의학박사 학위논문

Antigen-specific iTreg cells in minor histocompatibility antigen-mismatched islet transplantation model

부조직적합항원-불일치 췌도 이식 모델에서 유도된 항원-특이적 조절 T 림프구

2013 년 07 월

서울대학교 대학원

의학과 미생물학 과정

김 용 희

A thesis of the Degree of Doctor of Philosophy

부조직적합항원-불일치 쥐도 이식 모델에서 유도된 항원-특이적 조절 T 림프구

Antigen-specific iTreg cells in minor histocompatibility antigen-mismatched islet transplantation model

July 2013

The Department of Microbiology,
Seoul National University
College of Medicine
Yong-Hee Kim

Antigen-specific iTreg cells in minor histocompatibility antigen-mismatched islet transplantation model

by
Yong-Hee Kim

A thesis submitted to the Department of Microbiology in partial fulfillment of the requirement of the Degree of Doctor of Philosophy in Microbiology at Seoul National University College of Medicine

July 2013

Approved by Thesis Committee:

Professor _____ Chairman
Professor _____ Vice chairman
Professor _____
Professor _____
Professor _____

부조직적합항원-불일치 췌도 이식 모델에서 유도된 항원-특이적 조절 T 림프구

지도교수 박 정 규

이 논문을 의학박사 학위논문으로 제출함

2013 년 04 월

서울대학교 대학원

의학과 미생물학 전공

김 용 희

김용희의 의학박사 학위논문을 인준함

2013 년 07 월

위 원 장 (인)

부위원장 (인)

위 원 (인)

위 원 (인)

위 원 (인)

ABSTRACT

Introduction: Inducing immunological tolerance to transplant is the ultimate goal in transplantation field. It has been reported that the pancreatic islet transplantation across the minor histocompatibility of H-Y (male antigen) could induce immunological tolerance to male antigen in female C57BL/6 mice resulting in the acceptance of male skin graft without any immunological manipulation. In this study, I tried to find out the underlying mechanisms of the immune tolerance.

Methods: Female RAG2$^{-/-}$ Marilyn mice having transgenic TCR specific for H-Y peptide were transplanted with male skin on left flank or with male islet underneath the kidney capsule. Before and after the islet transplantation, CD4^{+} T cells were analyzed for expression of Foxp3 by flow cytometry. Subsequent male skin transplantations were operated to male islet recipients to verify the establishment of immunological tolerance.

Results: Female Marilyn mice rejected male skin graft within 24 days, but accepted male islet graft indefinitely and did not reject the subsequently transplanted male skin. Flow cytometric analysis of the peripheral blood of these mice revealed the emergence of CD4^{+}FoxP3^{+} regulatory T cells (Treg) which are normally absent in naïve Marilyn mice. These induced Treg cells were antigen-specific and played an important role in the prevention of the rejection of male skin graft. The suppressive function of inducible Treg (iTreg) cells was confirmed by *in vitro* Treg suppression assay. In addition, the positive correlation between the number of iTregs and the passenger

leukocytes accompanied by the transplanted male islets provide clues to the role of the passenger leukocytes in the iTreg induction.

Conclusions: Pancreatic islet transplantation across H-Y disparity induced iTregs and contributed to the establishment of transplantation tolerance. This model could be an asset for the characterization of de novo induced iTreg cells.

Keywords: Immune Tolerance, Islet Transplantation, Regulatory T Cells
Student number: 2007-22000

CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

RAG: recombination activating gene

TCR: T cell receptor

CD: cluster of differentiation

Foxp3: forkhead box P3

Treg: regulatory T cell

iTreg: inducible Treg

nTreg: natural Treg

TGF-β: transforming growth factor-β

HBSS: Hank's balanced salt solution

RPMI 1640: Rosewell Park Memorial Institute 1640

FBS: fetal bovine serum

IEQ: islet equivalent

PBS: phosphate buffered saline

EDTA: ethylenediaminetetraacteic acid

RBC: red blood cell

MACS: magnetic-activated cell sorting

FACS: fluorescence-activated cell sorting

BSA: bovine serum albumin

CTLA-4: cytotoxic T-lymphpcyte antigen 4

GITR: glucocorticoid-induced TNFR family related gene

MHC: major histocompatibility complex

APC: antigen –presenting cell

DC: dendritic cell

LN: lymph node

INTRODUCTION

Transplantation is a curative treatment for end-stage organ failures including type 1 diabetes. However, strong immunosuppressants should be applied to the recipients to prevent the rejection of grafts (1), and the resulting generalized immune suppressions may provoke life-threatening side effects, such as opportunistic infections and malignancies (2, 3). Therefore, specific suppression of immune responses by inducing immunological tolerance to the transplant is the ultimate goal in the field of transplantation. In several studies, it was shown that co-stimulatory blockade or co-receptor blockade regimen may induce transplantation tolerance (4-9). In these studies, regulatory T cells (Treg), in particular the inducible Treg (iTreg) cells played a crucial role (10).

The regulatory T cell (Treg) having suppressive function was proved by Sakaguchi and his colleagues (11). They first observed autoimmune oophoritis after neonatal thymectomy in mice (12). Next, they identified the requirement of T cells for the prevention of the organ-specific autoimmune diseases (13, 14) . Eventually, they found the suppressive subset of T cells, the $CD4^+CD25^+$ Treg cells. While the transfer of $CD4^+CD25^-$ cells into the athymic nude mouse caused an autoimmune disease, the co-transfer of $CD4^+CD25^+$ Treg cells prevented it (11). Although $CD4^+CD25^+$ cells mainly represent Treg cells, activated T cells also express CD25 (IL-2 receptor α). Thus, $CD4^+CD25^+$ double expression is not a lineage-specific marker of Treg cells. Many research groups endeavored to find the Treg-specific marker, and eventually, Foxp3 (forkhead box P3) had been identified as the lineage-specific marker of Treg cells. The Treg-specific expression of Foxp3 was identified, and it was found that forced expression of Foxp3 rendered naïve $CD4^+$ T cells suppressive (15-18).

Contrary to natural Treg (nTreg) cells generated during thymic T cell development, iTreg cells are induced in the periphery (19). Several investigators reported peripheral iTreg cell by various induction methods. Chen *et al*. (20) demonstrated *in vitro* conversion of $CD4^+CD25^-$ naïve T cells to $CD4^+CD25^+$ regulatory T cells through stimulation with T cell receptors (TCRs) and transforming growth factor (TGF-β) (20). Minute peptide antigen delivery by osmotic pump or oral antigen delivery has been shown to induce iTreg cells in mice (21, 22). Also, targeted antigen delivery to dendritic cells with antigen cross-linked anti-DEC-205 antibody could induce iTreg cells (23). In addition, skin graft with non-depleting anti-CD4 antibody induced iTreg cells for donor antigen (4).

In this study, minor histocompatibility antigen was used to study transplantation rejection and immunological tolerance against a specific antigen. Histocompatibility antigens are products of polymorphic genes recognized by the immune system following the exchange of organs or tissues between genetically different individuals (24). The major histocompatibility complex (MHC) which presents peptide antigens to T cells is most polymorphic between individuals, and the transplantation between MHC-mismatched individuals causes rapid graft rejection. However, graft rejection can also occur even when MHC-matched tissues are transplanted. This immune response is directed against minor histocompatibility antigens which are peptide products of ubiquitously expressed proteins. These peptides are expressed at the cell surface within the peptide binding groove of the MHC molecules, where they can be recognized by circulating $CD4^+$ and $CD8^+$ T cells (25). H-Y antigen is the most well-known minor histocompatibility antigen. H-Y antigen is encoded by a gene on the Y chromosome. H-Y antigen could be studied in every inbred mouse strain, since males were the natural congenic partner for females of the same strain (26). Minor

histocompatibility antigens, especially the H-Y antigens do in fact have clinical importance. The first clinical report was that rejection occurred in a female patient who received a bone-marrow graft from her HLA-identical brother (27). Furthermore, there were reports that male recipients of female stem-cell grafts had an increased risk of graft-versus-host disease (28, 29). Female recipients had an increased risk of rejection against male hematopoietic stem cell transplantation (30). In solid organ transplantation, female recipients of male organs had high rates of acute immunological rejection in corneal (31), cardiac (32, 33), hepatic (34), pulmonary (35) and renal (36, 37) transplantation. In a multi-center cohort study with 195,516 kidney allograft recipients, male donor and female recipient combination had the highest risk of failure compared with other gender combinations in 1 year and 10 years follow-up (38).

It has been known that the skin graft across H-Y minor histocompatibility antigen mismatch is normally rejected (26, 39). However, in our previous report (40), islet transplantation across H-Y barrier without any immunological manipulation induced H-Y antigen-specific immunological tolerance which prevented the rejection of subsequent male skin graft in C57BL/6 (B6) mouse model. In that study, the percentage of $CD4^{+}FoxP3^{+}$ Treg cell was increased after the male islet transplantation. It was speculated that the increased Treg cells would be iTreg cells induced by male islet transplantation, and that the immunological tolerance might be established by these iTreg cells.

In this study, male islet transplantations were conducted in $B6.RAG2^{-/-}$ Marilyn mice which have male antigen specific $CD4^{+}$ T cells but do not have $CD4^{+}FoxP3^{+}$ Treg cells. $CD4^{+}FoxP3^{+}$ Treg cells could be induced in this strain by the male islet transplantation. This induction of $CD4^{+}FoxP3^{+}$ iTreg cells was compatible with our hypothesis in the previous report; iTreg cell

induction by male islet transplantation without any immunological manipulation. The induced immunological tolerance was confirmed by the acceptance of subsequent male skin graft. The suppressive function of iTreg cells was confirmed by *in vitro* suppression assay. In addition, potential factors that influence the process of iTreg induction in this male islet transplantation were explored, and the passenger leukocytes accompanied by the transplanted male islets were found to be important determinants in the iTreg induction.

In fact, solid organs and cellular grafts contain bone marrow-derived hematopoietic cells of donor origin, namely the passenger leukocytes (41). Passenger leukocytes are bone marrow-derived hematopoietic cells that are carried within the interstitium of organ allografts (42). After implantation, they rapidly migrate to the recipient's lymphoid organs, where they can activate alloreactive T lymphocytes (43-45). Therefore, it was widely thought that passenger leukocytes can stimulate the host immune response to the allograft (42). Several studies have shown that depletion of these passenger leukocytes can lead to prolongation of graft survival (46-51). These findings have also led to clinical trials attempting to reduce the immunogenicity of allografts by eliminating passenger leukocytes (52). However, recipients that have achieved a long-term acceptance of liver(53) and kidney(54) allografts have been found to have a persistent population of donor-derived leukocytes in their peripheral lymphoid compartment, a state called 'microchimerism' (55, 56). Therefore, an essential role of this persistent microchimerism in long-term graft acceptance has been suggested (57, 58). It was speculated that passenger leukocytes may play an important role in the development of host unresponsiveness to donor antigens (42, 59).

Indeed, several studies have reported the essential role of the passenger leukocytes in the induction of transplantation tolerance. In some donor-

recipient combinations in pigs and rodents, liver allografts containing large numbers of donor-derived passenger leukocytes are spontaneously accepted (60, 61). However, when the passenger leukocyte-depleted liver grafts were transplanted, they were unable to induce an immunological tolerance (62). Abrogation of transplantation tolerance by depleting donor leukocytes not on day 18 but on day 0 revealed the essential role of passenger leukocytes in immunomodulatory effects on the induction phase of allograft acceptance (56). It has long been established that pre-transplant donor-specific blood transfusions (DST) result in the enhancement of vascularized allografts in rodents (63-65). However, DST alone did not induce tolerance, the combination of DST and allograft induced the inhibition of anti-donor response (66). When heart allografts were depleted of passenger leukocytes, tolerance induction in DST-treated recipients was abrogated. However, tolerance could be restored by injecting the recipients with donor dendritic cells (DCs) at the time of grafting (67). These studies imply the role of graft passenger leukocytes, most likely DCs in the induction of allograft tolerance.

DCs are professional antigen-presenting cells (APCs) that have been regarded as potent stimulators of innate and adaptive immunity. However, they also play major roles in directing the immune response toward immunological tolerance (68-73). Tolerogenic potential of DCs, especially of immature DCs, started being reported by Thomson and his colleagues (74-76). In their studies, immature donor bone marrow-derived DCs administrated 7 days before heart graft transplantation either alone or in combination with costimulatory blockade significantly prolonged the graft survival. Similar result which was reported by Lutz et al. (77) reinforced this notion. Subsequently, there have been numerous reports of indefinite allograft survival following administration of various types of donor-derived tolerogenic DCs (72, 78-84). Since tissue-resident DCs are immature in steady

states, the donor leukocytes present in the graft have been characterized mainly as immature DCs (85). DCs may exert their tolerogenic effects through a variety of mechanisms. In addition to their role in T cell anergy and deletion, DCs are pivotal in iTreg induction, where their ability to induce and interact with Treg cells is critical for their tolerogenic effect (68, 86).

In this study, a definite correlation between the iTreg cells and donor leukocytes was observed in male islet recipients. In addition, an obvious population of $CD11c^{+}MHCII^{+}$ DCs was identified in male islets as the passenger leukocytes. These donor-derived DCs in male islet transplantation may act as tolerogenic DCs to induce male antigen-specific iTreg cells and immunological tolerance to male antigens.

MATERIALS AND METHODS

1. Mice

Thy1.1 congenic B6.PL-Thy1^{a} mouse, B6.CD45.1 congenic mouse, C57BL/6 mouse (B6) and B6.RAG2$^{-/-}$ mouse were purchased from The Jackson Laboratory. B6.RAG2$^{-/-}$Marilyn mouse was kindly provided by Dr. Seung-Yong Seong. Foxp^{gfp} reporter mouse was kindly provided by Dr. Alexander Rudensky. B6.RAG2$^{-/-}$Marilyn mice were cross-bred with Foxp^{gfp} reporter mice to produce B6.RAG2$^{-/-}$Marilyn x Foxp^{gfp} reporter mouse. Mice were bred and housed in a specific pathogen-free facility. Animal studies were conducted under protocols approved by Seoul National University Institutional Animal Care and Use Committee (SNU-081117-4, SNU-090111-1 and SNU-110406-1).

2. Induction of diabetes mellitus and blood glucose monitoring

To induce diabetes mellitus, recipient Marilyn mice were intraperitoneally injected with 125 mg/kg of streptozotocin (Sigma, St. Louis, MO) in two consecutive days. After the injections, non-fasting blood glucose levels were monitored using a glucometer, OneTouch Ultra (LifeScan inc., Milpitas, CA) from blood obtained by tail snipping. Mice with two consecutive non-fasting blood glucose levels higher than 250 mg/dl were considered diabetic and selected for the islet transplantation.

3. Isolation and transplantation of pancreatic islets

Donor pancreases were injected with HBSS containing 0.5 mg/ml of Collagenage P (Roche, Mannheim, Germany) into the common bile duct which eventually flowed into the pancreatic duct. Inflated pancreases were

excised and incubated in a 37°C water bath for 20 minutes. Digested pancreases were filtered through a sieve and washed. Then, islets were purified from the digests using Euro-Ficoll gradients. Obtained islets were re-suspended with RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 2 mM L-glutamine, 50 μg/ml gentamycin and 100 μM non-essential amino acids (all purchased from Invitrogen).

