalitogenic T cell responses in experimental autoimmune encephalomyelitis. *J. Immunol.* **185,** 5953–5961 (2010).

25. Laurence, J. M. *et al.* Overexpression of indoleamine dioxygenase in rat liver allografts using a high-efficiency adeno-associated virus vector does not prevent acute rejection. *Liver Transplant.* **15,** 233–241 (2009).

26. Li, J. *et al.* Indoleamine 2,3-dioxygenase gene transfer prolongs cardiac allograft survival. *Am. J. Physiol. Heart Circ. Physiol.* **293,** H3415–H3423 (2007).

27. Yu, G. *et al.* Gene delivery of indoleamine 2,3-dioxygenase prolongs cardiac allograft survival by shaping the types of T-cell responses. *J. Gene Med.* **10,** 754–761 (2008).

28. Dellê, H. & Noronha, I. L. Induction of indoleamine 2,3-dioxygenase by gene delivery in allogeneic islets prolongs allograft survival. *Am. J. Transplant.* **10,** 1918–1924 (2010).

29. Alexander, A. M. *et al.* Endoleamine 2,3-dioxygenase expression in transplanted NOD islets prolongs graft survival after adoptive transfer of diabetogenic splenocytes. *Diabetes* **51,** 356–365 (2002).

30. Funeshima, N. *et al.* Inhibition of allogeneic T-cell responses by dendritic cells expressing

transduced indoleamine 2,3-dioxygenase. *J. Gene Med.* **7,** 565–575 (2005).

31. Yu, G. *et al.* Steady state dendritic cells with forced IDO expression induce skin allograft tolerance by upregulation of regulatory T cells. *Transpl. Immunol.* **18,** 208–219 (2008).

32. Xie, F. T. *et al.* IDO expressing dendritic cells suppress allograft rejection of small bowel transplantation in mice by expansion of Foxp3 regulatory T cells. *Transpl. Immunol.* (2015). doi:10.1016/j.trim.2015.05.003

33. Yan, M. L., Wang, Y. D., Tian, Y. F., Lai, Z. De & Yan, L. N. Inhibition of allogeneic T-cell response by Kupffer cells expressing indoleamine 2,3-dioxygenase. *World J. Gastroenterol.* **16,** 636–640 (2010).

34. Meisel, R. *et al.* Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2 , 3-dioxygenase – mediated tryptophan degradation. *Blood* **103,** 4619–4622 (2014).

35. Yu, J. *et al.* Myeloid-derived suppressor cells suppress antitumor immune responses through IDO expression and correlate with lymph node metastasis in patients with breast cancer. *J. Immunol.* **190,** 3783–97 (2013).

36. Li, Y. *et al.* Local expression of indoleamine 2,3-dioxygenase protects engraftment of xenogeneic skin substitute. *J. Invest. Dermatol.* **126,** 128–136 (2006).

37. Jalili, R. B. *et al.* Local expression of indoleamine 2,3 dioxygenase in syngeneic fibroblasts significantly prolongs survival of an engineered three-dimensional islet allograft. *Diabetes* **59,** 2219–2227 (2010).

38. Forouzandeh, F. *et al.* Local expression of indoleamine 2,3-dioxygenase suppresses T-cell-mediated rejection of an engineered bilayer skin substitute. *Wound Repair Regen.* **18,** 614–623 (2010).

39. Jalili, R. B. *et al.* Local expression of indoleamine 2,3 dioxygenase in syngeneic fibroblasts significantly prolongs survival of an engineered three-dimensional islet allograft. *Diabetes* **59,** 2219–2227 (2010).

40. Hosseini-Tabatabaei, A. *et al.* Immunoprotection and Functional Improvement of Allogeneic Islets in Diabetic Mice, Using a Stable Indoleamine 2,3-Dioxygenase Producing Scaffold. *Transplantation* **99,** 1341–1348 (2015).

41. Mazzoni, A. & Segal, D. M. Controlling the Toll road to dendritic cell polarization. *J. Leukoc. Biol.* **75,** 721–730 (2004).

