creative commons
COMMONS DEED

저작자표시-비영리-변경금지 2.0 대한민국

이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.

다음과 같은 조건을 따라야 합니다:

저작자표시. 귀하는 원저작자를 표시하여야 합니다.

비영리. 귀하는 이 저작물을 영리 목적으로 이용할 수 없습니다.

변경금지. 귀하는 이 저작물을 개작, 변형 또는 가공할 수 없습니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 이용허락규약(Legal Code)을 이해하기 쉽게 요약한 것입니다.

Disclaimer

dCollection

연세대학교
YONSEI UNIVERSITY

[UCI]I804:11046-000000515243

Effect of adipose-derived mesenchymal stem cells on regulatory T cell induction and skin allograft

Jong Won Hong

Department of Medicine

The Graduate School, Yonsei University

연세대학교
YONSEI UNIVERSITY

연세대학교
YONSEI UNIVERSITY

Effect of adipose-derived mesenchymal stem cells on regulatory T cell induction and skin allograft

Directed by Professor Dae Hyun Lew

The Doctoral Dissertation
submitted to the Department of Medicine
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

Jong Won Hong

December 2017

연세대학교
YONSEI UNIVERSITY

This certifies that the Doctoral Dissertation
of Jong Won Hong is approved.

Thesis Supervisor : Dae Hyun Lew

Thesis Committee Member#1 : Soon-Il Kim

Thesis Committee Member#2 : Jeon-Soo Shin

Thesis Committee Member#3: Hyeon Joo Jeong

Thesis Committee Member#4: Kyung Suck Koh

The Graduate School
Yonsei University

December 2017

연세대학교
YONSEI UNIVERSITY

ACKNOWLEDGEMENTS

Although I am a plastic surgeon, I became acquainted with abdominal transplantation by chance in a transplantation class. From that day on, I learnt about the fields of face and hand transplantation, which are composite tissue allotransplantations. I became convinced that it was necessary to study immunology and tolerance to contribute to the development of these fields. At that time, stem cell studies were booming, and most of the stem cell research started with bone marrow stem cells. The plastic surgery research community was interested in adipose-derived mesenchymal stem cells for wound healing or flap survival, and I also hoped to combine these two topics.

I really appreciate the support of Professor Dae Hyun Lew, who directed me throughout my Master's degree. I also appreciate a detail review and feedback of all thesis committee tutors. I am particularly grateful that he always valued my ideas and opinions. I also extend my thanks to researcher Lim Jung Hyun, who has facilitated a huge number of lab meetings and experiments over the last 6 years.

Most of all, I thank my parents and my elder sisters—who always cheer me up—with all my heart. A special thank you to my wife, Kim Hee Jeong, who was at my side throughout this process. Finally, I also thank Jooha, my lovely sweet girl, whom I love most in this world.

Winter 2017

Jong Won Hong

연세대학교
YONSEI UNIVERSITY

<TABLE OF CONTENTS>

ABSTRACT 1

I. INTRODUCTION 4
II. MATERIALS AND METHODS 7
1. *In vitro* study 7
A. Cell preparation 8
(A) Preparation of Ad-MSCs 8
(B) Preparation $CD4^+$ T cells 8
B. Induction of Tregs 9
(A) Co-culture method 9
(B) Transwell culture method 9
(C) Blocking of TGF-β and IL-10 9
C. Analysis methods 10
(A) FACS 10
(B) Analysis of Ad-MSC differentiation 11
(C) Semi-quantitative and real-time PCR 11
2. *In vivo* study 13
A. Animal model 13
(A) Skin allograft 13
(B) Delivery methods 14
B. Analysis methods 14
(A) Analysis of skin allograft survival 14
(B) Histology analysis 14
(C) Immunohistochemical stainining 16

연세대학교
YONSEI UNIVERSITY

(D) Mixed lymphocyte reactions 16
3. Statistics 17
III. RESULTS 18
1. *In vitro* study 18
A. Mesenchymal stem cell identification and differentiation 18
B. $CD25^+$ $FoxP3^+$ Treg induction 20
C. $CD25^+$ $FoxP3^+$ Treg variation in co-culture 21
D. $CD25^+$ $FoxP3^+$ Treg variation in transwell culture 22
E. Expression of CD25 and FoxP3 mRNA in co-culture 23
F. Comparison of TGF-β1 and IL-10 mRNA by cell type and culture condition 24
2. *In vivo* study 25
A. Survival of skin allograft 25
B. Histologic analysis 27
C. Mixed lymphocyte reaction analysis 30
IV. DISCUSSION 32
V. CONCLUSION 38

REFERENCES 39
ABSTRACT (IN KOREAN) 45
PUBLICATION LIST 48

연세대학교
YONSEI UNIVERSITY

LIST OF FIGURES

Figure 1. Ad-MSC surface marker analysis using FACS flow cytometry 18

Figure 2. Expression of $CD4^{+}CD25^{+}FoxP3^{+}$ Tregs 20

Figure 3. Changes of $CD4^{+}CD25^{+}FoxP3^{+}$ Tregs in co-culture 21

Figure 4. Changes of $CD4^{+}CD25^{+}FoxP3^{+}$ Tregs in transwell culture 22

Figure 5. Real-time PCR of CD25, FoxP3 23

Figure 6. Level of TGF-β1, IL-10 mRNA by cell type. 24

Figure 7. Survival area of skin allograft in Group 1 (IP injection) 25

Figure 8. Representative images on postoperative day 7 of skin allograft in Group 2 (local injection). 26

Figure 9. Survival analysis of skin allograft in Group 2. 26

Figure 10. Skin allograft histology and Banff classification .. 28

Figure 11. Inflammatory cell counts in IP injection group 29

Figure 12. Inflammatory cell counts in local injection group. 29

Figure 13. Immunohistochemical staining for FoxP3 30

Figure 14. Mixed lymphocyte reaction analysis 31

연세대학교
YONSEI UNIVERSITY

LIST OF TABLES

Table 1. *In vitro* study design: conditions of control and experimental groups ····· 7
Table 2. List of primer pairs ····· 12
Table 3. Modified Banff classification used for grading on biopsy ····· 15

연세대학교
YONSEI UNIVERSITY

ABSTRACT

Effect of adipose-derived mesenchymal stem cells on regulatory T cell induction and skin allograft

Jong Won Hong

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Dae Hyun Lew)

Purpose Immune tolerance induction has been considered as the best treatment to protect transplant patient form the complications of the long-term immunosuppression. One potential option is using mesenchymal stem cell (MSC) therapy to induce regulatory T cells (Tregs). There had been many researches to induce immune tolerance using MSC and they showed certain amount of positive effect to induce short-term immune tolerance only. But the exact mechanism of short-term immune tolerance with MSC therapy has not been elucidated yet.

The purpose of this study is to investigate the mechanism of Treg induction by adipose-derived MSCs (Ad-MSCs) as well as to determine the best delivery method such as direct stimulation of Tregs by MSCs or indirect stimulation via soluble factors in a clinical setting.