After overnight culture, islets were hand-picked under a microscopic view and then transferred to polyethylene tube to be used for transplantation. Recipient mice were anesthetized by the inhalant, isoflurane. Then, the left kidney was exposed and 250~300 islet equivalent number (IEQ) islets were delivered beneath the renal capsule. Unless otherwise described, islet recipients were female B6.RAG2$^{-/-}$Marilyn mice or B6.RAG2$^{-/-}$Marilyn x Foxp^{gfp} reporter mice.

4. Skin grafting

Full-thickness tail skins obtained from donors were transplanted to graft beds on the left flank of the anesthetized recipient mice and covered with Vaseline gauze and Band-Aid (Johnson&Johnson, New Brunswick, NJ). Bandages were removed after 7 days, and grafts were observed every 2 to 3 days for 3 week and weekly thereafter. The graft was scored as rejected when less than 30% of viable tissue remained and visible inflammation ended.

5. Flow cytometry

Peripheral blood was obtained from orbital venous sinus of anesthetized mice, and mixed with equal volume of blood buffer (PBS containing 100 U/ml heparin and 10 mM EDTA). Red blood cells were lysed with RBC lysis buffer (distilled water containing 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1

mM EDTA). After incubation with Fc receptor blocking antibody, 2.4G2, cells were stained for 30 min on ice with with a cocktail of antibodies for surface molecules. After washing twice with FACS buffer (PBS containing 0.5% BSA and 15.4 mM NaN_3), cells were fixed and permeabilized with FoxP3 Fixation/Permeabilization buffer set (eBioscience, San Diego, CA) according to the manufacturer's instructions. Then, intracellular FoxP3, CTLA-4, Helios and Ki-67 were stained with specific antibodies for 30 min on ice. After washing and re-suspending in FACS buffer, cells were acquired with a FACSCanto II (BD Biosciences, San Jose, CA) and analyzed with FACSDiva software (BD Biosciences) and Flowjo software (TreeStar, Ashland, OR)

Anti-CD4-PerCP-Cy5.5 (RM4-5), anti-CD4-APC-eFluor780 (RM4-5), anti-FoxP3-FITC (FJK-16s), anti-FoxP3-PE (FJK-16s), anti-FoxP3-APC (FJK-16s), anti-CD25-APC-Alexa Fluor 750 (PC61.5), anti-Thy1.1-APC (HIS51), anti-Thy1.2-FITC (53-2.1), anti-CD45.1-APC-eFluor 780 (A20), anti-CD45.2-APC-eFluor 780 (104), anti-GITR-FITC (DTA-1), anti-Ki-67-PerCP-eFluor710 (SolA15), anti-CD19-PE (1D3), anti-Helios-AlexaFluor 647 (22F6), anti-CD19-FITC (eBIo1D3), anti-MHC class II (I-A/I-E)-APC (M5/114.15.2), anti-CCR7-APC (4B12) and anti-PD-L1-PE (MIH5) antibodies were purchased from eBioscience. Anti-V_β6-FITC (RR4-7), anti-V_β6-PE (RR4-7), anti-Thy1.2-PE (53-2.1), anti-CD8α-APC-Cy7 (53-6.7), anti-CTLA-4-PE (UC10-4F10-11), anti-CD11c-PE (HL3), anti-MHC class II (I-A^b)-FITC (AF6-120.1), anti-CD45-PerCP (30-F11) and anti-CD45-APC (30-F11) antibodies were purchased from BD Biosciences. Anti-Neuropilin-1-PE (761705) and anti-Neuropilin-1-PerCP (polyconal) antibodies were purchased from R&D Systems (Minneapolis, MN). All isotype control antibodies were purchased from eBioscience.

To identify passenger leukocytes accompanied by the transplanted islets, overnight-cultured islets were hand-picked under a microscopic view, and

then dissociated into single cells by incubation with Accutase (PAA Laboratories, Piscataway, NJ) for 10 min at 37°C. Islet single cells were then stained, washed and analyzed by the flow cytometry method described above. To distinguish passenger leukocytes from islet beta cells, the zinc ion indicator FluoZin-3 (Life Technologies, Carlsbad, CA) were applied to stain islet beta cells. According to the manufacturer's instruction, FluoZin-3 staining was conducted at 37°C for 30 min, and then washed twice with RPMI 1640 media.

6. Cell sorting

Single cell suspensions were prepared from the spleen and lymph nodes of the mouse. Harvested spleen and lymph nodes were crushed with frosted slide glasses and then passed through a 70 μm strainer. Spleen cells were treated with RBC lysis buffer (Sigma-Aldrich, Buchs, Switzerland) to lyze red blood cells. For magnetic activated cell sorting (MACS), cells were washed with commercial running buffer (Miltenyi Biotec, Bergisch Gladbach, Germany) or handmade NaN_3-free buffer (2 mM EDTA and 2 % FBS in PBS solution). To enrich $CD4^+$ cells, cells were incubated with biotin-conjugated antibody cocktails and anti-biotin microbeads for negative selection (CD4+ T cell Isolation Kit II, Miltenyi Biotec) according to the manufacturer's instructions. Then, conjugated cells were sorted by AutoMACS Pro Separator (Miltenyi Biotec). For fluorescence-activated cell sorting (FACS), MACS-enriched $CD4^+$ cells were sorted by FACS Aria (BD Biosciences) according to the expression of GFP.

7. *In vitro* Treg suppression assay

Spleen and lymph node cells obtained from the male islet recipient naïve Marilyn mouse and $Foxp3^{gfp}$ reporter mouse were prepared as single cells.

$CD4^+$ cells were enriched by negative selection using AutoMACS Pro Separator. GFP^+ cells (Treg) or GFP^- cells (responder) were sorted with FACS Aria. $CD4^+Foxp3^-$ responder cells were labeled with 2 μM carboxyfluorescein succinimydil ester (CFSE) and plated onto a 96 well plate ($2\sim3x10^4$ responder cells/well). Responder cells were stimulated with irradiated (30 Gy) congenic B6.CD45.1 male splenocytes or plate-coated anti-CD3 antibody (3 μg/ml). Treg cells obtained from male islet recipient Marilyn x $Foxp3^{GFP}$ reporter mice or naïve $Foxp3^{GFP}$ reporter mouse were co-cultured with responder cells at various ratios.

RESULTS

Rejection of male skin and acceptance of male islet in female Marilyn mice

Male tissue transplantation was conducted in B6.RAG2$^{-/-}$Marilyn mice which have a transgenic TCR ($V_{\alpha}1.1$, $V_{\beta}6$) specific for the H-Y peptide (NAGFNSNRANSSRSS) (24) presented by MHC class II, I-A^b (87). Thus, no $CD8^+$ T cell or B cell is present in the female Marilyn mice, and all existing T cells are male antigen-specific $CD4^+$ T cells. Although $CD8^+$ T cells are considered the critical effecter cells in graft rejection, $CD4^+$ T cells have the ability to provoke rejection to skin grafts discordant for a single minor histocompatibility antigen in the absence of $CD8^+$ T cells or B cells (88). As with the A1.RAG1$^{-/-}$ mice in the above reference which are transgenic for a TCR specific for an H-Y peptide in the context of MHC class II, I-E^k (88), female B6.RAG2$^{-/-}$Marilyn mice rejected the male skin within 24 days (Fig. 1A).

In our previous report, male islet transplantation in wild type C57BL/6 (B6) mice induced transplantation tolerance to the male antigen, H-Y (40). In female Marilyn mouse, all lymphocytes are male antigen-specific $CD4^+$ T cells. Therefore, it was speculated that the immune response to the transplanted male tissue would be stronger in Marilyn mouse than that in the wild type mouse. However, female Marilyn mice did not reject the transplanted male islets. When the streptozotocin-induced diabetic Marilyn mice were transplanted with syngeneic male islets, recipients' hyperglycemia was normalized, and normal blood glucose levels were maintained in the majority of recipients (Fig. 1B). This implies that the grafted male islets are not only survived from rejection but also functioned effectively.

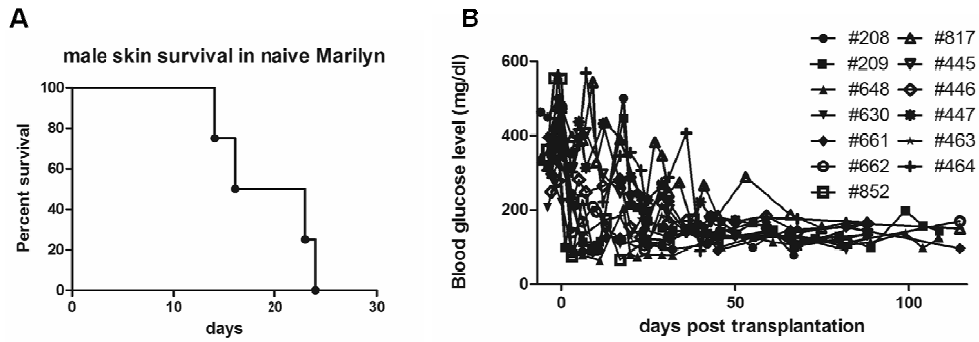


Figure 1. Naïve female Marilyn mouse rejects male skin but accepts male islets. Naïve female Marilyn mice were transplanted with syngeneic male skin or male islets. (A) Full-thickness tail skins were grafted on left flank of recipients. Percent survival of the transplanted male skin is depicted. Male skins were rejected within 24 days. Median survival day was 19.5 days (n=4). (B) 250~300 IEQ of male islets obtained from B6.Thy1.1 congenic mice were transplanted beneath the renal capsule of recipients which were rendered diabetic with intraperitoneal streptozotocin injection (125 mg/kg, 2 times). Non-fasting blood glucose levels were monitored. Transplanted male islets normalized blood glucose levels indefinitely. Data are sum of three representatives of at least nine independent experiments.

Regulatory T cell induction by male islet transplantation

In our previous report, tolerance to male skin in female C57BL/6 mice were induced by prior male islet transplantation and increased number of $CD4^+FoxP3^+$ regulatory T cell (Treg) was observed in those recipients (40). This increase in Treg cells may have resulted either from the expansion of pre-existing Treg cells or from *de novo* induction of inducible Treg (iTreg) cells. To distinguish the two possibilities, TCR-transgenic mice with RAG^- deficiency in which endogenous thymic $CD4^+FoxP3^+$ Treg cells are absent were adopted (23, 89). Indeed, there were no $CD4^+FoxP3^+$ Treg cells in naïve Marilyn mice (Fig. 2A). However, after the male islet transplantation, $CD4^+FoxP3^+$ T cells were detected in the recipient Marilyn mice (Fig. 2B). Since there were no $FoxP3^+$ Treg cells before the male islet transplantation, it was assumed that these $CD4^+FoxP3^+$ T cells are iTreg cells that have been converted from naïve $CD4^+FoxP3^-$ T cells by male antigen presentation.

To confirm that these Treg cells were induced by male antigen presentation, female islet transplantation was conducted in diabetic Marilyn mice as a control group. As expected, grafted female islets were not rejected and functioned well (Supplement 1). The presence of $FoxP3^+$ cells were also checked for in the female islet recipients. Unexpectedly, $CD4^+FoxP3^+$ T cells were detected after the female islet transplantation (Fig. 2C). However, commitment of Treg cells requires TCR engagement (90, 91). Since there was no presentation of male antigen to Marilyn $CD4^+$ T cells which have the male antigen-specific transgenic TCR, the possibility of conversion into iTreg cells was low. Therefore, it was speculated that these Treg cells may be the donor T cells which came with the transplanted islets.

Since B6.Thy1.1 congenic mice were used as the islet donors, $Thy1.2^+$ recipient T cells could be distinguished from $Thy1.1^+$ donor T cells. As expected, $CD4^+FoxP3^+$ T cells in the female islet recipients were $Thy1.1^+$

Thy1.2^{-} donor Treg cells (Fig. 3A), strongly suggesting that these Treg cells are passenger lymphocytes accompanied by the transplanted islets. Therefore, it was concluded that there was no *de novo* induction of iTreg cells from recipient CD4^{+} T cells in female islet recipients. Male islet recipients also had some Thy1.1^{+}Thy1.2^{-} donor-origin Treg cells. However, there was a definite population of recipient-origin Thy1.2^{+}CD4^{+}FoxP3^{+} Treg cells in male islet recipients (Fig. 3B). Therefore, there was *de novo* conversion of recipient CD4^{+}FoxP3^{-} T cells to CD4^{+}FoxP3^{+} Treg cells in the male islet recipients.

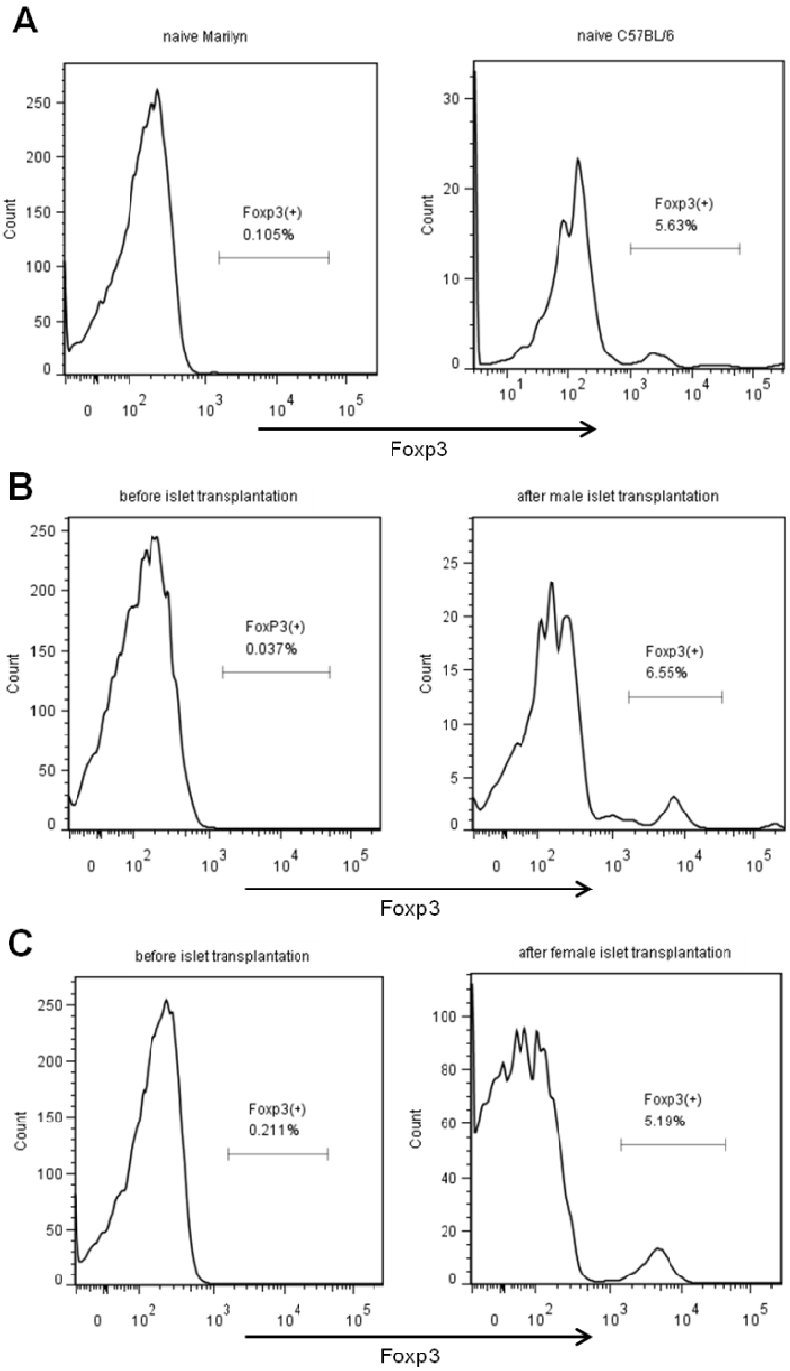


Figure 2. $CD4^{+}FoxP3^{+}$ Treg cells newly emerged after the islet transplantation. Peripheral blood obtained from orbital venous sinus of naïve (A), male islet recipient (B) and female islet recipient (C) Marilyn mice were stained with fluorescence conjugated antibodies. Histograms are gated on

$CD4^+$ cells (A) In contrast to wild type C57BL/6 mice (right panel) naïve Marilyn mice did not contain $CD4^+$ $FoxP3^+$ T cells (left panel). (B-C) There was no $CD4^+FoxP3^+$ T cell before the islet transplantations (left panels). 4~5 weeks after the islet transplantation (right panels), $CD4^+FoxP3^+$ T cells were detected in peripheral blood of the recipients. Data are representative of at least seven independent experiments.