42. Klechevsky, E. *et al.* Functional Specializations of Human Epidermal Langerhans Cells and CD14+ Dermal Dendritic Cells. *Immunity* **29,** 497–510 (2008).

43. Saalbach, A. *et al.* Dermal fibroblasts induce maturation of dendritic cells. *J. Immunol.* **178,** 4966–4974 (2007).

44. Saalbach, A. *et al.* Dermal fibroblasts promote the migration of dendritic cells. *J. Invest. Dermatol.* **130,** 444–454 (2010).

45. Schirmer, C., Klein, C., Bergen, M. Von, Simon, J. C. & Saalbach, A. Human fibroblasts support the expansion of IL-17 – producing T cells via up-regulation of IL-23 production by dendritic cells. *Blood* **116,** 1715–1725 (2010).

46. Berthier, R. *et al.* Fibroblasts inhibit the production of interleukin-12p70 by murine dendritic cells. *Immunology* **108,** 391–400 (2003).

47. Shiraishi, H. *et al.* Prostaglandin E2 is a major soluble factor produced by stromal cells for preventing inflammatory cytokine production from dendritic cells. *Int. Immunol.* **20,** 1219–1229 (2008).

48. Chiesa, S. *et al.* Mesenchymal stem cells impair in vivo T-cell priming by dendritic cells. *Proc. Natl. Acad. Sci.* **108,** 17384–17389 (2011).

49. Li, Y.-P. *et al.* Human mesenchymal stem cells license adult CD34+ hemopoietic progenitor cells to differentiate into regulatory dendritic cells through activation of the Notch pathway. *J. Immunol.* **180,** 1598–1608 (2008).

50. Zhang, B., Liu, R., Shi, D. & Liu, X. Mesenchymal stem cells induce mature dendritic cells into a novel Jagged-2–dependent regulatory dendritic cell population. … **113,** 46–57 (2009).

51. Wang, M. *et al.* The immunomodulatory activity of human umbilical cord blood-derived mesenchymal stem cells in vitro. *Immunology* **126,** 220–232 (2009).

52. Dixon, K. O., Rossmann, L., Kamerling, S. W. A. & Kooten, C. Van. Human renal fibroblasts generate dendritic cells with a unique regulatory profile. *Immunol. Cell Biol.* **92,** 688–698 (2014).

53. Steinman, R. M., Hawiger, D. & Nussenzweig, M. C. Tolerogenic dendritic cells. *Annu. Rev. Immunol.* **21,** 685–711 (2003).

54. Morelli, A. E. & Thomson, A. W. Tolerogenic dendritic cells and the quest for transplant tolerance. *Nat. Rev. Immunol.* **7,** 610–621 (2007).

55. Rezakhanlou, A. M. *et al.* Highly efficient stable expression of indoleamine 2,3 dioxygenase gene in primary fibroblasts. *Biol. Proced. Online* **12,** 107–112 (2010).

56. Lutz, M. B. *et al.* An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* **223,** 77–92 (1999).

57. Wen, Y. J., Min, R., Tricot, G., Barlogie, B. & Yi, Q. Tumor lysate-specific cytotoxic T lymphocytes in multiple myeloma: Promising effector cells for immunotherapy. *Blood* **99,** 3280–3285 (2002).

58. Lutz, M. B. *et al.* An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* **223,** 77–92 (1999).

59. Hosseini-Tabatabaei, A. *et al.* Mechanism underlying defective interferon gamma-induced IDO expression in non-obese diabetic mouse fibroblasts. *PLoS One* **7,** (2012).

60. Behboudi, S., Chao, D., Klenerman, P. & Austyn, J. The effects of DNA containing CpG motif on dendritic cells. *Immunology* **99,** 361–366 (2000).

61. Wen, Y. J., Min, R., Tricot, G., Barlogie, B. & Yi, Q. Tumor lysate-specific cytotoxic T lymphocytes in multiple myeloma: Promising effector cells for immunotherapy. *Blood* **99,** 3280–3285 (2002).

62. Ma, J. Z. I. *et al.* Murine CD4+ T cell responses are inhibited by Cytotoxic T cell-mediated killing of dendritic cells and are restored by antigen transfer. *PLoS One* **7,** (2012).