연세대학교
YONSEI UNIVERSITY

Materials and Methods In vitro study, $CD4^+$ T cells were cultured without Ad-MSCs (control group) or with Ad MSCs. $CD4^+$ T cells culture with Ad-MSC were performed by direct contact (co-culture group) or inhibition of direction contact (transwell culture group). Control group, co-culture group and transwell culture group were analyzed for $CD4^+CD25^+FoxP3^+$ Treg levels by FACS. $CD4^+CD25^+FoxP3^+$ Treg variation was also analyzed to block TGF-β and/or IL-10 signaling. CD25, FoxP3, TGF-β, and IL-10 mRNA were accessed with real-time PCR. I*n vivo* studies, 2×2 cm full thickness skin allograft was performed in mouse model with 2.0×10^6 Ad-MSCs injection through either intraperitoneal (IP) or direct injection to skin allograft (local injection). Survival area was measured as a survival analysis tool. Inflammatory cell counts were used for histological evalutation. The mixed lymphocyte reaction (MLR) tests were also performed for immunological evaluation.

Results Treg numbers were increased in both co-culture and trans-well culture groups in contrast to no changes in control group. Treg induction was reduced by the inhibition of TGF-β and IL-10 in both culture groups. But Treg induction remained increased compared to the control group although TGF-β and/or IL-10 were blocked. Expressions of CD25 and FoxP3 mRNA were also increased in the co-culture group. In addition, expressions of TGF-β and IL-10 mRNA were higher in Ad-MSCs than $CD4^+$ T cells cultured alone. Co-culturing increased TGF-β and IL-10 mRNA levels in $CD4^+$ T cells. *In vivo study*, only direct Ad-MSC injections to skin graft showed increased skin graft survival (at a 50% survival rate). However, MLR reactivity and inflammatory cell counts were decreased in both Ad-MSC injection groups compared to controls.

연세대학교
YONSEI UNIVERSITY

Conclusion Ad-MSCs induced Treg differentiation regardless of direct contact with $CD4^+$ T cells. Ad-MSCs also have a positive effect on increasing expression of TGF-β and IL-10 in $CD4^+$ T cells. TGF-β and IL-10 secreted from adipose stem cells affected Treg induction. Finally, skin allograft survival was higher in the local direct injection of Ad-MSCs group than in the IP injection group. Finally, it was observed that Ad-MSC injections can be effective on immune tolerance for 14 days in the aspect of MLR and FoxP3 in histology. But clinically meaningful effects regarding graft rejection were only seen in the early days after skin allograft.

Key words : Adipsoe-derived mesenchymal stem cell, Immune tolerance, Regulatory T cell, Skin allograft

연세대학교
YONSEI UNIVERSITY

Effect of adipose-derived mesenchymal stem cells on regulatory T cell induction and allogenic skin graft

Jong Won Hong

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Dae Hyun Lew)

I. Introduction

The use of immunosuppressive drugs is essential for organ transplantation to avoid rejection, however, immunosuppressants can have undesirable side effects, such as hypertension and diabetes.[1] Vascularized composite tissue allotransplantation (VCA) is an allogenic complex tissue transplantation that is based on the concept of 'like with like', focusing on functional organs,[2-4] and has recently been performed in procedures such as facial transplantation and hand transplantation. However, the use of potentially harmful immunosuppressants for VCA procedures is controversial, as they do not involve vital organ transplantation.[5] As an alternative, achieving immune tolerance through immune regulation has been suggested for overcoming the complications of immunosuppression.[6-8]

There are many ways to induce immune tolerance in allotransplantation, one of which is through stem cell therapy. In the early years of stem cell research, bone marrow and cord blood were the primary sources of stem

연세대학교
YONSEI UNIVERSITY

cells;[9] however, immunomodulatory research has been performed using mesenchymal stem cells (MSCs). While initial studies using MSCs mainly used MSCs derived from bone marrow (BM-MSCs),[10-12] MSCs are present in all tissues and diverse sources have been investigated. For example, adipose tissue contains a large amount of stem cells, and research utilizing adipose-derived mesenchymal stem cells (Ad-MSCs) has also been performed.[13] In particular, Ad-MSCs have many advantages, such as low donor morbidity, and a large number can be extracted using an easier procedure than for other types of MSCs.[14,15] The development of fat grafting and liposuction have also aided in Ad-MSC research.

Traditionally, little distinction has been made between different types of stem cells; however, current studies are being conducted to classify the unique characteristics of specific types.[16] Moreover, while many features are shared amongst these cells, differences in surface marker expression and proliferation rate have been identified.[6,17-19] In this study, we sought to further clarify the characteristics of Ad-MSCs and investigate their effects on immune tolerance through *in vitro* and *in vivo* studies.

One of the mechanisms of immune tolerance in which MSCs are implicated is the induction of regulatory T cell (Treg).[10,20-22] It is thought that these effects are mediated by direct contact with T cells and/or through cytokine secretion.[10,16,23-25] However, these effects are not yet clear *in vivo*. The mechanism by which MSCs cause Treg induction is important to understand because it may determine the delivery method that must be used to achieve an effective therapy. Specifically, if cell-cell contact is the primary mechanism of Treg induction, a delivery method that allows for direct contact with immune cells will be more effective.

연세대학교
YONSEI UNIVERSITY

Conversely, if cytokine secretion is more important, more diverse methods of delivery may be used to achieve the anticipated paracrine effects.

To this end, an *in vitro* study was conducted to investigate the mechanism by which Ad-MSCs mediate Tregs, and to observe the effects of both cell-cell contact and soluble factors on immune tolerance. In addition, differences in immune tolerance according to the delivery method using skin graft models were investigated. I attempted to prevent contact between MSCs and immune cells, so that potential paracrine effects induced by soluble factors could be examined, using intraperitoneal (IP) injections, instead of intravenous (IV) injections. Conversely, to enhance contact between Ad-MSCs and immune response cells, direct injections around the recipient area was performed. Therefore, immune modulation *in vivo* by controlling cell contact with the injection method was investigated.

연세대학교
YONSEI UNIVERSITY

II. Materials and Methods

1. *In vitro* study

Culture studies were performed using $CD4^+$ T cells and Ad-MSCs to determine whether Ad-MSCs could elicit Treg induction. This study utilized two primary experimental groups: the first consisted of $CD4^+$ T cells that were co-cultured directly with Ad-MSCs, and the second was a transwell culture that contained $CD4^+$ T cells and Ad-MSCs and allowed for the diffusion of soluble factors, but not for direct interaction. In addition, a control group consisting of only $CD4^+$ T cells was included. Finally, blocking tests were also performed in some cultures using anti-transforming growth factor (TGF)-β and/or anti-interleukin (IL)-10 antibodies. In total, there were eight experimental groups and one control group, which are shown in Table 1.

Table 1. *In vitro* study design: conditions of control and experimental groups

	Control group	Experimental group							
		Co-culture group				Transwell culture group			
		1	2	3	4	5	6	7	8
$CD4^+$ T cell	+	+	+	+	+	+	+	+	+
Ad-MSCs	-	+	+	+	+	+	+	+	+
Anti TGF-β antibody	-	-	+	-	+	-	+	-	+
Anti IL-10 antibody	-	-	-	+	+	-	-	+	+

연세대학교
YONSEI UNIVERSITY

A. Cell preparation

(A) Preparation of Ad-MSCs

Ad-MSCs were isolated from normal human fat tissues obtained via liposuction. The process was performed with the approval of our institutional review board (Gangnam Severance Hospital, IRB#2012-1867). In brief, adipose tissues were washed with phosphate buffered saline (PBS; Gibco) to remove blood cells, dead tissue, and other debris. They were enzymatically digested at 37℃ for 30 minutes with sterile 0.075% type IA collagenase (Sigma, St. Louis, MO, USA) at a concentration of 0.5 mg/mL in PBS. Samples were centrifuged at 1200*g* for 5 min, and then cell pellets were resuspended and filtered through a 100 μm nylon filter (Cell Strainer, Becton Dickinson, Franklin Lakes, NJ, USA). Cells were cultured in 100 mm culture dishes at 37°C in a 5% humidified CO_2 incubator. After 24 hours, non-adherent cells were removed. Cells were expanded through 3-5 passages.[26,27]