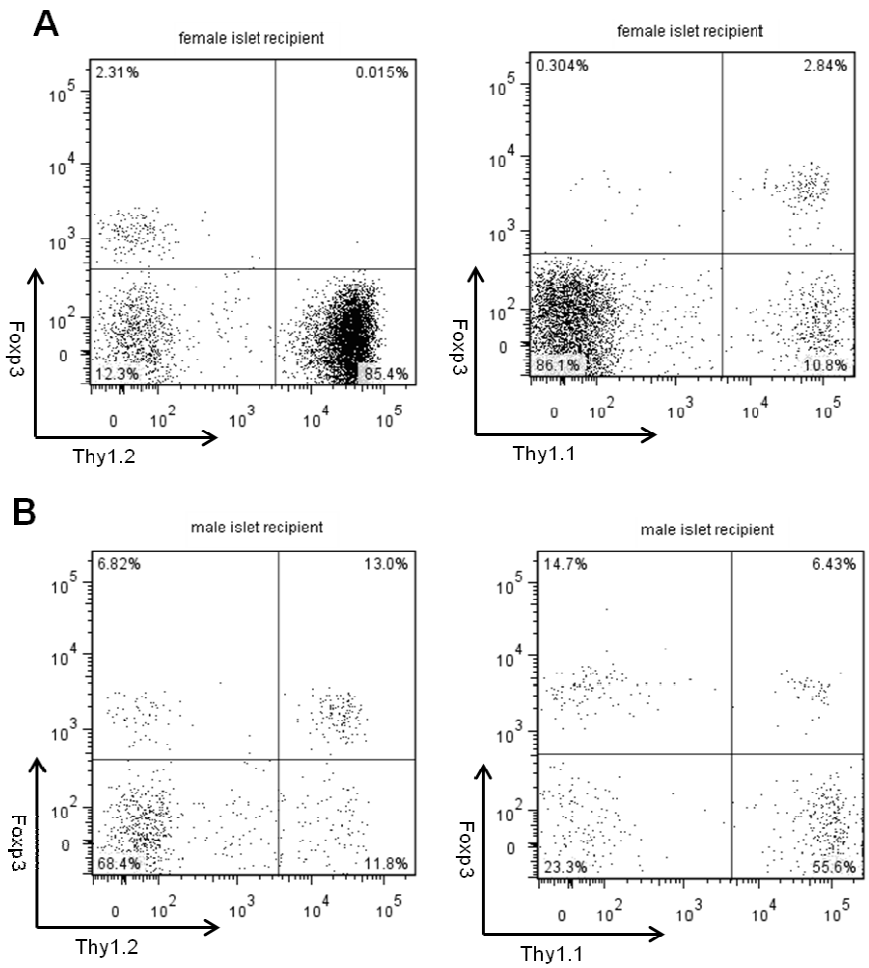


Figure 3. Conversion of $CD4^+FoxP3^-$ T cells to $CD4^+FoxP3^+$ Treg cells occurred in male islet recipients. 7~8 weeks after the islet transplantation, peripheral blood obtained from orbital venous sinus of the female islet recipients (A) and the male islet recipients (B) were stained with fluorescence-conjugated antibodies for FoxP3 and congenic markers, Thy1.1 (donors) and Thy1.2 (recipients). Dot plots are gated in $CD4^+$ cells. There were $Thy1.1^+FoxP3^+$ donor-origin Treg cells in both islet recipients. However, newly converted $Thy1.2^+$ $FoxP3^+$ recipient-origin iTreg cells were present in male islet recipients. Data are representative of at least seven independent experiments.

Expression of characteristic Treg cell markers in *de novo* induced Treg cells

In addition to FoxP3, some other known Treg markers were analyzed. The iTreg cells induced by the male islet transplantation expressed comparable levels of CTLA-4 and CD25 with Treg cells obtained from naïve C57BL/6 mouse (Fig. 4A,B), and all the *de novo* induced iTreg cells were $GITR^+$ (Fig. 4C). In addition to the congenic marker Thy1.2, the recipient-origin Treg cells' TCR with the specific type of variable β chain was also checked for. Marilyn's transgenic TCR has Vβ6 and Vα1.1 type. As expected, all $Thy1.2^+$ converted iTreg cells had transgenic $V\beta6^+$ TCR (Fig. 4D). These iTreg cells which have male antigen-specific transgenic TCRs can be considered as the antigen-specific Treg cells against the male antigen. In a recent study, Ki-67 was suggested as a marker for activated Treg cells (92). The converted iTreg cells which were continuously encountering the specific male antigen by the transplanted male islets had higher levels of Ki-67 than Treg cells obtained from naïve C57BL/6 mice (Fig. 4E).

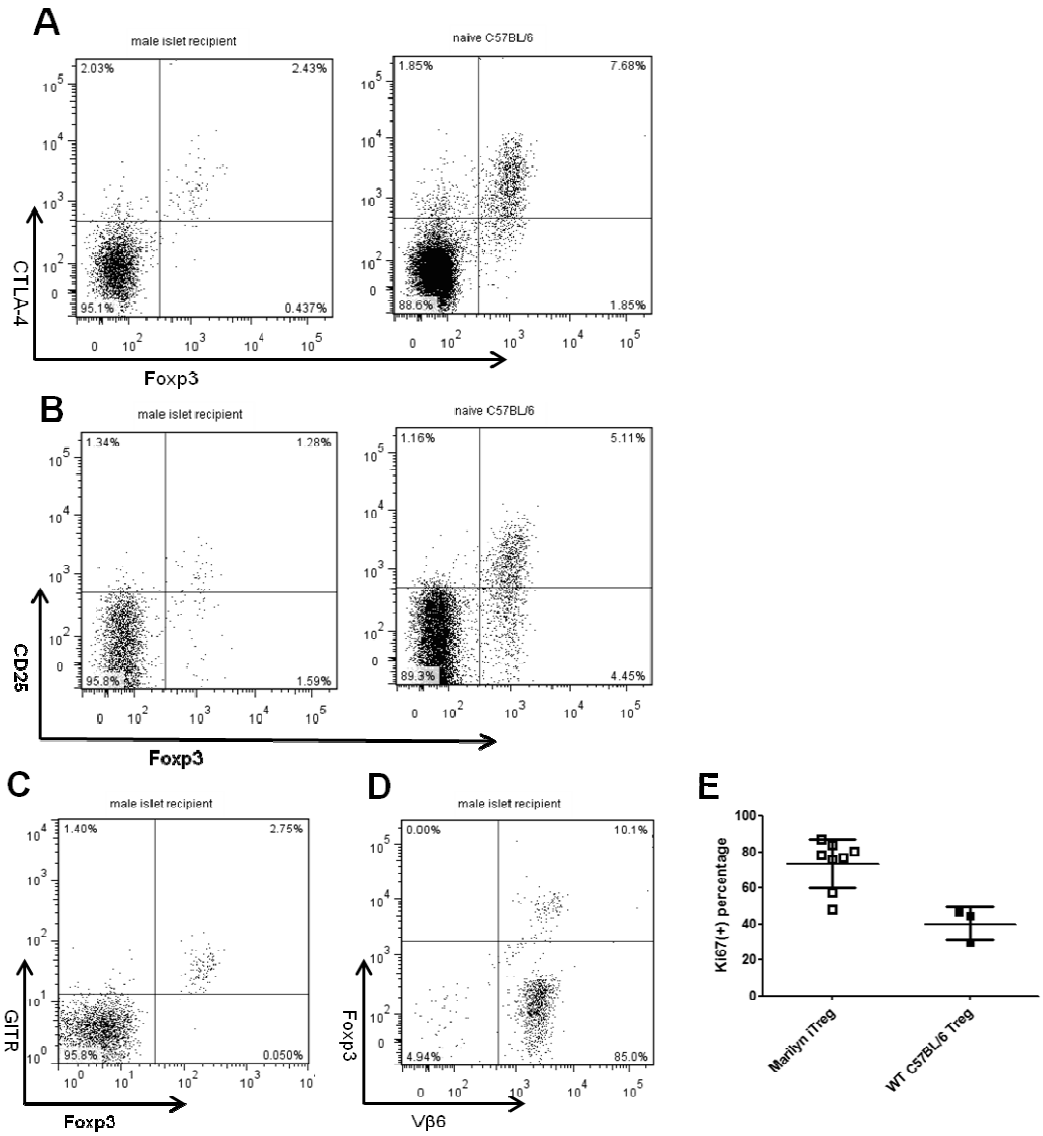


Figure 4. Induced Treg cells have characteristic markers of Treg cells and male antigen-specific transgenic TCR. 7~8 weeks after the islet transplantation, peripheral blood obtained from orbital venous sinus of the recipients were stained with fluorescence conjugated antibodies. (A-D) Dot plots are gated on recipient origin $CD4^+$ cells. Data are representative of at least three independent experiments. (E) Percentage of Ki-67^+ cells in the recipient-origin iTreg cells of islet recipients (open squares) or in Treg cells of naïve C57BL/6 mice are depicted. Each dot represents an individual mouse. Values are plotted as mean ± standard deviation.

In order to distinguish iTreg cells from natural Treg (nTreg) cells, a specific marker is required. Thornton *et al.* reported that Helios, a member of Ikaros transcription factor family is a potential marker of nTreg cells (93). Although it was still controversial (94-96), Helios expression of iTreg cells induced by male islet transplantation was assessed. It was expected that iTreg cells will not express the suggested nTreg marker, Helios. However, there was no significant difference in Helios expression between iTreg cells from male islet recipient and Treg cells from naïve C57BL/6 mouse. Most $CD4^{+}Foxp3^{+}$ cells in both groups expressed high levels of Helios (Fig. 5A). Akimova *et al.* disagreed with the idea that Helios is a nTreg marker, and they insisted that Helios is a marker of T cell activation and proliferation (95). To address this question, double staining for Helios and Ki-67 on iTreg cells induced by the male islet transplantation was conducted. In naïve C57BL/6 mouse, there was no obvious correlation between Ki-67 and Helios expression of Treg cells. However, most iTreg cells from male islet recipients expressed Ki-67 together with Helios (Fig. 5B). In a more recent study, Weiss *et al.* and Yadav *et al.* reported that nTreg cells expressed high levels of neuropilin 1 (Nrp1), but, mucosa-generated iTreg cells and spontaneously generated iTreg cells were $Nrp1^{low}$ (97, 98). Unlike Helios, a discernible pattern of Nrp1 expression could be observed in iTreg cells induced by male islet transplantation. Percentage of iTreg cells expressing high levels of Nrp1 was significantly lower than that of conventional Treg cells of naïve C57BL/6 mouse (Fig. 5C). Although both articles suggested Helios and Nrp1 as independent nTreg markers (93, 97, 98), the expression of these two molecules did not show high coincidence. In iTreg cells induced by the male islet transplantation, relatively high percentage $Nrp1^{low}Foxp3^{+}$ cells were $Helios^{+}$ (Fig. 5D).

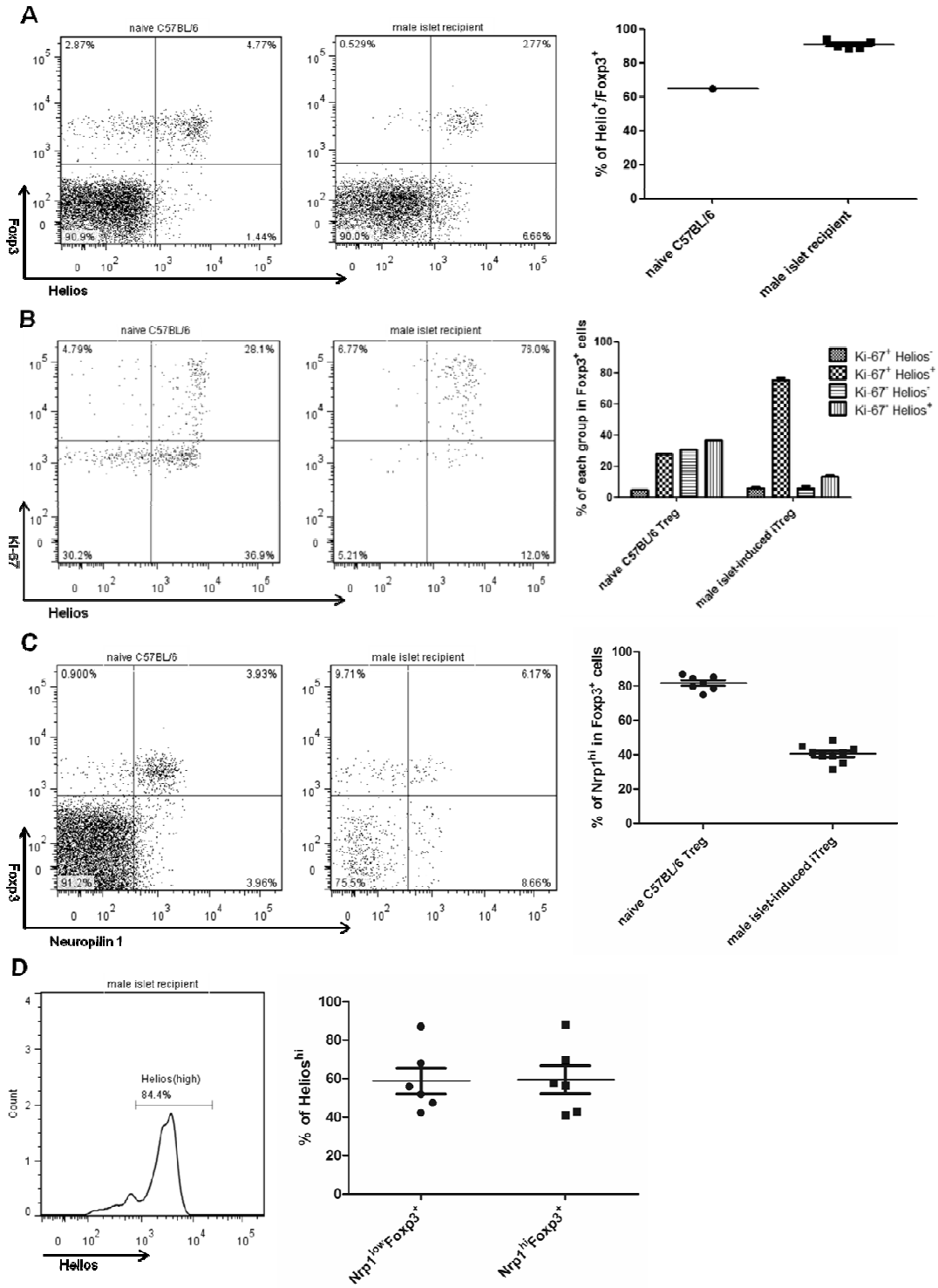


Figure 5. Verification of differential markers between male islet-induced iTreg cells and nTreg cells. Peripheral blood obtained from orbital venous sinus of

the male islet recipients and naïve C57BL/6 mice were stained with fluorescence conjugated antibodies (A-D). (A) Dot plots are gated in recipient-origin $CD4^+$ cells. Not only naïve C57BL/6 Treg cells (left panel) but also male islet-induced iTreg cells (middle panel) have high percentage of $Helios^+$ cells. Dot plots are representative of two independent experiments. Percentages of $Helios^+$ cells in each group of $CD4^+Foxp3^+$ cells are depicted (right panel). Each dot represents an individual mouse. (B) Dot plots are gated in naïve C57BL/6 Treg cells (left panel) or male islet-induced iTreg cells (middle panel). Percentages of cells in each quadrant are depicted (right panel). Male islet recipient contains high percentage of $Ki\text{-}67^+Helios^+$ iTreg cells. (C) Dot plots are gated in recipient-origin $CD4^+$ cells. When compared with naïve C57BL/6 Treg cells (left panel), male islet-induced iTreg cells (middle panel) have lower percentage of $Neuropilin1^{hi}$ cells. Dot plots are representative of four independent experiments. Percentages of $Neuropilin1^{hi}$ cells in each group of $CD4^+Foxp3^+$ Treg cells are depicted (right panel). Each dot represents an individual mouse. (D) Helios expression of $Neuropilin1^{low}Foxp3^+$ cells in (C) is depicted (left panel). Correlation of Helios and Neuropilin1 expression is analyzed by percentage of $Helios^{hi}$ cells in $Nrp1^{low}$ or $Nrp1^{hi}$ $Foxp3^+$ cells (right panel).