63. Höpken, U. E. *et al.* The ratio between dendritic cells and T cells determines the outcome of their encounter: Proliferation versus deletion. *Eur. J. Immunol.* **35,** 2851–2863 (2005).

64. Zaher, S. S., Germain, C., Fu, H., Larkin, D. F. P. & George, A. J. T. 3-hydroxykynurenine suppresses CD4+ T-cell proliferation, induces T-regulatory-cell development, and prolongs corneal allograft survival. *Investig. Ophthalmol. Vis. Sci.* **52,** 2640–2648 (2011).

65. Bauer, T. M. *et al.* Studying the immunosuppressive role of indoleamine 2,3-dioxygenase: Tryptophan metabolites suppress rat allogeneic T-cell responses in vitro and in vivo. *Transpl. Int.* **18,** 95–100 (2005).

66. Khosravi-Maharlooei M, Pakyari M, B Jalili R, Salimi-Elizei S, Lai JC, Poormasjedi-Meibod M, Kilani RT, Dutz J, G. A. Tolerogenic Effect of Mouse Fibroblasts on Dendritic Cells. Epub ahead of print (2016).

67. Liu, H., Liu, L., Fletcher, B. S. & Visner, G. A. Novel action of indoleamine 2,3-dioxygenase attenuating acute lung allograft injury. *Am. J. Respir. Crit. Care Med.* **173,** 566–572 (2006).

68. Zhang Y, Jalili RB, Kilani RT, Elizei SS, Farrokhi A, Khosravi-Maharlooei M, Warnock GL, Ao Z, Marzban L, G. A. IDO-Expressing Fibroblasts Protect Islet beta Cells from Immunological Attack and Reverse Hyperglycemia in Non-Obese Diabetic Mice. Epub ahead of print (2016).

69. Lafferty, K. J. & Cunningham, A. J. A new analysis of allogeneic interactions. *Aust. J. Exp. Biol. Med. Sci.* **53,** 27–42 (1975).

70. Thangavelu, G., Smolarchuk, C. & Anderson, C. C. Co-inhibitory molecules: Controlling the effectors or controlling the controllers? *Self/Nonself* **1,** 77–88 (2010).

71. Zhong, X. *et al.* PD-L2 expression extends beyond dendritic cells/macrophages to B1 cells enriched for VH11/VH12 and phosphatidylcholine binding. *Eur. J. Immunol.* **37,** 2405–2410 (2007).

72. Yi, K. H. & Chen, L. Fine tuning the immune response through B7-H3 and B7-H4. *Immunological Reviews* **229,** 145–151 (2009).

73. Maldonado, R. A. & von Andrian, U. H. *How Tolerogenic Dendritic Cells Induce Regulatory T Cells*. *Advances in Immunology* **108,** (2010).

74. Brooks, D. G., Walsh, K. B., Elsaesser, H. & Oldstone, M. B. A. IL-10 directly suppresses CD4 but not CD8 T cell effector and memory responses following acute viral infection. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 3018–3023 (2010).

75. Forouzandeh, F., Jalili, R. B., Germain, M., Duronio, V. & Ghahary, a. Differential immunosuppressive effect of indoleamine 2,3-dioxygenase (IDO) on primary human CD4+ and CD8+ T cells. *Mol. Cell. Biochem.* **309,** 1–7 (2008).

76. Mellor, A. L. *et al.* Cutting edge: induced indoleamine 2,3 dioxygenase expression in dendritic cell subsets suppresses T cell clonal expansion. *J. Immunol.* **171,** 1652–1655 (2003).

77. Munn, D. H. *et al.* Prevention of allogeneic fetal rejection by tryptophan catabolism. *Science* **281,** 1191–1193 (1998).

78. Terness, P. *et al.* Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. *J. Exp. Med.* **196,** 447–457 (2002).

79. Ehst, B. D., Ingulli, E. & Jenkins, M. K. Development of a novel transgenic mouse for the study of interactions between CD4 and CD8 T cells during graft rejection. *Am. J. Transplant* **3,** 1355–1362 (2003).