(B) Preparation of $CD4^+$ T cells

Human peripheral blood mononuclear cells (PBMCs) from a healthy individual were isolated using Ficoll-Hypaque density gradient centrifugation. $CD4^+$ T cells from the PBMCs were isolated using a $CD4^+$ T cell isolation kit (Miltenyi Biotec, Germany).[10] To this end, the PBMCs were counted and then 1.0×10^7 cells were resuspended in 80 μL of magnetic-activated cell sorting (MACS) buffer (0.5% bovine serum albumin [BSA] in PBS). 20 μL of CD4 microbeads (Miltenyi Biotec) to the aspirated cells were added. The cell suspension were incubated for 15 min at 4℃ and then applied the prepared cells to an MS column

연세대학교
YONSEI UNIVERSITY

(Miltenyi Biotec) in which a strong magnetic field was induced by the MACS separator. The labeled cells were collected on the tube after removing the column from the separator.

B. Induction of Tregs

(A) Co-culture method

A total of 1.0×10^5 Ad-MSCs were seeded per well, in a six-well culture plate. Then, we added 3.0×10^5 $CD4^+$ T cells to each well and incubated the cultures for 72 h at 37℃. Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Welgene, Korea) containing 10% fetal bovine serum (FBS; GE, Utah, USA) and 1% penicillin/streptomycin (Thermo, IL, USA).

(B) Transwell culture method

$CD4^+$ T cells were cultured with Ad-MSCs as described above, with the exception that transwell permeable supports (0.4 μm pores, Corning, Tewksbury, Massachusetts, USA) were used, which allowed the two cell types to be separated. $CD4^+$ T cells were cultured in the insert, and Ad-MSCs were seeded beneath the insert in the well.

(C) Blocking of TGF-β and IL-10

To determine the effects of TGF-β and IL-10 on immune tolerance, as mediated by AD-MSCs, TGF-β and/or IL-10 were neutralized using the following antibodies: anti-TGF-β (10 μg/mL, R&D systems, MN, USA) and/or anti-IL-10 (0.05 μg/mL, R&D systems), as shown in Table 1. TGF-β and IL-10 blocking tests were performed for both the co-cultured and transwell cultured groups. All cultures were grown for either 72 h

연세대학교
YONSEI UNIVERSITY

and then analyzed via fluorescence-activated cell sorting (FACS), or for 24 h and then analyzed using real-time PCR.

C. Analysis methods

(A) FACS

To identify Ad-MSC surface markers and confirm Treg induction, the cells were analyzed using FACS. Specifically, the following membrane-associated molecules were assessed as positive markers[28,29]: CD13 (anti-human PE, eBioscience, San Diego, CA, USA), CD44 (anti-human Alexa Fluor 488, Molecular Probes, MD, USA), and CD105 (anti-human Alexa Fluor 488, Molecular Probes). Conversely, CD31 (anti-human PE, BD Pharmingen, San Jose, USA), CD34 (anti-human APC, Molecular Probes), and CD45 (anti-human PreCP-Cy5-5-A, Molecular Probes) were used as negative markers. Human isotype-matched IgG served as a negative control (BD Pharmingen)

After culturing for 72 h (as described above), the $CD4^+$ T cells were labeled for surface CD4 and CD25, as well as for intracellular FoxP3, washed them with blocking buffer (0.5% BSA in PBS), and then centrifuged. CD4-FITC (anti-human CD4, Miltenyi Biotec) and CD25-PE (anti-human CD25, BD Pharmingen) antibodies were added to the cells and incubated them for 10 min. Next, cells were washed, fixed with 100 μL of 2% paraformaldehyde, and then permeabilized by incubating with 0.2% Tween 20 in PBS for 10 min. After the blocking process, a FoxP3-APC antibody (anti-human Foxp3, eBioscience, CA, USA) were added and incubated the mixtures at room temperature for 30 min in the dark. Finally, the cells in FACS buffer (0.5% BSA in PBS) were resuspended at a concentration of 1.0×10^5 cells/mL. FACS were

연세대학교
YONSEI UNIVERSITY

performed using a BD FACS Canto II Flow Cytometer and the FACS DIVA software (BD Biosciences, USA).

(B) Analysis of Ad-MSC differentiation

Differentiation of Ad-MSCs was confirmed through analyses of adipogenesis and osteogenesis. Unless otherwise specified, all the reagents in this section came from Sigma. Isolated and expanded Ad-MSCs were cultured in conditioned media for three weeks. To induce adipogenesis, media consisting of 10% FBS (Gibco, Garand Island, NY, USA), 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 10 ug/mL insulin, and 100 μM indomethacin in high glucose-Dulbecco's Modified Eagle medium (DMEM) were used. The cells were stained with fresh Oil red-O solution to confirm the lipid droplets, as previously described.[26] To achieve osteogenesis, Ad-MSCs were cultured in osteogenic-conditioned media for three weeks, consisting of 10% FBS, 10 mM β–glycerolphosphate, 0.1uM dexamethasone, and 0.2 mM ascorbic acid in low glucose-DMEM.[26] The cells were stained Alizarin red S to detect calcium deposits.

(C) Semi-quantitative PCR and real-time PCR

After culturing for 24 h (as described in a previous section), semi-quantitative PCR were performed to analyze $CD4^+$ T cells for levels of CD25, FoxP3, IL-10, TGF-β1, TGF-β2, and TGF-β3. It was not observed any differences between the monoculture (control group) and co-culture with respect to TGF-β2, TGF-β3 levels, therefore it was not included them in the real-time PCR analysis, but rather assessed only CD25, FoxP3, IL-10, and TGF-β1.

연세대학교
YONSEI UNIVERSITY

Total RNA from the co-cultured $CD4^+$ T cell were isolated using TRIzol reagent (Invitrogen, CA, USA). The primer sequences are summarized in Table 2. Real-time PCR was performed with an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, CA, USA). Target amplification was checked using dissociation curves, and the data was analyzed using the competitive method ($2^{-\Delta\Delta Ct}$).

Table 2. List of primer pairs

Gene	Sequence
GAPDH	forward 5'-ACT AGG CGC TCA CTG TTC TCT-3' (21mer)
	reverse 5'-CGA CCA AAT CCG TTG ACT CC-3' (20mer)
CD25	forward 5'-TCT GCC ACT CGG AAC ACA AC-3' (20mer)
	reverse 5'-TTG AAA ATC TGG AAG GCT CGC-3' (21mer)
FoxP3	forward 5'-ACT GGG GTC TTC TCC CTC AA-3' (20mer)
	reverse 5'-AAA GGG TGC TGT CCT TCC TG-3' (20mer)
TGF-β1	forward 5'-GCT GTA TTT AAG GAC ACC CGT-3' (21mer)
	reverse 5'-ATG ACA CAG AGA TCC GCA GTC-3' (21mer)
IL-10	forward 5'-TCC ATT CCA AGC CTG ACC AC-3' (20mer)
	reverse 5'-AGC AGT TAG GAA GCC CCA AG-3' (20mer)

연세대학교
YONSEI UNIVERSITY

2. *In vivo* study

Skin allografts in mice were performed to compare graft survival between different methods of Ad-MSC treatment. The experimental groups were as follows: Group 1 received an IP injection of Ad-MSCs, and Group 2 received local injections around the skin graft site. To compare the treatment outcomes, diverse analyses were performed for graft survival and size, hematoxylin and eosin (H&E) staining, immunohistochemical staining, inflammatory cell counts, and mixed lymphocyte reactions (MLRs).