Male skin protection by male antigen-specific iTreg cells in male islet recipients

The conversion into iTreg cells induced by male islet transplantation was observed in female Marilyn mouse. It was speculated that the male islet recipients would have acquired immunological tolerance to male antigen by these iTreg cells. To test whether the recipients had acquired the tolerance, male skin transplantation was conducted in male islet recipients and female islet recipients. Although female islet recipients have Treg cells, these Treg cells are of donor-origin and are not male antigen-specific. Therefore, these antigen-non-specific polyclonal Treg cells were expected to not protect the male skin from rejection, and indeed the female islet recipients rejected the male skin within 30 days. On the other hand, male islet recipients successfully accepted the male skin (Fig. 6A). On days 10~20, there were some inflammation on the male skin, but this inflammation subsided in several days, and new hair growth on the male skin could be observed eventually (Fig. 6B). Male islet recipients and female islet recipients both had donor-origin Treg cells. However, male islet recipients had *de novo* induced male antigen-specific recipient-origin iTreg cells as well as donor-origin Treg cells. This difference might yield different outcomes in subsequent male skin transplantation. *De novo* induced male antigen-specific recipient-origin iTreg cells were sufficient to protect the male skin from being rejected. As shown in Figure 1A, naïve female Marilyn mouse rejected the male skin, however, after the male islet transplantation, recipients accepted the male skin. Therefore, it was concluded that iTreg cell induction by male islet transplantation induced immunological tolerance to the male antigen.

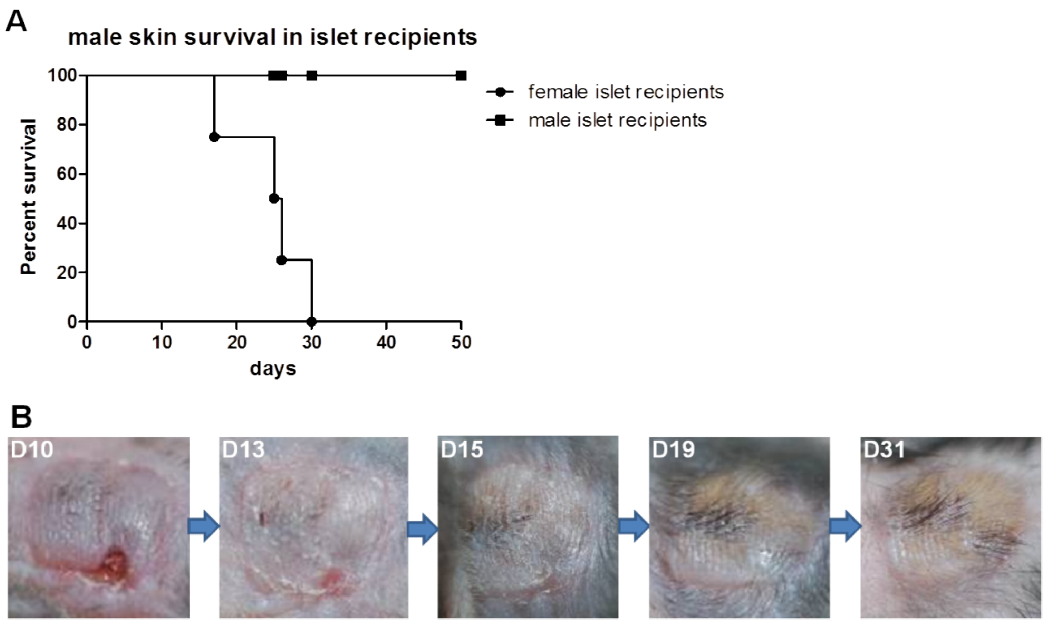


Figure 6. Male skins were accepted in male islet recipients but not in female islet recipients. (A) Male islet recipients (squares, n=4) or female islet recipients (circles, n=4) were transplanted with full thickness male skin on the left flank. Percent survival of grafted male skin is depicted. In female islet recipients, male skins were rejected within 30 days. Median survival day was 25.5 days. On the other hand, male skins survived indefinitely in male islet recipients. (B) Graft status pictures over time courses. Numbers in each left upper corner of picture represents the number of days that have passed after skin transplantation. Pictures of representative male islet recipient are arranged. In the graft bed, the left part is the female skin and the right part is the male skin. Inflammation started at day 10, subsided in several days, and new hair growth on male skin could be observed eventually.

In vitro Treg suppression assay to confirm the iTreg cells' role in immunological tolerance to the male antigen

The male antigen-specific iTreg cells in male islet recipients may prevent immune rejection of the male islet and skin. However, this probability is just an indirect evidence of the iTreg cells' role in tolerance induction. More direct evidence to confirm their role was needed. Therefore, an attempt was made to prove the suppressive function of iTreg cells in the *in vitro* Treg suppression assay. $CD4^{+}Foxp3^{-}$ cells were obtained from the male islet recipient ('primed' responder) or naïve Marilyn mouse ('naive' responder), and then stimulated with male antigen-expressing irradiated splenocytes or plate-coated anti-CD3 antibody. Suppressive function of added iTreg cells obtained from the male islet recipient (iTreg) was compared with antigen-non-specific Treg cells obtained from naïve $Foxp3^{gfp}$ reporter mouse (nTreg). Marilyn $CD4^{+}$ responder cell proliferation was inhibited by the co-cultured iTreg cells (Fig. 7A). Proliferation of responder cells diminished due to the co-cultured iTreg cells irrespective of stimulation conditions or responder types (Fig. 7B). When 'primed' responder cells were stimulated with anti-CD3 antibodies, iTreg cells and polyclonal Treg cells exhibited comparable suppressive function (Fig. 7C). However, when the male antigen-specific responder cells were stimulated with male antigen-expressing splenocytes, male antigen-specific iTreg cells exerted a much potent suppressive function compared to that of antigen-non-specific polyclonal Treg cells (Fig. 7D). Under such stimulation condition, polyclonal Treg cells exhibited their suppressive function only at high concentrations. On the other hand, male antigen-specific iTreg cells suppressed male-antigen reactive Marilyn $CD4^{+}$ T cells' proliferation almost completely at high concentrations and the dose-dependent effect could be observed (Fig. 7D). Although antigen-non-specific polyclonal Treg cells suppressed proliferation of naïve Marilyn $CD4^{+}$ responders stimulated with

anti-CD3 antibodies, those Treg cells could not suppress the male antigen-induced proliferation of naïve Marilyn $CD4^+$ responders (Fig. 7E). On the other hand, male antigen-specific iTreg cells effectively suppressed male antigen-induced proliferation of 'naïve' Marilyn $CD4^+$ cells, and the suppression was almost complete at high concentrations and somewhat dose-dependent (Fig. 7E). When the male antigen-reactive responders were stimulated with male antigen-expressing splenocytes, male antigen-specific iTreg cells exhibited superior suppressive function to both 'primed' and 'naïve' responders compared to that of antigen-non-specific polyclonal Treg cells.

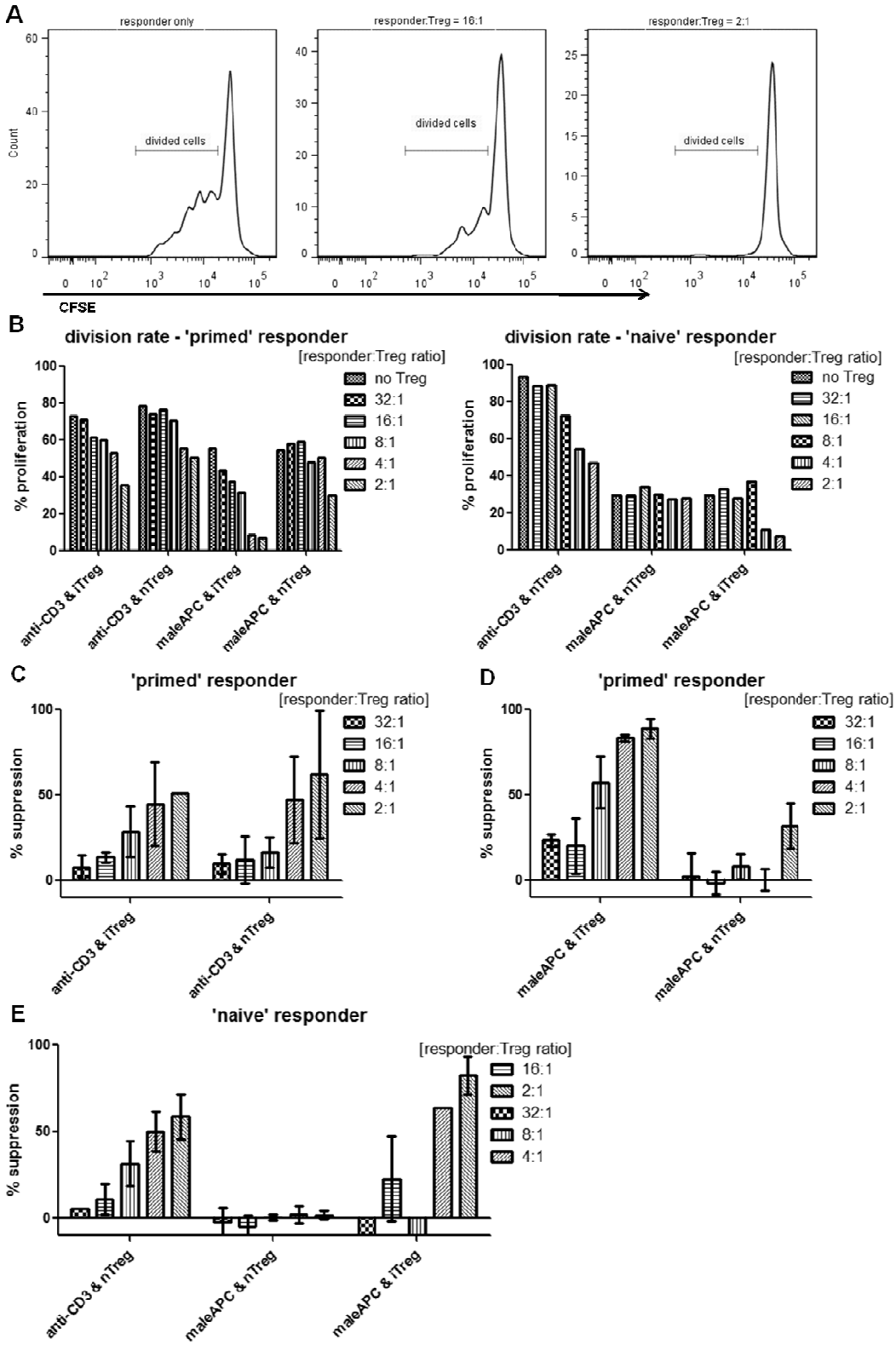
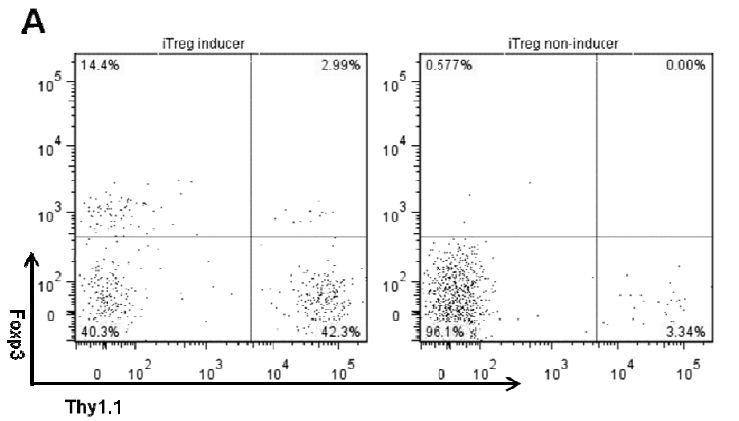

A
responder only
responder:Treg = 16:1
responder:Treg = 2:1
Count
divided cells
CFSE
B
division rate - 'primed' responder
division rate - 'naive' responder
[responder:Treg ratio]
no Treg
32:1
16:1
8:1
4:1
2:1
% proliferation
anti-CD3 & iTreg
anti-CD3 & nTreg
maleAPC & iTreg
maleAPC & nTreg
C
'primed' responder
% suppression
D
'primed' responder
E
'naive' responder

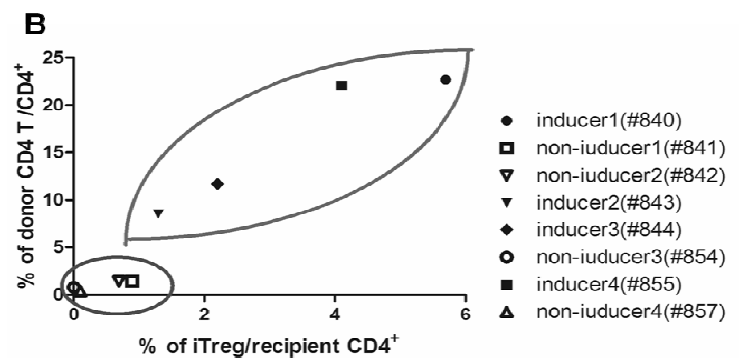
Figure 7. Suppressive function of iTreg cells induced by male islet transplantation was confirmed by *in vitro* Treg suppression assay. $CD4^+Foxp3^-$ responder cells obtained from the male islet recipient or naïve Marilyn mouse were stimulated with irradiated (30 Gy) congenic male splenocytes or plate-coated anti-CD3 antibodies (3 μg/ml). Responder cells were labeled with 2 μM CFSE (carboxyfluorescein succinimydil ester). The iTreg cells obtained from male islet recipient Marilyn x $Foxp3^{gfp}$ reporter mice or conventional Treg cells (mainly nTreg, polyclonal) obtained from naïve $Foxp3^{gfp}$ reporter mouse were co-cultured with $2\sim3x10^4$ responder cells in a round bottom 96 well plate with various ratios. Responder cell proliferation was visualized by CFSE dilution. (A) Histograms are representative of CFSE intensities of responder cells stimulated with male splenocytes without (left panel) or with 1/16 ratio of iTreg cells (middle panel) or 1/2 ratio of iTreg cells. Histograms are gated in $CD45.2^+CD4^+$ responder cells to distinguish them from CD45.1 congenic male splenocytes and GFP^+ Treg cells. (B-E) Proliferation of $CD4^+Foxp3^-$ responder cells obtained from the male islet recipient (B-D) and from naïve Marilyn mice (B,E) are analyzed. Percentage of divided cells (B) derived from histograms and percent suppressions (((proliferation without Treg – proliferation with each ratio of Treg)/ proliferation without Treg) x100 %) are depicted (C-E). The graph is a representative of four independent experiments (A-B) or a sum of the four independent experiments (C-E). Abbreviations; maleAPC; stimulation with male splenocytes, anti-CD3; stimulation with plate-coated anti-CD3 antibodies., iTreg; male antigen-specific iTreg cells obtained from male islet recipient Marilyn x $Foxp3^{gfp}$ reporter mouse, nTreg; male antigen-non-specific polyclonal Treg cells obtained from $Foxp3^{gfp}$ reporter mouse.