A. Animal model

(A) Skin allograft

Skin allografting in a mouse model were performed to evaluate the effect of Ad-MSC immune tolerance. The protocol was approved by the Yonsei University Animal Experimental Center (#2015-0108). The recipients of the grafts were 30g BALB/C (H-2d) mice. Mice were first anesthetized using 0.03 mL of Zoletil® 50 (Virbac, Carros, France) and 0.01 mL of Rompun (Bayer Korea Ltd, Seoul, Korea). After removal of hair using a depilatory, an MHC-mismatched skin allograft from C57BL/6 (H-2b) mice was performed on the warmer: 2×2 cm dorsal full-thickness skin from C57BL/6 mice was harvested and transplanted to the dorsum of the BALB/C mice. A suture was performed using #5-0 nylon and a simple dressing was done.

연세대학교
YONSEI UNIVERSITY

(B) Delivery methods

Two skin allograft model were designed depending on the Ad-MSC delivery method. Group 1 received Ad-MSCs via an IP injection, and Group 2 received Ad-MSCs via local injection during the skin allograft operation. Both experimental groups received 1.0×10^6 Ad-MSCs in 0.2 mL of PBS, whereas the Controls received 0.2 mL of PBS only.

A total of 21 BALB/C mice were used in Group 1 for the day 14 analysis, and 23 were used in Group 2. An additional 25 BALB/C mice were used in Group 1, and 20 mice in Group 2, for the day 3 analysis.

B. Analysis methods

(A) Analysis of skin allograft survival

The status of postoperative mice was checked daily, and pictures were taken for two weeks to analyze skin allograft survival size and detect rejections. In addition, the grafted skin was sampled at postoperative days 3 and 14. The change in skin graft survival size were calculated using IMT iSolution Lite (ver. 21.1, serial No. 818967021, IMT i-Solution, Canada). The survival analysis was performed using both 100% rejection and 50% rejection.

(B) Histologic analysis

On postoperative days 3 and 14, skin graft tissue was harvested. Specimens were embedded in paraffin blocks following fixation with a 4% paraformaldehyde solution. Subsequently, 5 μm sections were obtained, deparaffinized, and the sections were dehydrated with graded alcohol, then rehydrated. The section was stained with hematoxylin for 4

연세대학교
YONSEI UNIVERSITY

min at room temperature. After treating with 0.3% acid alcohol and rinsing, the sections were stained with eosin for 2 min. After dehydrating again with graded alcohol, the slides were mounted.

Histology was analyzed for rejection of the graft and inflammatory cell counts. The acute/active skin rejection scoring system was classified in five grades (Table 3), according to a modified Banff classification.[30,31] For inflammatory cell counts, each H&E-stained slide was visualized using a high power field (×400) with an Olympus Bx40 microscope (Olympus, Japan). Three randomly selected fields were photographed for each slide with an Olympus DP 71 camera (Olympus, Japan) that was connected to the microscope and the digital image processing software, DP Manager (ver. 3., Olympus, Japan). The total number of inflammatory cells in each image were counted, and cell counts were calculated in the dermis and subcutaneous layer.

Table 3. Modified Banff classification used for grading on biopsy[31]

Grade	Histologic features
I	Mild to moderate lymphocytic perivascular infiltrate in the superficial to middle dermis
II	Moderate lymphocytic perivascular infiltrate in the superficial to middle dermis Mild epidermal interface changes with or without spongiosis without keratinocyte death
III	Moderate to severe lymphocytic perivascular infiltrate filling the dermis Interface inflammation with at least clusters of at least two apoptotic keratinocytes
IV	Grade Ⅲ with epidermal necrosis

연세대학교
YONSEI UNIVERSITY

(C) Immunohistochemical staining

Immunohistochemical staining was performed on 5 μm sections of formalin-fixed, paraffin-embedded blocks. The slides were first deparaffinized and rehydrated. For heat-induced antigen retrieval, slides were heated in citrate buffer (pH 6.0) for 10 min in a microwave, then incubated with 3% hydrogen peroxide for 10 min. After washing with tris-buffered saline containing Tween 20 (TBST), the slides were incubated with a normal goat serum (Vector laboratories Inc., CA, USA) blocking solution for 1 hr at room temperature. Slides were treated with a FoxP3 primary antibody at a 1:10 dilution (eBioscience) and incubated overnight at 4℃. Then, the slides were washed with TBST, a biotinylated, goat anti-rat secondary antibody (1;1000, Vector laboratories Inc) was applied, and slides were incubated for 1 h at room temperature. The slides were again washed with TBST, and then incubated with a Vectastain elite ABC reagent (Vector laboratories Inc) for 30 min at room temperature. After incubation, the slides were washed with TBST and diaminobenzidine (DAB, Vector laboratories Inc) was applied. Finally, slides were counterstained with Harris Hematoxylin to detect cell nuclei and then dehydrated.[32]

(D) Mixed lymphocyte reactions

Spleens were harvested from Group 1 and Control mice on the 14th postoperative day, and MLRs were performed using the harvested spleen tissues. Responder cells were isolated using a naive CD4^{+} isolation kit (Miltenyi Biotec) and labeled with 5 μM carboxyfluorescein succinimidyl ester (CFSE; Molecular probe, CFDA SE, Oregon, USA). To this end, 2.0 × 10^{5} responder cells were seeded per well in a 96-well

연세대학교
YONSEI UNIVERSITY

round form plate. Stimulator cells from C57BL/6 were treated with 50 μg/mL of mitomycin C (Sigma, St. Louis USA) and incubated them for 20 min at 37℃ in the dark. The cell suspension were then washed with complete media three times. Finally, 1.0×10^5 treated stimulator cells were seeded per well in the 96-well round form plate and incubated the mixed lymphocyte culture for four days.

3. Statistical analysis

Paired t-tests were used to analyze the CD25 and FoxP3 data in the co-culture experiments. Wilcoxon signed-rank tests were used to analyze the results of the real-time PCR. Mann-Whitney U tests were performed to analyze MLR results. Two-sample t-tests were used to analyze the skin allograft survival areas. Survival was analyzed using the Kaplan-Meier method. All statistical analyses were conducted using SPSS version 23 (IBM SPSS Statistics). Statistical significance was set at $p<0.05$.