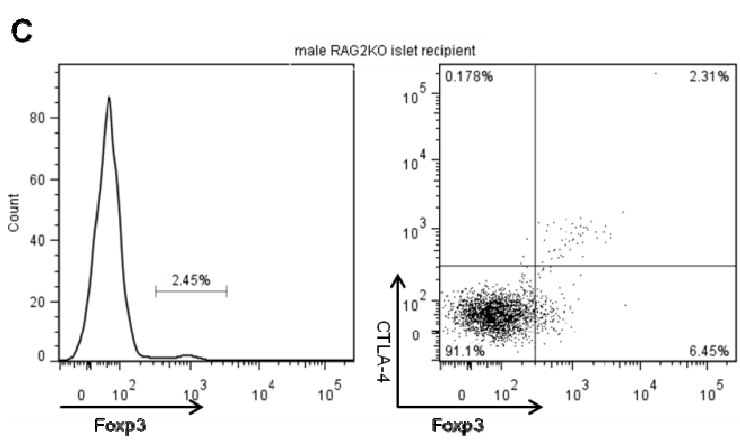
Role of the passenger leukocytes in iTreg cell induction

How could male islet transplantation induce iTreg cells, and what is the underlying mechanism of iTreg induction? In fact, induction of iTreg cells was not achieved in all male islet transplantation recipients. The induction rate of iTreg cells was about 50~60%. It was curious about the factor which is responsible for determining whether iTreg cells are induced or not. There was an interesting indication in the recipients in which iTreg cells were induced. As described in Figure 3, recipient-origin $CD4^+Foxp3^+$ iTreg cells must be distinguished from donor T cells by a congenic marker, Thy1. In other words, there were passenger lymphocytes accompanied by the male islet graft. In fact, solid organ and cellular grafts contain bone marrow-derived hematopoietic cells of donor origin, the passenger leukocytes (41). Interestingly, the recipients which had iTreg cells showed a tendency to harbor a greater numbers of donor-origin $Thy1.1^+$ T cells than that in other recipients in which iTreg cells were not induced (Fig. 8A). In a representative cohort of 8 male islet recipients, 4 mice induced iTreg cells and the other 4 did not. The percentages of iTreg cells and donor-origin $CD4^+$ T cells percentage among the $CD4^+$ cells were analyzed. As demonstrated in the in Figure 8A, there was a definite correlation between the percentage of iTreg cells and donor $CD4^+$ T cells. The iTreg-inducer male islet recipients had 8.5 % ~ 22.7 % of donor-origin $CD4^+$ T cells. On the other hand, the iTreg non-inducer recipients only had 0.4% ~ 1.4% of donor-origin $CD4^+$ T cells (Fig. 8B). To verify whether the passenger lymphocytes are essential for the induction of iTreg cells by male islet transplantation, lymphocyte-deficient $B6.RAG2^{-/-}$ mice were used as male islet donors instead of the congenic B6 mice. Among the 6 female Marilyn recipients transplanted with $B6.RAG2^{-/-}$ male islet, 2 mice induced iTreg cells and the other 4 mice did not (Fig. 8C). Therefore, the passenger 'lymphocyte' accompanied with donor islets was not

essential in inducing iTreg cells. However, in Figure 8A and B, the focus was on donor $CD4^+$ T cells only. In addition to $CD4^+$ T cells, other donor-origin lymphocyte populations such as $CD8^+$ T cells and B cells also exist in the hand-picked islets (Fig. 8D). Although only lymphocytes were considered here, the passenger cells were 'leukocytes', not just 'lymphocytes'. Other donor-origin leukocytes would also be accompanied by the male islets. These non-lymphocyte-passenger leukocytes may play a role in iTreg induction by male islet transplantation, especially the antigen-presenting cells (APCs) such as dendritic cells (DCs). Next, the analysis of islet passenger leukocytes was focused on passenger APCs. The majority of $CD45^+$ passenger leukocytes expressed MHC class II. This MHC class II expression implies the direct role of donor APCs in the male antigen presentation to Marilyn $CD4^+$ T cells. Although the majority of $MHCII^+$ cells were $CD19^+$ B lymphocytes, there was a definite population of $CD11c^+MHCII^+$ DCs which was not seen in the isotype control antibody staining sample (Fig. 8E). The majority of $CD11c^+MHCII^+$ DCs were PD-$L1^+$ and some were $CCR7^+$ (Fig. 8F). Taking into account the known functions of CCR7(99) and PD-L1(100-103), CCR7 expression may enable the DCs to migrate to the draining lymph nodes (LNs), and to encounter male antigen-specific $CD4^+$ T cells easily. Then, PD-L1 expression may facilitate the differentiation of naïve Marilyn $CD4^+$ T cells into iTreg cells.


A
iTreg inducer
iTreg non-inducer
14.4%
2.99%
40.3%
42.3%
0.577%
0.00%
96.1%
3.34%
Foxp3
Thy1.1


B
% of donor CD4 T /CD4+
% of iTreg/recipient CD4+
inducer1(#840)
non-iuducer1(#841)
non-iuducer2(#842)
inducer2(#843)
inducer3(#844)
non-iuducer3(#854)
inducer4(#855)
non-iuducer4(#857)


C
male RAG2KO islet recipient
Count
2.45%
Foxp3
0.178%
2.31%
91.1%
6.45%
CTLA-4
Foxp3

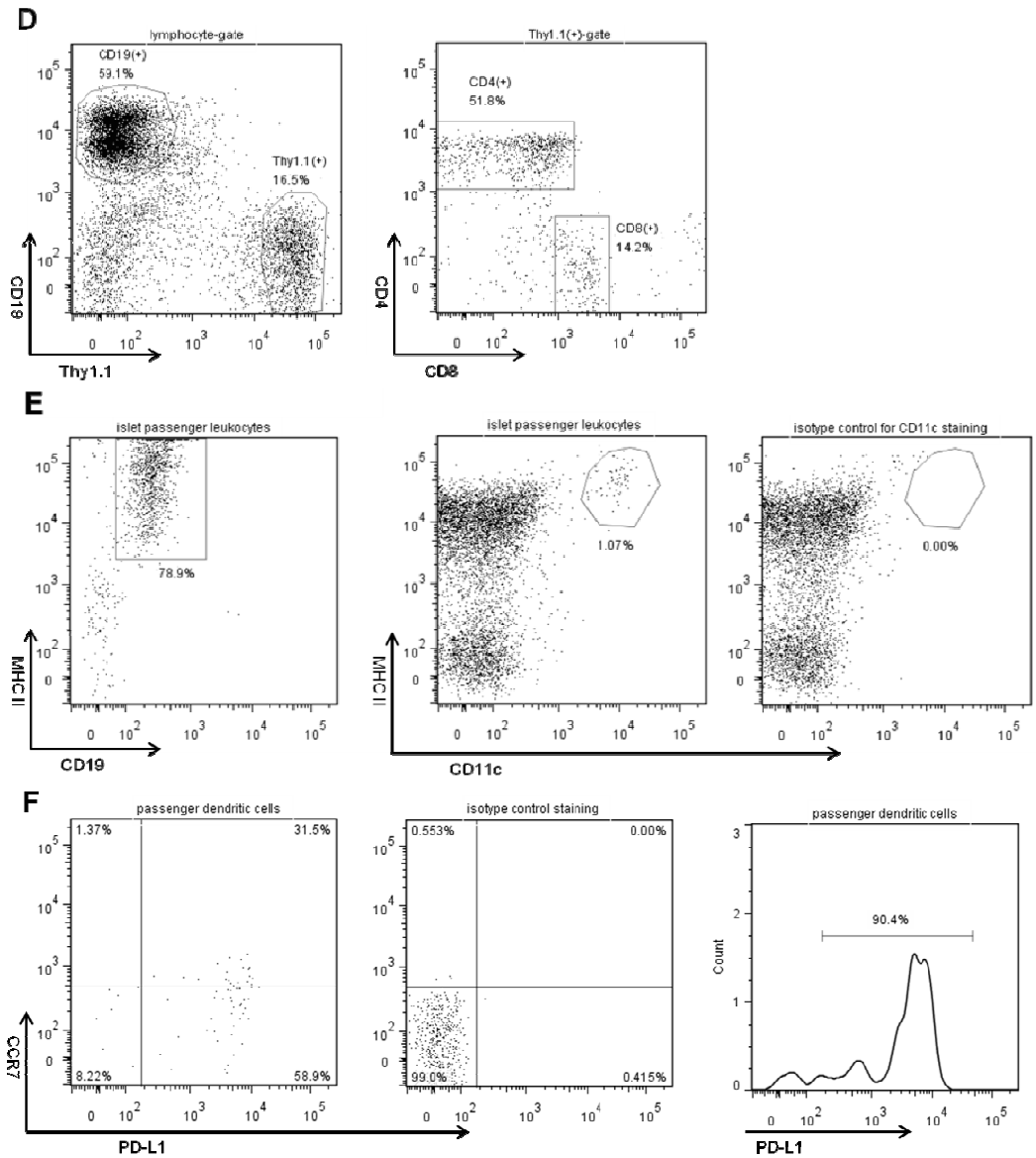


Figure 8. Passenger leukocytes may play a critical role in iTreg cell induction by the male islet transplantation. (A-C) Male Thy1.1 B6 congenic islets (A-B) or male B6.RAG2$^{-/-}$ islets (C) were transplanted to female Marilyn mice. (A-B) In a representative batch of transplantation (n=8), representations of iTreg inducers (A, left panel, n=4) and iTreg non-inducers (A, right panel, n=4) were analyzed with dot plots gated in CD4^{+} cells with distinction of Thy1.1^{+} donor-origin T cells. (B) For a correlation analysis, the percentages of iTreg cells (Foxp3^{+}/Thy1.2^{+}CD4^{+}) and donor CD4^{+} cell percentage (Thy1.1^{+}/CD4^{+}) in the

blood of 8 recipients (4 iTreg inducers and 4 non-inducers) are depicted. Filled symbols represent iTreg inducers, open symbols represent iTreg non-inducers. R^2 = 0.9314. (C) Among 6 recipients for male B6.RAG2$^{-/-}$ islets in 2 independent experiments, a representation of 2 iTreg-inducers is depicted on $CD4^+$ cell gating. (D-F) After an overnight culture as the transplantation protocol, hand-picked islets were prepared as single cells by Accutase treatment, and analyzed for their expression of (D) lymphocyte lineage markers or (E-F) MHCII, CD11c, CD19, CCR7 and PD-L1. Dot plots are gated on $CD45^+$ cells (D-E) or $CD45^+CD11c^+MHCII^+$ cells (F).

DISCUSSION

In this study, it was demonstrated that $CD4^+FoxP3^+$ regulatory T cells could be induced from naïve $CD4^+$ T cells by male islet transplantation. Since Marilyn mice did not have $FoxP3^+$ Treg cells before the islet transplantation, the newly emerged recipient-origin $CD4^+FoxP3^+$ Treg cells can be considered as inducible Treg (iTreg) cells. In our previous report (40), it was speculated that the increased $CD4^+FoxP3^+$ Treg cells in wild type female C57BL/6 recipient by male islet transplantation resulted from iTreg cell induction. Therefore, our hypothesis could be proved by the experiments described in this report. Furthermore, immunological tolerance to the male antigen could be achieved by male islet transplantation without any immunological manipulation. Certain studies showed that some mouse models may naturally accept foreign tissue without the need for additional immune suppression (104-106). In those studies, pre-existing $CD4^+FoxP3^+$ Treg cells were essential. Therefore, one might argue against our previous report (40) which states that induced tolerance is mediated by newly-emerging recipient-origin $CD4^+FoxP3^+$ Treg cells. However, according to the results obtained from this study, *de novo* induced iTreg cells were essential for the induction of immunological tolerance to the male tissues.

In Figure 6, whereas female islet recipients rejected the male skin, male islet recipients accepted it. Both of these two groups had male antigen-specific effecter $CD4^+$ T cells and passenger lymphocytes including antigen-non-specific Treg cells. One difference between the two groups is the existence of male antigen-specific iTreg cells in male islet recipients. Antigen-specifically activated Treg cells can exert a more potent suppressive function than antigen-non-specific Treg cells (107-109). Due to the presence of male

antigen-specific iTreg cells, the male islet recipients did not reject the male skin. In female islet recipients, the median survival day of the male skin was prolonged to 25.5 days from 19.5 days in naïve Marilyn mice (Fig. 1, 6). This delay of rejection might have resulted from the bystander effect of antigen-non-specific polyclonal donor-origin Treg cells in female islet recipients. Indeed, the potent suppressive function of antigen-specific iTreg cells could be observed as shown in Figure 7. Antigen-specific iTreg cells almost completely inhibited the proliferation of responder cells stimulated with the specific male antigen. Polyclonal Treg cells acting mainly through bystander effect had a limited suppressive function compared to that of potent antigen-specific iTreg cells. As illustrated in Figure 7E, while antigen-non-specific polyclonal Treg cells were able to suppress the proliferation of 'naïve' Marilyn $CD4^+$ responders stimulated with anti-CD3 antibodies, when the responders were stimulated with irradiated male splenocytes used as antigen-presenting cells (APCs), the polyclonal Treg cells were not able to suppress the male antigen specific responders. Similarly, a limited suppressive function of polyclonal Treg cells was observed for the 'primed' responders stimulated with male splenocytes (Fig. 7D). When the male-antigen specific responders were stimulated by pan-T cell stimulating anti-CD3 antibody, the polyclonal Treg cells had a suppressive function that was comparable to that of male-antigen specific Treg cells (Fig. 7C). On the other hand, if the stimulation was induced more physiologically by the male antigen-expressing APCs, only the male antigen-specific Treg cells were able to exert a potent suppressive function. During baseline proliferation in the absence of Treg cells, $CD4^+Foxp3^-$ responder cells from the male islet recipients proliferated more vigorously than responder cells from naïve Marilyn mice (Fig. 7B). This implies that $CD4^+Foxp3^-$ cells in male islet recipients are not anergic cells, but indeed 'primed' effector cells. Therefore, it was speculated that male skin

protection in male islet recipients did not result from dysfunctioning of $CD4^{+}Foxp3^{-}$ effector cells. Thus, it is obvious that these 'primed' effector cells were being actively suppressed by the co-existing iTreg cells in the male islet recipients *in vivo*.