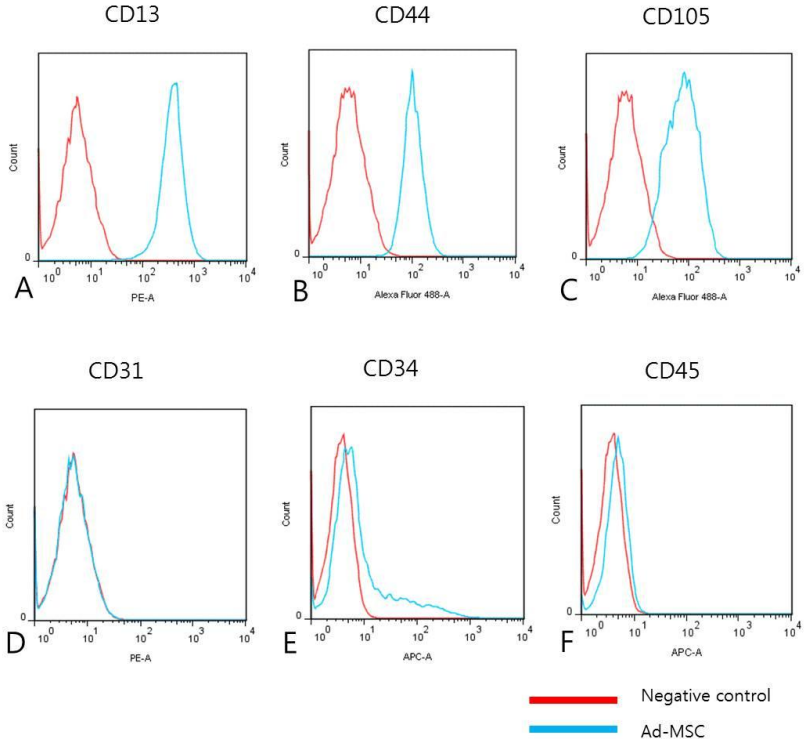
연세대학교
YONSEI UNIVERSITY

III. Results

1. *In vitro* study

A. Mesenchymal stem cell identification and differentiation

It was observed that the Ad-MSC cells grew and formed clusters after approximately 21 days in culture. After re-culturing the cells for 3–5 passages, we checked the positive and negative MSC markers using FACS (Fig. 1 A-F). It was found that the positive markers, CD13, CD44, and CD105, were expressed, whereas the negative markers CD31, CD34, and CD45 were not expressed; therefore, it was able to confirm the differentiation of Ad-MSCs. Furthermore, adipogenesis and osteogenesis were confirmed in Ad-MSCs by Oil red-O and Alisarin red S staining for lipid droplets and calcium deposits, respectively (Fig. 1 G, H).



연세대학교
YONSEI UNIVERSITY

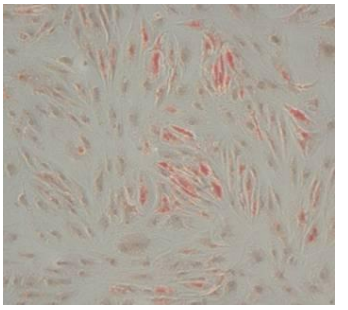
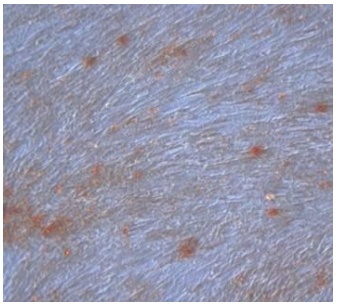
G  H 

Figure 1. Ad-MSC surface marker analysis using FACS flow cytometry. (A-F) Cell surface markers of MSCs were determined. Ad-MSC signals are shown as blue lines, whereas the isotype-matched control antibodies (negative control to assess the background signal) are shown as the red lines. (G) Lipid droplets were stained a red color using an Oil red-O solution to detect adipogenesis (×100). (H) Calcium deposits were stained red using Alizarin red S to detect osteogenesis (× 200).

연세대학교
YONSEI UNIVERSITY

B. $CD4^+CD25^+FoxP3^+$ Tregs induction

After analyzing with FACS, it was found that the expression of $CD4^+CD25^+FoxP3^+$ Tregs in both the co-culture and transwell culture groups was increased compared to the control group ($p<0.001$ and $p=0.008$, respectively, paired t-test; Fig. 2). The mean percentage of Tregs in the co-culture model was 4.46 ± 1.63 (n = 16), whereas in the transwell culture model the percent was 3.46 ± 1.75 (n = 17). In addition, $CD4^+CD25^+FoxP3^+$ Tregs in the co-culture group showed more increased pattern than transwell culture group, but this was not statistically meaningful.

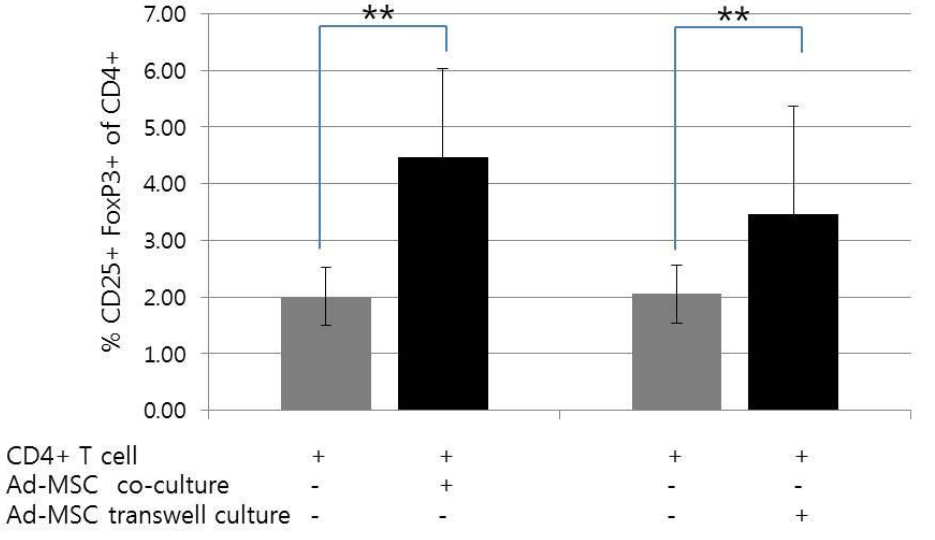


Figure 2. Expression of $CD4^+CD25^+FoxP3^+$ Tregs, as assessed by FACS, according to the culture method. $CD4^+$ T cells cultured with Ad-MSCs were induced into $CD4^+CD25^+FoxP3^+$ Tregs in both experimental groups, compared to controls. $^{**}p<0.01$

연세대학교
YONSEI UNIVERSITY

C. $CD4^+CD25^+FoxP3^+$ Tregs variation in co-culture

The results of the FACS analysis revealed that the number of $CD4^+CD25^+FoxP3^+$ Tregs was higher when T cells were co-cultured with Ad-MSCs, compared to when they were cultured alone ($p<0.001$, paired t-test). Furthermore, under conditions in which TGF-β or IL-10 signaling was blocked with antibodies, the proportion of Tregs was decreased compared to the unblocked group ($p<0.001$ for both, paired t-test; n = 16). Moreover, blocking both TGF-β and IL-10 further decreased the proportion of $CD4^+CD25^+FoxP3^+$, compared to the unblocked group ($p<0.001$, paired t-test, n = 16; Fig. 3). TGF-β or IL-10 blocking were performed only in the presence of Ad-MSCs, however, it was observed that Treg induction was higher under either of the blocked conditions than for the $CD4^+$ T cell monoculture ($p<0.001$ and $p=0.013$, paired t-test)

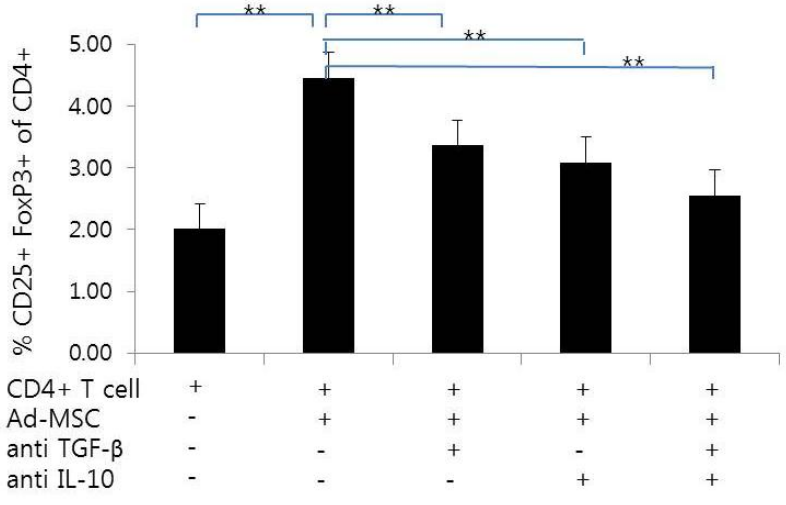


Figure 3. Change of $CD4^+CD25^+FoxP3^+$ Treg in co-culture. $CD4^+CD25^+FoxP3^+$ Tregs increased in the co-cultured group and decreased in the double-block group. **$p<0.01$

연세대학교
YONSEI UNIVERSITY

D. $CD4^+CD25^+FoxP3^+$ Tregs variation in transwell culture

Similar to the co-culture model, the results of the FACS analysis revealed that the expression Treg cells in all transwell culture experimental groups (wherein the $CD4^+$ cells were cultured in the presence of Ad-MSCs) were increased compared with the control group (p=0.008). In addition, blocking of either TGF-β or IL-10 signaling decreased the proportion of Tregs (p=0.02 and 0.01, respectively, paired t-test, n = 17; Fig. 4). The $CD4^+$ T cell monoculture (Control group) with the blocking conditions were also compared. It was found that the proportion of Tregs in cultures that contained Ad-MSCs, but were blocked with an anti-TGF-β antibody, an anti-IL-10 antibody, or both, was higher than for the $CD4^+$ T cell monoculture (p=0.007, p=0.045, p=0.029, respectively, paired t-test).

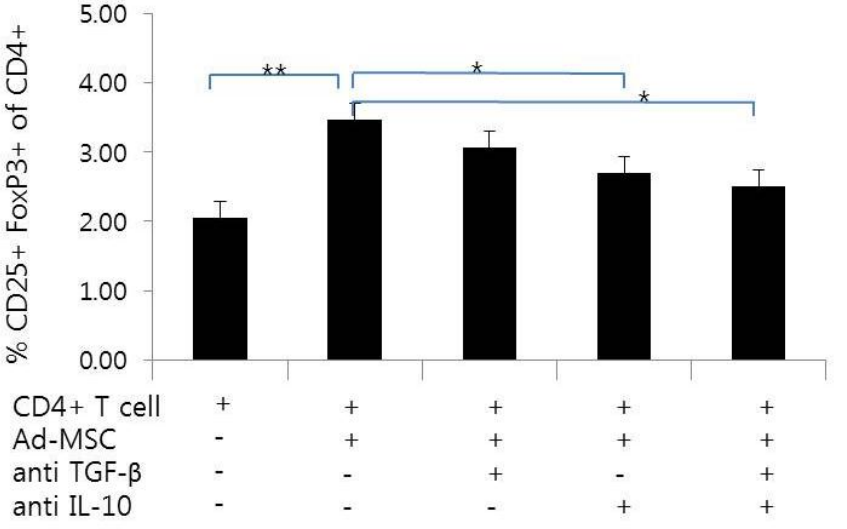


Figure 4. Change of $CD4^+CD25^+FoxP3^+$ Tregs in the transwell culture. $CD4^+CD25^+FoxP3^+$ Tregs increased in the transwell culture group (p<0.001) and decreased in the double-block group. *p<0.05, **p<0.01

연세대학교
YONSEI UNIVERSITY

E. Expression of CD25 and FoxP3 mRNA in co-culture

After performing semi-quantitative PCR, it was observed that the CD25 and FoxP3 bands from cultures in which $CD4^+$ T cell were co-cultured with Ad-MSCs were more intense than those from the control. Real-time PCR confirmed that CD25 and FoxP3 mRNA levels were higher in the co-cultures containing both $CD4^+$ T cells and Ad-MSCs than those in the monoculture (CD25, p=0.006; FoxP3, p=0.002; Wilcoxon signed-rank tests; Fig. 5).

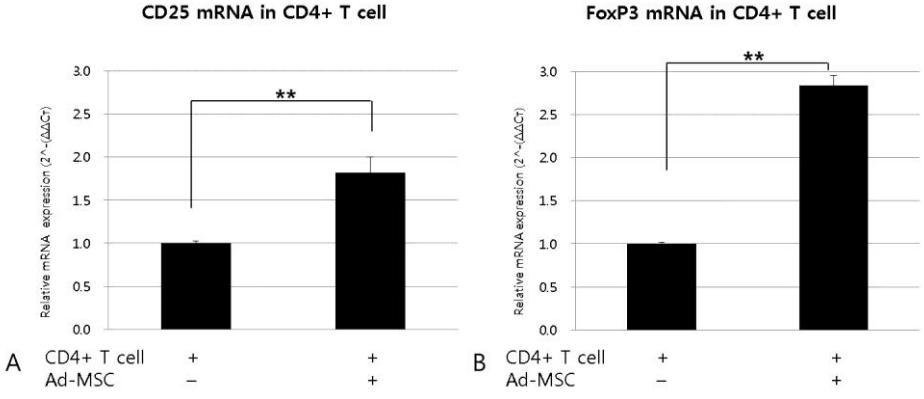


Figure 5. Real-time PCR results for the expression of CD25 (A) and FoxP3 (B) mRNA in the co-culture method. Expression is shown relative to the control group ($CD4^+$ T cells only). *p<0.05, **p<0.01, Wilcoxon signed-rank test, n = 7.

연세대학교
YONSEI UNIVERSITY

F. Comparison of TGF-β1 and IL-10 mRNA by cell type and culture condition

It was found that the levels of TGF-β1 and IL-10 mRNA were higher in Ad-MSCs than in $CD4^+$ T cells. (n = 6; TGF-β1, p=0.02; IL-10, p=0.02, Wilcoxon signed-rank test; Fig. 6). Furthermore, expression of TGF-β1 and IL-10 mRNA was higher in $CD4^+$ T cells when they were co-cultured with Ad-MSCs than when cultured alone (n = 6; TGF-β1, p=0.04; IL-10, p=0.008, Wilcoxon signed-rank test).

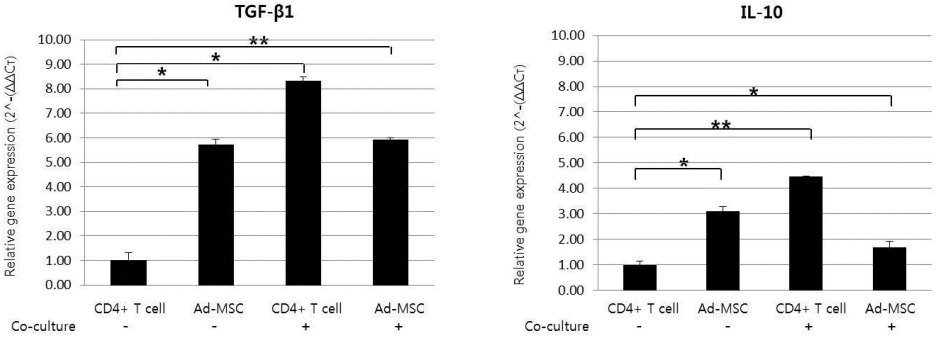


Figure 6. Levels of TGF-β1 and IL-10 mRNA by cell type were analyzed by real-time PCR in $CD4^+$ T cells and/or Ad-MSCs cultured either alone or together. 'Co-culture' indicates that the cells were cultured together using the co-culture model. *p<0.05, **p<0.01

연세대학교
YONSEI UNIVERSITY

1. *In vivo* study

A. Survival of skin allograft

Following skin graft surgeries, the survival of the graft were analyzed by creating a Kaplan-Meier curve. The data is shown in Fig. 7. In Group 1 (IP injection of Ad-MSCs), it was observed that the area of graft survival was slightly increased compared to the PBS-injected control, but the difference was not statistically significant.