Several investigators have reported *in vivo* iTreg conversions (4, 21-23), however, this is the first report showing that the transplantation itself without any immunological manipulation can induce iTreg cells. The role of the passenger leukocytes should not be overlooked in the mechanism of this physiologic iTreg induction. Passenger APCs, especially the dendritic cells (DCs) may function as the tolerogenic DCs. DCs are professional APCs that have been regarded as key instigators of innate and adaptive immunity. However, they also play major roles in directing the immune response toward tolerance (68-73). Especially, their ability to induce and interact with Treg cells is critical for their tolerogenic effect (68, 86, 110).

In addition, the unique feature of antigen presentation in the male islet transplantation may favor the iTreg induction. Alloantigen recognition of graft-specific T cells occurs by two pathways. In the direct pathway, intact donor MHC molecules expressed on donor APCs are directly recognized by host T cells. Alternatively, in the indirect pathway, recipient APCs process graft-derived material and present peptides to alloreactive host T cells (111, 112). In MHC-matched transplantation such as H-Y-mismatched experiments, minor histocompatibility antigen disparate grafts have MHC molecules that are identical to that of the recipient which differ only by a limited set of bound self-peptides (113). Consequently, donor APCs present minor histocompatibility antigen peptides on syngeneic MHC molecules as the host APCs do in the indirect pathway. Therefore, in the context of MHC class II and loaded H-Y peptide, the way of allorecognition in the direct pathway of H-Y-mismatched transplantation is the same with that in the indirect pathway.

Indirect presentation may be important in driving a chronic rejection (114), so any strategy that tolerizes through this route may have beneficial effects on late graft loss (115).Therefore, induction of iTreg cells by the allorecognition with syngeneic MHC molecule have great clinical significance.

Yamada *et al*. (116) identified the requisiteness of the indirect pathway for transplantation tolerance induced by anti-CD40L and CTLA4-Ig treatment in MHC-mismatched skin or cardiac transplantation. They used B6.II-4$^+$ mice, which are MHC class II-deficient mice expressing an MHC II transgene only on the thymic epithelium. Although these mice have normal numbers of peripheral CD4$^+$ cells, MHCII$^+$ APCs are absent in the periphery (117). When being used as recipients, these mice could not mount the indirect response and could not induce transplantation tolerance via costimulatory blockade (116). As the active regulatory T cells had been known to be induced by costimulatory blockade (118, 119), Yamada et al. agreed with the suggestion raised by Waldmann and collegues (115) that the indirect pathway is the predominant mode of allorecognition by Treg cells. Although this was even before there were reports demonstrating the peripheral induction of iTreg cells (4, 21), Yamada *et al*. suggested the possibility of regulatory cell induction through the recognition of indirect determinants. Following studies have also shown the requirement for alloantigen presentation through the indirect pathway in the transplantation tolerance induction protocols (120, 121).

In our H-Y-mismatched islet transplantation model, Marilyn CD4$^+$ T cells are specific for the indirect determinant presented by syngeneic MHC class II (I-A^b) molecule. The naïve Marilyn CD4$^+$ T cells could be converted to iTreg cells by recognizing the indirect peptide determinant on host APCs or donor APCs. Passenger leukocytes may play critical roles in both pathways. The syngeneic MHC II-expressing donor APCs could directly present the H-Y-peptide antigens to the naïve CD4$^+$ T cells, and other leukocytes could

effectively deliver the H-Y-peptide antigens to the lymph nodes (LNs) and host APCs. Furthermore, CCR7 expression (Fig. 8F) may facilitate the migration. In fact, almost immediately upon transplantation, APCs from the donor tissue migrate from the graft to the draining LNs of the host (122). Therefore, prompt migration of the passenger leukocytes and active communication with host immune cells may possibly happen in the draining LNs. These efficient presentations of the indirect determinant to the naïve $CD4^+$ T cells may favor the conversion to the iTreg cells. As mentioned earlier, tolerogenic DCs have been receiving spotlight as tolerance-inducing tools. Donor DCs or recipient-derived DCs pulsed with donor cell-derived antigens including allopeptides, have been used to promote transplantation tolerance (123-127). In the male islet transplantation, the passenger leukocytes may play a role as the infused recipient-derived DCs pulsed with donor peptide antigen or facilitate the donor peptide delivery to recipient DCs.

Demonstration of iTreg induction with male immature DCs in A1.RAG1$^{-/-}$ TCR transgenic mice reported by Yates *et al.*(128) strongly supports the speculation of iTreg induction by the donor APCs accompanied by male islets. In that report, intravenous injection of bone marrow-derived male immature DCs induced iTreg cells *de novo* in female A1.RAG1$^{-/-}$ mice which are transgenic for a TCR specific for the Y-chromosome-derived Dby epitope in the context of I-E^K (88). The iTreg cell induction could protect the subsequent male skin grafts indefinitely (128). In male islet transplantation, the passenger APCs would play a role as injected immature DCs and induce iTreg cells to establish a transplantation tolerance. Other studies also support the role of passenger leukocytes in the induction of transplantation tolerance. In rats, the spontaneous acceptance of MHC-incompatible liver grafts was prompted by "passenger leukocytes", and depletion of these cells by donor irradiation resulted in a rapid graft rejection (129, 130). Reports that show migration of

potentially tolerogenic costimulatory molecule-deficient APCs from the graft could result in tolerance also reinforce the speculation of iTreg induction (131, 132).

Usually, the induction of Treg cells is achieved through antigen presentation in the absence of costimulatory signals or cytokines, either alone or in combination with the production of tolerogenic factors, such as IL-10, TGF-β, retinoic acid and programmed death ligands (23, 69). Antigen presentation in male islet transplantation might occur without costimulatory signals or cytokines. The absence of MHC-mismatch in male islet transplantation eliminates the chance of direct recognition of disparate MHC molecule by the direct pathway which is considered to be the main mechanism that leads to acute graft rejection (133, 134). Peptide antigen presentation on syngenic MHC molecules in H-Y-mismatched transplantation may provide a quiescent environment that does not provoke costimulatory signals or cytokines, and such environment may favor the induction of iTreg cells. In addition, PD-L1 expression on donor APCs (Fig. 8F) may facilitate the iTreg induction (100). However, in contrast to the male islet graft, male skin graft was rejected within 24 days (Figure 1A). In the context of the immunogenicity, skin is considered to be the most immunogenic tissue (135, 136). The characteristics of APCs in the donor skin would explain the reason for this high immunogenicity. Skin contains numerous Langerhans cells which are professional APCs with the capacity to migrate from the graft and efficiently stimulate recipient T cells (122, 137). Besides, Langerhans cells are potent stimulators which do not require costimulation for the activation of T cells (121). Even the absence of B7-1 and B7-2 on skin-derived Langerhans cells did not affect their ability to induce T cell proliferation *in vitro* (121). Therefore, male skin-derived Langerhans cells may contribute to the rejection of the male skin grafts even in the absence of costiumlatory signals.

Although Thornton *et al.* reported that Helios expression distinguishes natural Treg cells (nTreg) from iTreg cells (93), this is still controversial as a result of its inconsistent expression (94-96). Indeed, iTreg cells in male islet recipients expressed higher levels of Helios than Treg cells in naïve C57BL/6 mouse (Fig. 5A). This higher level of expression may reflect the activated status of iTreg cells by continuous antigen contact from the grafted male islets, and this is compatible with the idea that Helios is a marker of T cell activation and proliferation (95). Recently, Weiss *et al.* and Yadav *et al.* reported that nTreg cells express high levels of Neuropilin 1 (Nrp1), while mucosa-generated iTreg cells and spontaneously generated iTreg cells are $Nrp1^{low}$ (97, 98). Although iTreg cells induced by male islet transplantation show compatible low-Nrp1 expression profile (Fig. 5C), there are still no definite markers to distinguish iTreg cells from nTreg cells. In our experimental model, because there were no thymic nTreg cells in the recipients, recipient-origin Treg cells are the iTreg cells. Unlike other *in vivo* iTreg generation models, our iTreg cells were generated in the absence of any immunological manipulation. Since they are generated under a more physiological condition, these Treg cells may represent the genuine iTreg cells in wild type animals more properly. Therefore, it is a good model system to study iTreg cells. Since the iTreg cells were generated in a monoclonal T cell mouse model, an extremely homogenous population of iTreg cells can be obtained. In a further study, comparative analysis of mRNA and microRNA sequencing profile in the iTreg cells vs. naïve C57BL/6 Treg cells will be conducted. The purpose will be to find specific and definite markers that distinguish iTreg cells from nTreg cells.

REFERENCES

1. Wood, K. J., A. Bushell, and J. Hester. 2012. Regulatory immune cells in transplantation. *Nature reviews. Immunology* 12: 417-430.
2. Lodhi, S. A., K. E. Lamb, and H. U. Meier-Kriesche. 2011. Solid organ allograft survival improvement in the United States: the long-term does not mirror the dramatic short-term success. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 11: 1226-1235.
3. Meier-Kriesche, H. U., J. D. Schold, T. R. Srinivas, and B. Kaplan. 2004. Lack of improvement in renal allograft survival despite a marked decrease in acute rejection rates over the most recent era. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 4: 378-383.
4. Cobbold, S. P., R. Castejon, E. Adams, D. Zelenika, L. Graca, S. Humm, and H. Waldmann. 2004. Induction of foxP3+ regulatory T cells in the periphery of T cell receptor transgenic mice tolerized to transplants. *J Immunol* 172: 6003-6010.
5. Lechler, R. I., O. A. Garden, and L. A. Turka. 2003. The complementary roles of deletion and regulation in transplantation tolerance. *Nature reviews. Immunology* 3: 147-158.
6. Li, X. C., T. B. Strom, L. A. Turka, and A. D. Wells. 2001. T cell death and transplantation tolerance. *Immunity* 14: 407-416.
7. Scully, R., S. Qin, S. Cobbold, and H. Waldmann. 1994. Mechanisms in CD4 antibody-mediated transplantation tolerance: kinetics of induction, antigen dependency and role of regulatory T cells. *European journal of immunology* 24: 2383-2392.

8. Waldmann, H., and S. Cobbold. 2001. Regulating the immune response to transplants. a role for CD4+ regulatory cells? *Immunity* 14: 399-406.
9. Qin, S. X., S. Cobbold, R. Benjamin, and H. Waldmann. 1989. Induction of classical transplantation tolerance in the adult. *J Exp Med* 169: 779-794.
10. Kendal, A. R., Y. Chen, F. S. Regateiro, J. Ma, E. Adams, S. P. Cobbold, S. Hori, and H. Waldmann. 2011. Sustained suppression by Foxp3+ regulatory T cells is vital for infectious transplantation tolerance. *J Exp Med* 208: 2043-2053.
11. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155: 1151-1164.
12. Nishizuka, Y., and T. Sakakura. 1969. Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. *Science* 166: 753-755.
13. Sakaguchi, S., T. Takahashi, and Y. Nishizuka. 1982. Study on cellular events in post-thymectomy autoimmune oophoritis in mice. II. Requirement of Lyt-1 cells in normal female mice for the prevention of oophoritis. *J Exp Med* 156: 1577-1586.
14. Sakaguchi, S., K. Fukuma, K. Kuribayashi, and T. Masuda. 1985. Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. *J Exp Med* 161: 72-87.
15. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057-1061.

16. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4: 330-336.

17. Khattri, R., T. Cox, S. A. Yasayko, and F. Ramsdell. 2003. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* 4: 337-342.

18. Walker, M. R., D. J. Kasprowicz, V. H. Gersuk, A. Benard, M. Van Landeghen, J. H. Buckner, and S. F. Ziegler. 2003. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. *J Clin Invest* 112: 1437-1443.

19. Curotto de Lafaille, M. A., and J. J. Lafaille. 2009. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity* 30: 626-635.

20. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198: 1875-1886.

21. Apostolou, I., and H. von Boehmer. 2004. In vivo instruction of suppressor commitment in naive T cells. *J Exp Med* 199: 1401-1408.

22. Mucida, D., N. Kutchukhidze, A. Erazo, M. Russo, J. J. Lafaille, and M. A. Curotto de Lafaille. 2005. Oral tolerance in the absence of naturally occurring Tregs. *J Clin Invest* 115: 1923-1933.

23. Kretschmer, K., I. Apostolou, D. Hawiger, K. Khazaie, M. C. Nussenzweig, and H. von Boehmer. 2005. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 6: 1219-1227.

24. Scott, D., C. Addey, P. Ellis, E. James, M. J. Mitchell, N. Saut, S. Jurcevic, and E. Simpson. 2000. Dendritic cells permit identification of genes encoding MHC class II-restricted epitopes of transplantation antigens. *Immunity* 12: 711-720.

25. Roopenian, D. C. 1992. What are minor histocompatibility loci? A new look at an old question. *Immunology today* 13: 7-10.

26. Simpson, E., D. Scott, and P. Chandler. 1997. The male-specific histocompatibility antigen, H-Y: a history of transplantation, immune response genes, sex determination and expression cloning. *Annual review of immunology* 15: 39-61.

27. Goulmy, E., A. Termijtelen, B. A. Bradley, and J. J. van Rood. 1977. Y-antigen killing by T cells of women is restricted by HLA. *Nature* 266: 544-545.

28. Miklos, D. B., H. T. Kim, K. H. Miller, L. Guo, E. Zorn, S. J. Lee, E. P. Hochberg, C. J. Wu, E. P. Alyea, C. Cutler, V. Ho, R. J. Soiffer, J. H. Antin, and J. Ritz. 2005. Antibody responses to H-Y minor histocompatibility antigens correlate with chronic graft-versus-host disease and disease remission. *Blood* 105: 2973-2978.

29. Gratwohl, A., J. Hermans, D. Niederwieser, A. van Biezen, H. C. van Houwelingen, and J. Apperley. 2001. Female donors influence transplant-related mortality and relapse incidence in male recipients of sibling blood and marrow transplants. *The hematology journal : the official journal of the European Haematology Association / EHA* 2: 363-370.

30. Stern, M., J. R. Passweg, A. Locasciulli, G. Socie, H. Schrezenmeier, A. N. Bekassy, M. Fuehrer, J. Hows, E. T. Korthof, S. McCann, A. Tichelli, N. C. Zoumbos, J. C. Marsh, A. Bacigalupo, and A. Gratwohl. 2006. Influence of donor/recipient sex matching on outcome of allogeneic hematopoietic stem cell transplantation for aplastic anemia. *Transplantation* 82: 218-226.

31. Bohringer, D., E. Spierings, J. Enczmann, S. Bohringer, R. Sundmacher, E. Goulmy, and T. Reinhard. 2006. Matching of the minor histocompatibility antigen HLA-A1/H-Y may improve prognosis in corneal transplantation. *Transplantation* 82: 1037-1041.

32. Kawauchi, M., S. R. Gundry, J. A. de Begona, D. A. Fullerton, A. J. Razzouk, M. M. Boucek, S. Nehlsen-Cannarella, and L. L. Bailey.