In Group 2 (local injection), the experimental animals appeared to have a larger area of the graft survival than the controls (Fig 8). Kaplan-Meier curve were used to analyze survival (50% rejection) and a statistically significant improvement in the Group 2 animals were found compared to controls (p=0.04; log-rank, Mantel–Cox; Fig. 9).

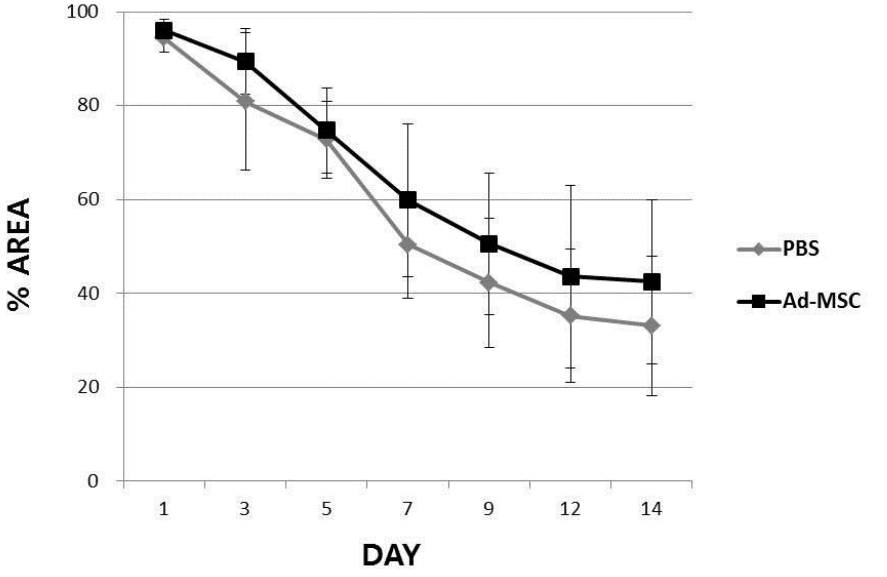


Figure 7. Survival area of the skin allograft in Group 1 animals (IP injection of Ad-MSCs) was compared to PBS-injected controls as a function of postoperative day. (control, n = 11; Group 1, n = 10).

연세대학교
YONSEI UNIVERSITY

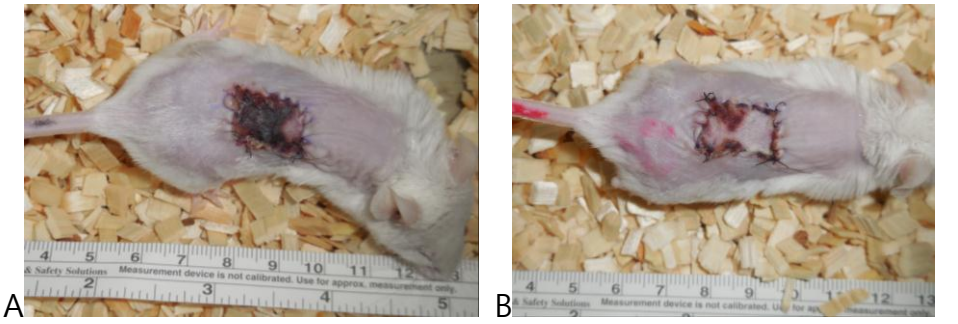


Figure 8. Representative images taken on postoperative day 7 of the skin allograft for the Control group (A) and for Group 2 (local injection, panel B) Over 50% of the area was being rejected by the host and undergoing necrosis in the Control. In contrast, most of skin graft area was surviving in Group 2.

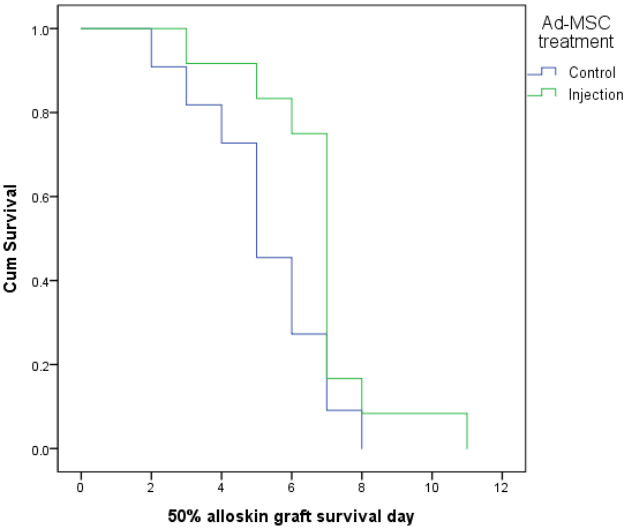


Figure 9. Survival analysis of skin allograft in Group 2 (local injection) animals by Kaplan-Meier curve (50% rejection) as a function of postoperative day. Local injection of Ad-MSCs was more effective at promoting survival than the control group (p =0.04; log-rank, Mantel–Cox; control group, n = 11, experimental group n = 12).

연세대학교
YONSEI UNIVERSITY

B. Histologic analysis

A histologic analysis was performed on samples taken from group 1 (IP injection) and group 2 (local injection). It was found that inflammatory cells infiltrated the dermis on postoperative days 3 and 14 (Fig. 10). Most samples had a histologic grade of II or III on day 3, whereas by day 14 they had progressed to grade III or IV. It was observed no noticeable differences between the control and Ad-MSC injection groups. In addition, irregular and thick distribution of collagen, as well as epidermolysis, were observed on day 3.

On postoperative day 3, it was noted extravasation of red blood cells, On day 14, several samples exhibited karyolysis of the dermis, but there was no meaningful difference between groups.

Distribution of inflammatory cells was different in dermis and subcutaneous layer (Fig. 10). Therefore, the inflammatory cells on divided area, dermis and subcutaneous layer were counted. 3 different sites on each dermis and subcutaneous layer were captured. There was statistical difference on subcutaneous layer inflammatory cell counts on 3 day in group 1 IP injection (p=0.004, Fig. 11). Inflammatory cell counts in group 2 (local injection) were performed same method. The results of inflammatory cell counts were similar to group 1. There was statistical difference on subcutaneous layer inflammatory cell counts on 3 day in group 1 IP injection (p=0.04, Fig. 12). Ad-MSC IP injection group had cells of FoxP3 positive cells on immunohistochemical staining (Fig. 13).