1993. Male donor into female recipient increases the risk of pediatric heart allograft rejection. *The Annals of thoracic surgery* 55: 716-718.

33. Prendergast, T. W., S. Furukawa, A. J. Beyer, 3rd, B. J. Browne, H. J. Eisen, and V. Jeevanandam. 1998. The role of gender in heart transplantation. *The Annals of thoracic surgery* 65: 88-94.

34. Candinas, D., B. K. Gunson, P. Nightingale, S. Hubscher, P. McMaster, and J. M. Neuberger. 1995. Sex mismatch as a risk factor for chronic rejection of liver allografts. *Lancet* 346: 1117-1121.

35. Roberts, D. H., J. C. Wain, Y. Chang, and L. C. Ginns. 2004. Donor-recipient gender mismatch in lung transplantation: impact on obliterative bronchiolitis and survival. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 23: 1252-1259.

36. Scholz, D., and M. Mebel. 1983. [The significance of sex determined antigens for kidney transplantation prognosis--an analysis of 500 transplantations at a center]. *Zeitschrift fur Urologie und Nephrologie* 76: 209-217.

37. Goulmy, E., B. A. Bradley, Q. Lansbergen, and J. J. van Rood. 1978. The importance of H-Y incompatibility in human organ transplantation. *Transplantation* 25: 315-319.

38. Gratwohl, A., B. Dohler, M. Stern, and G. Opelz. 2008. H-Y as a minor histocompatibility antigen in kidney transplantation: a retrospective cohort study. *Lancet* 372: 49-53.

39. Millrain, M., P. Chandler, F. Dazzi, D. Scott, E. Simpson, and P. J. Dyson. 2001. Examination of HY response: T cell expansion, immunodominance, and cross-priming revealed by HY tetramer analysis. *J Immunol* 167: 3756-3764.

40. Yoon, I. H., S. E. Choi, Y. H. Kim, S. H. Yang, J. H. Park, C. S. Park, Y. Kim, J. S. Kim, S. J. Kim, E. Simpson, and C. G. Park. 2008. Pancreatic islets induce CD4(+) [corrected] CD25(-)Foxp3(+) [corrected] T-cell regulated tolerance to HY-mismatched skin grafts. *Transplantation* 86: 1352-1360.

41. Hart, D. N., and J. W. Fabre. 1981. Demonstration and characterization of Ia-positive dendritic cells in the interstitial connective tissues of rat heart and other tissues, but not brain. *J Exp Med* 154: 347-361.

42. Eiref, S. D., W. Zhang, S. H. Popma, L. J. Shah, J. S. Moore, and B. R. Rosengard. 1997. Creation of chimeric hearts: a tool for testing the "passenger leukocyte" hypothesis. *The Annals of thoracic surgery* 64: 628-633.

43. Larsen, C. P., P. J. Morris, and J. M. Austyn. 1990. Migration of dendritic leukocytes from cardiac allografts into host spleens. A novel pathway for initiation of rejection. *J Exp Med* 171: 307-314.

44. Bishop, G. A., J. Sun, D. J. DeCruz, K. L. Rokahr, J. D. Sedgwick, A. G. Sheil, N. D. Gallagher, and G. W. McCaughan. 1996. Tolerance to rat liver allografts. III. Donor cell migration and tolerance-associated cytokine production in peripheral lymphoid tissues. *J Immunol* 156: 4925-4931.

45. Kreisel, D., H. Petrowsky, A. M. Krasinskas, A. S. Krupnick, W. Y. Szeto, A. D. McLean, S. H. Popma, A. E. Gelman, M. K. Traum, E. E. Furth, J. S. Moore, and B. R. Rosengard. 2002. The role of passenger leukocyte genotype in rejection and acceptance of rat liver allografts. *Transplantation* 73: 1501-1507.

46. Hegre, O. D., R. J. Ketchum, H. Popiela, C. R. Eide, R. M. Meloche, J. R. Serie, and W. V. Moore. 1989. Allotransplantation of culture-isolated neonatal rat islet tissue. Absence of MHC class II positive antigen-presenting cells in nonimmunogenic islets. *Diabetes* 38: 146-151.

47. Lafferty, K. J., M. A. Cooley, J. Woolnough, and K. Z. Walker. 1975. Thyroid allograft immunogenicity is reduced after a period in organ culture. *Science* 188: 259-261.

48. Lechler, R. I., and J. R. Batchelor. 1982. Restoration of immunogenicity to passenger cell-depleted kidney allografts by the addition of donor strain dendritic cells. *J Exp Med* 155: 31-41.

49. Stegall, M. D., K. Tezuka, S. F. Oluwole, K. Engelstad, M. X. Jing, J. Andrew, and M. A. Hardy. 1990. Interstitial class II-positive cell depletion by donor pretreatment with gamma irradiation. Evidence of differential immunogenicity between vascularized cardiac allografts and islets. *Transplantation* 49: 246-251.

50. Stuart, F. P., T. Garrick, A. Holter, A. Lynch, and E. Bastien. 1971. Delayed rejection of renal allografts in the rat and dog by reduction of passenger leukocytes. *Surgery* 70: 128-134.

51. Hart, D. N., C. G. Winearls, and J. W. Fabre. 1980. Graft adaptation: studies on possible mechanisms in long-term surviving rat renal allografts. *Transplantation* 30: 73-80.

52. Goldberg, L. C., J. A. Bradley, J. Connolly, P. J. Friend, D. B. Oliveira, N. R. Parrott, R. S. Rodger, D. Taube, and M. G. Thick. 1995. Anti-CD45 monoclonal antibody perfusion of human renal allografts prior to transplantation. A safety and immunohistological study. CD45 Study Group. *Transplantation* 59: 1285-1293.

53. Starzl, T. E., A. J. Demetris, N. Murase, S. Ildstad, C. Ricordi, and M. Trucco. 1992. Cell migration, chimerism, and graft acceptance. *Lancet* 339: 1579-1582.

54. Starzl, T. E., A. J. Demetris, M. Trucco, A. Zeevi, H. Ramos, P. Terasaki, W. A. Rudert, M. Kocova, C. Ricordi, S. Ildstad, and et al. 1993. Chimerism and donor-specific nonreactivity 27 to 29 years after kidney allotransplantation. *Transplantation* 55: 1272-1277.

55. Starzl, T. E., A. J. Demetris, M. Trucco, N. Murase, C. Ricordi, S. Ildstad, H. Ramos, S. Todo, A. Tzakis, J. J. Fung, and et al. 1993. Cell migration and chimerism after whole-organ transplantation: the basis of graft acceptance. *Hepatology* 17: 1127-1152.

56. Ko, S., A. Deiwick, M. D. Jager, A. Dinkel, F. Rohde, R. Fischer, T. Y. Tsui, K. L. Rittmann, K. Wonigeit, and H. J. Schlitt. 1999. The functional relevance of passenger leukocytes and microchimerism for heart allograft acceptance in the rat. *Nature medicine* 5: 1292-1297.

57. Starzl, T. E., A. J. Demetris, N. Murase, A. W. Thomson, M. Trucco, and C. Ricordi. 1993. Donor cell chimerism permitted by immunosuppressive drugs: a new view of organ transplantation. *Immunology today* 14: 326-332.

58. Starzl, T. E., and R. M. Zinkernagel. 1998. Antigen localization and migration in immunity and tolerance. *The New England journal of medicine* 339: 1905-1913.

59. Starzl, T. E., A. J. Demetris, N. Murase, M. Trucco, A. W. Thomson, and A. S. Rao. 1996. The lost chord: microchimerism and allograft survival. *Immunology today* 17: 577-584; discussion 588.

60. Zimmermann, F. A., H. S. Davies, P. P. Knoll, J. M. Gokel, and T. Schmidt. 1984. Orthotopic liver allografts in the rat. The influence of strain combination on the fate of the graft. *Transplantation* 37: 406-410.

61. Qian, S., A. J. Demetris, N. Murase, A. S. Rao, J. J. Fung, and T. E. Starzl. 1994. Murine liver allograft transplantation: tolerance and donor cell chimerism. *Hepatology* 19: 916-924.

62. Sriwatanawongsa, V., H. S. Davies, and R. Y. Calne. 1995. The essential roles of parenchymal tissues and passenger leukocytes in the tolerance induced by liver grafting in rats. *Nature medicine* 1: 428-432.

63. Fabre, J. W., and P. J. Morris. 1972. The effect of donor strain blood pretreatment on renal allograft rejection in rats. *Transplantation* 14: 608-617.

64. Peugh, W. N., K. J. Wood, and P. J. Morris. 1988. Genetic aspects of the blood transfusion effect. *Transplantation* 46: 438-443.

65. Soulillou, J. P., F. Blandin, E. Gunther, and V. Lemoine. 1984. Genetics of the blood transfusion effect on heart allografts in rats. *Transplantation* 38: 63-67.

66. Chevalier, S., H. Lacroix, J. F. Moreau, and J. P. Soulillou. 1987. Blood transfusion plus allograft--but not blood transfusion alone--

induce IL 2-producing suppressor cells in Lew-1A recipients of LEW-1W heart allograft. *Transplantation proceedings* 19: 544-546.

67. Josien, R., M. Heslan, S. Brouard, J. P. Soulillou, and M. C. Cuturi. 1998. Critical requirement for graft passenger leukocytes in allograft tolerance induced by donor blood transfusion. *Blood* 92: 4539-4544.

68. Ezzelarab, M., and A. W. Thomson. 2011. Tolerogenic dendritic cells and their role in transplantation. *Semin Immunol* 23: 252-263.

69. Maldonado, R. A., and U. H. von Andrian. 2010. How tolerogenic dendritic cells induce regulatory T cells. *Advances in immunology* 108: 111-165.

70. Steinman, R. M., D. Hawiger, and M. C. Nussenzweig. 2003. Tolerogenic dendritic cells. *Annual review of immunology* 21: 685-711.

71. van Kooten, C., G. Lombardi, K. A. Gelderman, P. Sagoo, M. Buckland, R. Lechler, and M. C. Cuturi. 2011. Dendritic cells as a tool to induce transplantation tolerance: obstacles and opportunities. *Transplantation* 91: 2-7.

72. Morelli, A. E., and A. W. Thomson. 2007. Tolerogenic dendritic cells and the quest for transplant tolerance. *Nature reviews. Immunology* 7: 610-621.

73. Divito, S. J., Z. Wang, W. J. Shufesky, Q. Liu, O. A. Tkacheva, A. Montecalvo, G. Erdos, A. T. Larregina, and A. E. Morelli. 2010. Endogenous dendritic cells mediate the effects of intravenously injected therapeutic immunosuppressive dendritic cells in transplantation. *Blood* 116: 2694-2705.

74. Lu, L., W. Li, F. Fu, F. G. Chambers, S. Qian, J. J. Fung, and A. W. Thomson. 1997. Blockade of the CD40-CD40 ligand pathway potentiates the capacity of donor-derived dendritic cell progenitors to induce long-term cardiac allograft survival. *Transplantation* 64: 1808-1815.

75. Fu, F., Y. Li, S. Qian, L. Lu, F. Chambers, T. E. Starzl, J. J. Fung, and A. W. Thomson. 1996. Costimulatory molecule-deficient dendritic

cell progenitors (MHC class II+, CD80dim, CD86-) prolong cardiac allograft survival in nonimmunosuppressed recipients. *Transplantation* 62: 659-665.

76. Rastellini, C., L. Lu, C. Ricordi, T. E. Starzl, A. S. Rao, and A. W. Thomson. 1995. Granulocyte/macrophage colony-stimulating factor-stimulated hepatic dendritic cell progenitors prolong pancreatic islet allograft survival. *Transplantation* 60: 1366-1370.

77. Lutz, M. B., R. M. Suri, M. Niimi, A. L. Ogilvie, N. A. Kukutsch, S. Rossner, G. Schuler, and J. M. Austyn. 2000. Immature dendritic cells generated with low doses of GM-CSF in the absence of IL-4 are maturation resistant and prolong allograft survival in vivo. *European journal of immunology* 30: 1813-1822.

78. Beriou, G., A. Moreau, and M. C. Cuturi. 2012. Tolerogenic dendritic cells: applications for solid organ transplantation. *Current opinion in organ transplantation* 17: 42-47.

79. DePaz, H. A., O. O. Oluwole, A. O. Adeyeri, P. Witkowski, M. X. Jin, M. A. Hardy, and S. F. Oluwole. 2003. Immature rat myeloid dendritic cells generated in low-dose granulocyte macrophage-colony stimulating factor prolong donor-specific rat cardiac allograft survival. *Transplantation* 75: 521-528.

80. Wang, Q., M. Zhang, G. Ding, Y. Liu, Y. Sun, J. Wang, W. Zhang, Z. Fu, and X. Cao. 2003. Anti-ICAM-1 antibody and CTLA-4Ig synergistically enhance immature dendritic cells to induce donor-specific immune tolerance in vivo. *Immunology letters* 90: 33-42.

81. Garrod, K. R., C. K. Chang, F. C. Liu, T. V. Brennan, R. D. Foster, and S. M. Kang. 2006. Targeted lymphoid homing of dendritic cells is required for prolongation of allograft survival. *J Immunol* 177: 863-868.

82. Lan, Y. Y., Z. Wang, G. Raimondi, W. Wu, B. L. Colvin, A. de Creus, and A. W. Thomson. 2006. "Alternatively activated" dendritic cells preferentially secrete IL-10, expand Foxp3+CD4+ T cells, and induce

long-term organ allograft survival in combination with CTLA4-Ig. *J Immunol* 177: 5868-5877.

83. Griffin, M. D., W. Lutz, V. A. Phan, L. A. Bachman, D. J. McKean, and R. Kumar. 2001. Dendritic cell modulation by 1alpha,25 dihydroxyvitamin D3 and its analogs: a vitamin D receptor-dependent pathway that promotes a persistent state of immaturity in vitro and in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 98: 6800-6805.

84. Fugier-Vivier, I. J., F. Rezzoug, Y. Huang, A. J. Graul-Layman, C. L. Schanie, H. Xu, P. M. Chilton, and S. T. Ildstad. 2005. Plasmacytoid precursor dendritic cells facilitate allogeneic hematopoietic stem cell engraftment. *J Exp Med* 201: 373-383.

85. Wood, K. J. 2003. Passenger leukocytes and microchimerism: what role in tolerance induction? *Transplantation* 75: 17S-20S.

86. Cong, Y., A. Konrad, N. Iqbal, R. D. Hatton, C. T. Weaver, and C. O. Elson. 2005. Generation of antigen-specific, Foxp3-expressing CD4+ regulatory T cells by inhibition of APC proteosome function. *J Immunol* 174: 2787-2795.

87. Lantz, O., I. Grandjean, P. Matzinger, and J. P. Di Santo. 2000. Gamma chain required for naive CD4+ T cell survival but not for antigen proliferation. *Nat Immunol* 1: 54-58.

88. Zelenika, D., E. Adams, A. Mellor, E. Simpson, P. Chandler, B. Stockinger, H. Waldmann, and S. P. Cobbold. 1998. Rejection of H-Y disparate skin grafts by monospecific CD4+ Th1 and Th2 cells: no requirement for CD8+ T cells or B cells. *J Immunol* 161: 1868-1874.

89. Itoh, M., T. Takahashi, N. Sakaguchi, Y. Kuniyasu, J. Shimizu, F. Otsuka, and S. Sakaguchi. 1999. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* 162: 5317-5326.

90. Josefowicz, S. Z., and A. Rudensky. 2009. Control of regulatory T cell lineage commitment and maintenance. *Immunity* 30: 616-625.

91. Sakaguchi, S., T. Yamaguchi, T. Nomura, and M. Ono. 2008. Regulatory T cells and immune tolerance. *Cell* 133: 775-787.

92. Rosenblum, M. D., I. K. Gratz, J. S. Paw, K. Lee, A. Marshak-Rothstein, and A. K. Abbas. 2011. Response to self antigen imprints regulatory memory in tissues. *Nature* 480: 538-542.

93. Thornton, A. M., P. E. Korty, D. Q. Tran, E. A. Wohlfert, P. E. Murray, Y. Belkaid, and E. M. Shevach. 2010. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J Immunol* 184: 3433-3441.

94. Verhagen, J., and D. C. Wraith. 2010. Comment on "Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells". *J Immunol* 185: 7129; author reply 7130.

95. Akimova, T., U. H. Beier, L. Wang, M. H. Levine, and W. W. Hancock. 2011. Helios expression is a marker of T cell activation and proliferation. *PLoS One* 6: e24226.

96. Gottschalk, R. A., E. Corse, and J. P. Allison. 2012. Expression of Helios in peripherally induced Foxp3+ regulatory T cells. *J Immunol* 188: 976-980.

97. Weiss, J. M., A. M. Bilate, M. Gobert, Y. Ding, M. A. Curotto de Lafaille, C. N. Parkhurst, H. Xiong, J. Dolpady, A. B. Frey, M. G. Ruocco, Y. Yang, S. Floess, J. Huehn, S. Oh, M. O. Li, R. E. Niec, A. Y. Rudensky, M. L. Dustin, D. R. Littman, and J. J. Lafaille. 2012. Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. *J Exp Med* 209: 1723-1742, S1721.

98. Yadav, M., C. Louvet, D. Davini, J. M. Gardner, M. Martinez-Llordella, S. Bailey-Bucktrout, B. A. Anthony, F. M. Sverdrup, R. Head, D. J. Kuster, P. Ruminski, D. Weiss, D. Von Schack, and J. A. Bluestone. 2012. Neuropilin-1 distinguishes natural and inducible

regulatory T cells among regulatory T cell subsets in vivo. *J Exp Med* 209: 1713-1722, S1711-1719.

99. Alvarez, D., E. H. Vollmann, and U. H. von Andrian. 2008. Mechanisms and consequences of dendritic cell migration. *Immunity* 29: 325-342.

100. Francisco, L. M., V. H. Salinas, K. E. Brown, V. K. Vanguri, G. J. Freeman, V. K. Kuchroo, and A. H. Sharpe. 2009. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med* 206: 3015-3029.

101. Keir, M. E., L. M. Francisco, and A. H. Sharpe. 2007. PD-1 and its ligands in T-cell immunity. *Current opinion in immunology* 19: 309-314.

102. Wang, L., K. Pino-Lagos, V. C. de Vries, I. Guleria, M. H. Sayegh, and R. J. Noelle. 2008. Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3+CD4+ regulatory T cells. *Proceedings of the National Academy of Sciences of the United States of America* 105: 9331-9336.

103. Francisco, L. M., P. T. Sage, and A. H. Sharpe. 2010. The PD-1 pathway in tolerance and autoimmunity. *Immunological reviews* 236: 219-242.

104. Benghiat, F. S., L. Graca, M. Y. Braun, S. Detienne, F. Moore, S. Buonocore, V. Flamand, H. Waldmann, M. Goldman, and A. Le Moine. 2005. Critical influence of natural regulatory CD25+ T cells on the fate of allografts in the absence of immunosuppression. *Transplantation* 79: 648-654.

105. Robertson, N. J., J. G. Chai, M. Millrain, D. Scott, F. Hashim, E. Manktelow, F. Lemonnier, E. Simpson, and J. Dyson. 2007. Natural regulation of immunity to minor histocompatibility antigens. *J Immunol* 178: 3558-3565.

106. Miyajima, M., C. M. Chase, A. Alessandrini, E. A. Farkash, P. Della Pelle, G. Benichou, J. A. Graham, J. C. Madsen, P. S. Russell, and R. B. Colvin. 2011. Early acceptance of renal allografts in mice is

dependent on foxp3(+) cells. *The American journal of pathology* 178: 1635-1645.

107. Samy, E. T., L. A. Parker, C. P. Sharp, and K. S. Tung. 2005. Continuous control of autoimmune disease by antigen-dependent polyclonal CD4+CD25+ regulatory T cells in the regional lymph node. *J Exp Med* 202: 771-781.

108. Tang, Q., J. Y. Adams, A. J. Tooley, M. Bi, B. T. Fife, P. Serra, P. Santamaria, R. M. Locksley, M. F. Krummel, and J. A. Bluestone. 2006. Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. *Nat Immunol* 7: 83-92.

109. Tarbell, K. V., S. Yamazaki, K. Olson, P. Toy, and R. M. Steinman. 2004. CD25+ CD4+ T cells, expanded with dendritic cells presenting a single autoantigenic peptide, suppress autoimmune diabetes. *J Exp Med* 199: 1467-1477.

110. Thomson, A. W., H. R. Turnquist, A. F. Zahorchak, and G. Raimondi. 2009. Tolerogenic dendritic cell-regulatory T-cell interaction and the promotion of transplant tolerance. *Transplantation* 87: S86-90.

111. Jiang, S., O. Herrera, and R. I. Lechler. 2004. New spectrum of allorecognition pathways: implications for graft rejection and transplantation tolerance. *Current opinion in immunology* 16: 550-557.

112. Fernandes, E., H. D. Goold, A. Kissenpfennig, B. Malissen, J. Dyson, and C. L. Bennett. 2011. The role of direct presentation by donor dendritic cells in rejection of minor histocompatibility antigen-mismatched skin and hematopoietic cell grafts. *Transplantation* 91: 154-160.

113. Simpson, E., D. Scott, E. James, G. Lombardi, K. Cwynarski, F. Dazzi, M. Millrain, and P. J. Dyson. 2002. Minor H antigens: genes and peptides. *Transplant immunology* 10: 115-123.

114. Vella, J. P., L. Vos, C. B. Carpenter, and M. H. Sayegh. 1997. Role of indirect allorecognition in experimental late acute rejection. *Transplantation* 64: 1823-1828.

115. Wise, M. P., F. Bemelman, S. P. Cobbold, and H. Waldmann. 1998. Linked suppression of skin graft rejection can operate through indirect recognition. *J Immunol* 161: 5813-5816.

116. Yamada, A., A. Chandraker, T. M. Laufer, A. J. Gerth, M. H. Sayegh, and H. Auchincloss, Jr. 2001. Recipient MHC class II expression is required to achieve long-term survival of murine cardiac allografts after costimulatory blockade. *J Immunol* 167: 5522-5526.

117. Laufer, T. M., J. DeKoning, J. S. Markowitz, D. Lo, and L. H. Glimcher. 1996. Unopposed positive selection and autoreactivity in mice expressing class II MHC only on thymic cortex. *Nature* 383: 81-85.

118. Honey, K., S. P. Cobbold, and H. Waldmann. 1999. CD40 ligand blockade induces CD4+ T cell tolerance and linked suppression. *J Immunol* 163: 4805-4810.

119. Graca, L., K. Honey, E. Adams, S. P. Cobbold, and H. Waldmann. 2000. Cutting edge: anti-CD154 therapeutic antibodies induce infectious transplantation tolerance. *J Immunol* 165: 4783-4786.

120. Niederkorn, J. Y., and E. Mayhew. 2002. Phenotypic analysis of oral tolerance to alloantigens: evidence that the indirect pathway of antigen presentation is involved. *Transplantation* 73: 1493-1500.

121. Rulifson, I. C., G. L. Szot, E. Palmer, and J. A. Bluestone. 2002. Inability to induce tolerance through direct antigen presentation. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2: 510-519.

122. Richters, C. D., E. van Gelderop, J. S. du Pont, M. J. Hoekstra, R. W. Kreis, and E. W. Kamperdijk. 1999. Migration of dendritic cells to the draining lymph node after allogeneic or congeneic rat skin transplantation. *Transplantation* 67: 828-832.

123. Garrovillo, M., A. Ali, and S. F. Oluwole. 1999. Indirect allorecognition in acquired thymic tolerance: induction of donor-

specific tolerance to rat cardiac allografts by allopeptide-pulsed host dendritic cells. *Transplantation* 68: 1827-1834.

124. Ali, A., M. Garrovillo, M. X. Jin, M. A. Hardy, and S. F. Oluwole. 2000. Major histocompatibility complex class I peptide-pulsed host dendritic cells induce antigen-specific acquired thymic tolerance to islet cells. *Transplantation* 69: 221-226.

125. Beriou, G., H. Peche, C. Guillonneau, E. Merieau, and M. C. Cuturi. 2005. Donor-specific allograft tolerance by administration of recipient-derived immature dendritic cells and suboptimal immunosuppression. *Transplantation* 79: 969-972.

126. Hill, M., P. Thebault, M. Segovia, C. Louvet, G. Beriou, G. Tilly, E. Merieau, I. Anegon, E. Chiffoleau, and M. C. Cuturi. 2011. Cell therapy with autologous tolerogenic dendritic cells induces allograft tolerance through interferon-gamma and epstein-barr virus-induced gene 3. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 11: 2036-2045.

127. Ochando, J. C., C. Homma, Y. Yang, A. Hidalgo, A. Garin, F. Tacke, V. Angeli, Y. Li, P. Boros, Y. Ding, R. Jessberger, G. Trinchieri, S. A. Lira, G. J. Randolph, and J. S. Bromberg. 2006. Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. *Nat Immunol* 7: 652-662.

128. Yates, S. F., A. M. Paterson, K. F. Nolan, S. P. Cobbold, N. J. Saunders, H. Waldmann, and P. J. Fairchild. 2007. Induction of regulatory T cells and dominant tolerance by dendritic cells incapable of full activation. *J Immunol* 179: 967-976.

129. Sun, J., G. W. McCaughan, N. D. Gallagher, A. G. Sheil, and G. A. Bishop. 1995. Deletion of spontaneous rat liver allograft acceptance by donor irradiation. *Transplantation* 60: 233-236.

130. Tu, Y., T. Arima, and M. W. Flye. 1997. Rejection of spontaneously accepted rat liver allografts with recipientinterleukin-2 treatment or donor irradiation. *Transplantation* 63: 177-181.

131. Steptoe, R. J., F. Fu, W. Li, M. L. Drakes, L. Lu, A. J. Demetris, S. Qian, H. J. McKenna, and A. W. Thomson. 1997. Augmentation of dendritic cells in murine organ donors by Flt3 ligand alters the balance between transplant tolerance and immunity. *J Immunol* 159: 5483-5491.

132. Thomson, A. W., L. Lu, N. Murase, A. J. Demetris, A. S. Rao, and T. E. Starzl. 1995. Microchimerism, dendritic cell progenitors and transplantation tolerance. *Stem Cells* 13: 622-639.

133. Suchin, E. J., P. B. Langmuir, E. Palmer, M. H. Sayegh, A. D. Wells, and L. A. Turka. 2001. Quantifying the frequency of alloreactive T cells in vivo: new answers to an old question. *J Immunol* 166: 973-981.

134. Lindahl, K. F., and D. B. Wilson. 1977. Histocompatibility antigen-activated cytotoxic T lymphocytes. II. Estimates of the frequency and specificity of precursors. *J Exp Med* 145: 508-522.

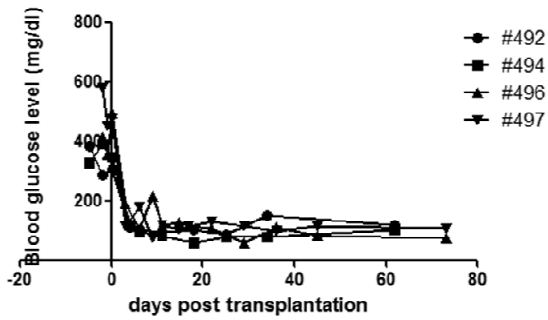
135. Hildemann, W. H., M. Morgan, and L. Frautnick. 1970. Immunogenetic components of weaker histoincompatibility systems in mice. *Transplantation proceedings* 2: 24-31.

136. Jones, N. D., S. E. Turvey, A. Van Maurik, M. Hara, C. I. Kingsley, C. H. Smith, A. L. Mellor, P. J. Morris, and K. J. Wood. 2001. Differential susceptibility of heart, skin, and islet allografts to T cell-mediated rejection. *J Immunol* 166: 2824-2830.

137. Larsen, C. P., R. M. Steinman, M. Witmer-Pack, D. F. Hankins, P. J. Morris, and J. M. Austyn. 1990. Migration and maturation of Langerhans cells in skin transplants and explants. *J Exp Med* 172: 1483-1493.

SUPPLEMENT

Supplement 1



Female islet transplantation normalized diabetic hyperglycemia of female Marilyn recipients.

국문 초록

서론: 이식편에 대한 면역관용의 유도는 이식분야에서의 궁극적인 목표이다. 부조직적합항원인 H-Y 항원 (남성 항원)에 불일치가 있는 췌도 이식이 다른 면역학적 조작이 없이도 암컷 C57BL/6 생쥐에서 남성 항원에 대한 면역관용을 유도할 수 있고 수컷 피부이식에 대한 수용을 유도할 수 있음이 알려져 있고 이 연구에서는 그 면역관용의 기전에 대해 알아보고자 했다.

방법: HY peptide 에 특이적인 유전자삽입 T 림프구 수용체를 가진 암컷 RAG2$^{-/-}$ Marilyn 생쥐의 왼쪽 옆구리에 수컷 피부를 이식하거나 신장피막 밑에 수컷 췌도를 이식하였다. 췌도 이식 전후로 CD4^{+} T 림프구의 Foxp3 발현을 유세포분석기로 분석하였다. 면역관용의 유도여부를 확인하기 위해서, 수컷 췌도 수용자 생쥐에 추가적인 수컷 피부 이식을 실시하였다.

결과: 암컷 Marilyn 생쥐는 수컷 피부에 대해 24 일 이내에 거부반응을 일으켰지만, 수컷 췌도 이식편은 성공적으로 생착하였다. 그리고 수컷 췌도를 이식받은 암컷 Marilyn 생쥐는 수컷 피부에 거부반응을 일으키지 않았다. 이들 생쥐의 말초혈액을 유세포분석기로 확인해보니, naive Marilyn 생쥐에는 없던 CD4^{+}Foxp3^{+} 조절 T 림프구에 생성되어 있었다. 이 유도 조절 T 림프구가 항원특이적이며 수컷 피부 이식편에 대한 거부반응을 막는 것에 중요한 역할을 하

는 것을 확인할 수 있었다. 이 유도 조절 T 림프구의 면역억제 능력을 생체 밖 시험을 통해 재확인하였다. 또한, 췌도 이식 시에 동반되는 백혈구와 유도 조절 T 림프구 수와의 양적 상관관계를 통하여, 유도 조절 T 림프구의 유도에서의 동반 백혈구의 역할에 대한 단서를 얻을 수 있었다.

결론: H-Y 항원에 불일치가 있는 췌도 이식이 유도 조절 T 림프구를 유도하였으며 이식 면역관용을 확립하였다. 이 모델은 새로이 생성되는 유도 조절 T 림프구의 특성을 연구하는 자산이 될 수 있을 것이다.

주요어 : 면역관용, 췌도 이식, 조절 T 림프구

학 번 : 2007-22000