연세대학교
YONSEI UNIVERSITY

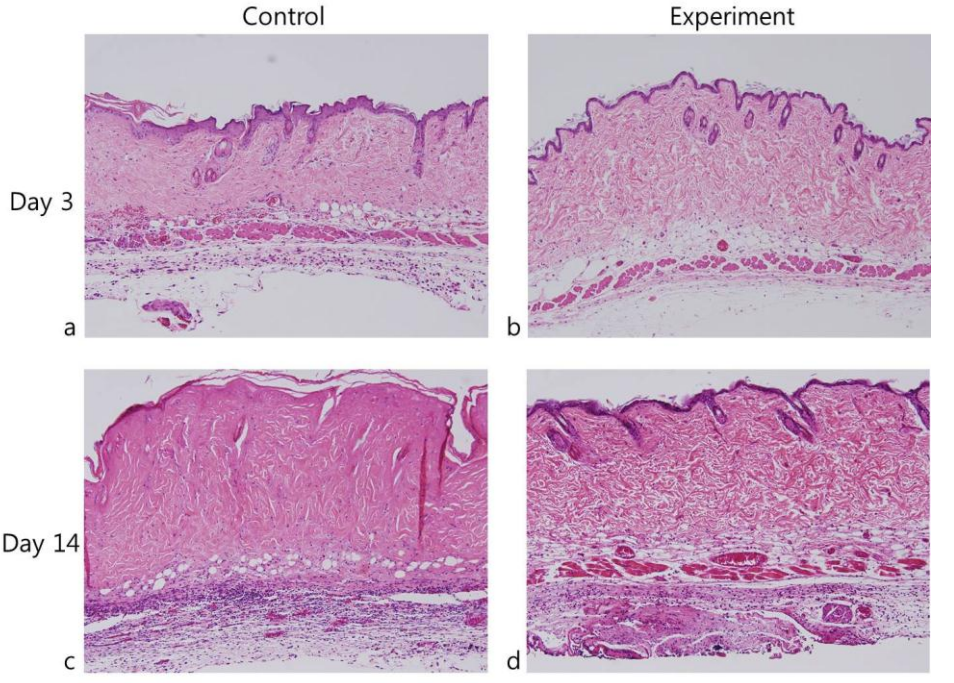


Figure 10. Skin allograft histology and Banff classification. H&E staining was performed on skin graft samples from Controls (a) and Group 1 animals (b) on day 3. Lymphocyte infiltration was observed in the papillary dermis, and inflammatory cell infiltration occurred to a greater extent in the subcutaneous layer, including the reticular dermis. Banff Grade II histology was also performed. Inflammatory cells were found in the dermis and subcutaneous layer. (c) Control group, day 14. Epidermolysis and coarse collagen distribution was found. Dense inflammatory cell infiltration was seen in the subcutaneous layer with necrosis (Grade IV). (d) Group 1, day 14. Histologic findings were similar to the control group (Grade IV). (Magnification x100)

연세대학교
YONSEI UNIVERSITY

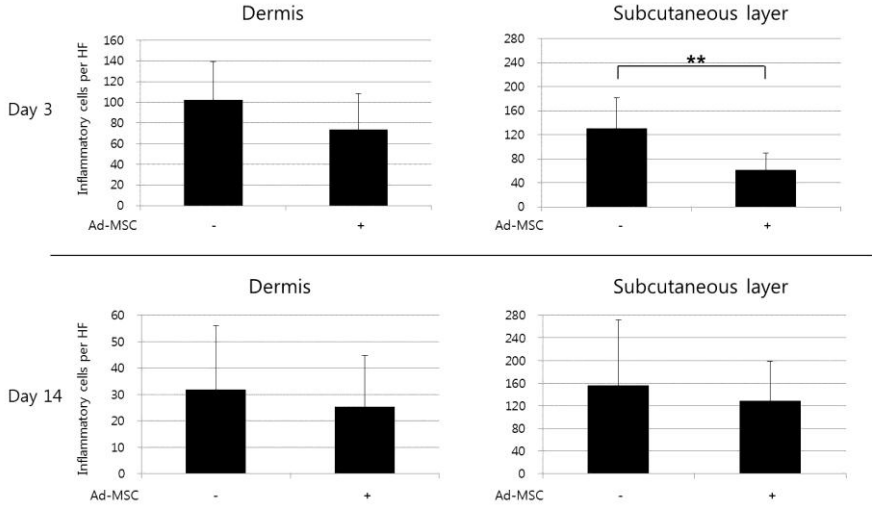


Figure 11. Inflammatory cell counts in the dermis and subcutaneous layer in IP injection group. Inflammatory cells were counted in images obtained using a high power field (x 400) on day 3 and day 14. Three sites were randomly selected for each slide, in the dermis and subcutaneous layer for the total inflammatory cell counts. ***p*<0.01, t-test.

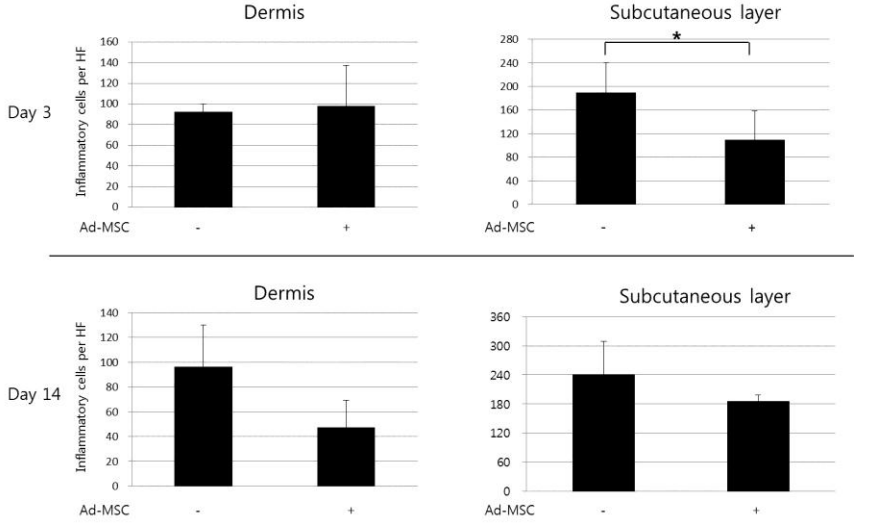


Figure 12. Inflammatory cell counts in the dermis and subcutaneous layer in local injection group. Inflammatory cells in subcutaneous layer on day 3 were lower than control group. **p*<0.05, t-test.

연세대학교
YONSEI UNIVERSITY

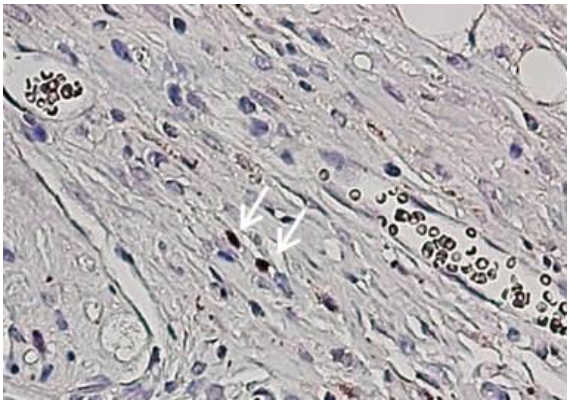


Figure 13. Immunohistochemical staining was performed for FoxP3 and a representative image is shown for Group 1. FoxP3 positive cells can be seen in the subcutaneous layer (arrow, × 400).

C. Mixed lymphocyte reaction analysis

MLR analysis were performed of splenocytes from BALB/C mice that received skin allografts, either with or without injection of Ad-MSCs. The responder cells were the splenocytes of mice that had received grafts, and the stimulator cells were the splenocytes of C57BL/6 mice. $CD4^+$ splenocytes were analyzed using CD4-APC and CFSE staining. In group 1 for IP injection, splenocytes from mice that received Ad-MSCs showed decreased lymphocyte reaction compared with splenocytes from mice that did not receive Ad-MSCs (p=0.002, Mann-Whitney U test; Fig. 14).

연세대학교
YONSEI UNIVERSITY

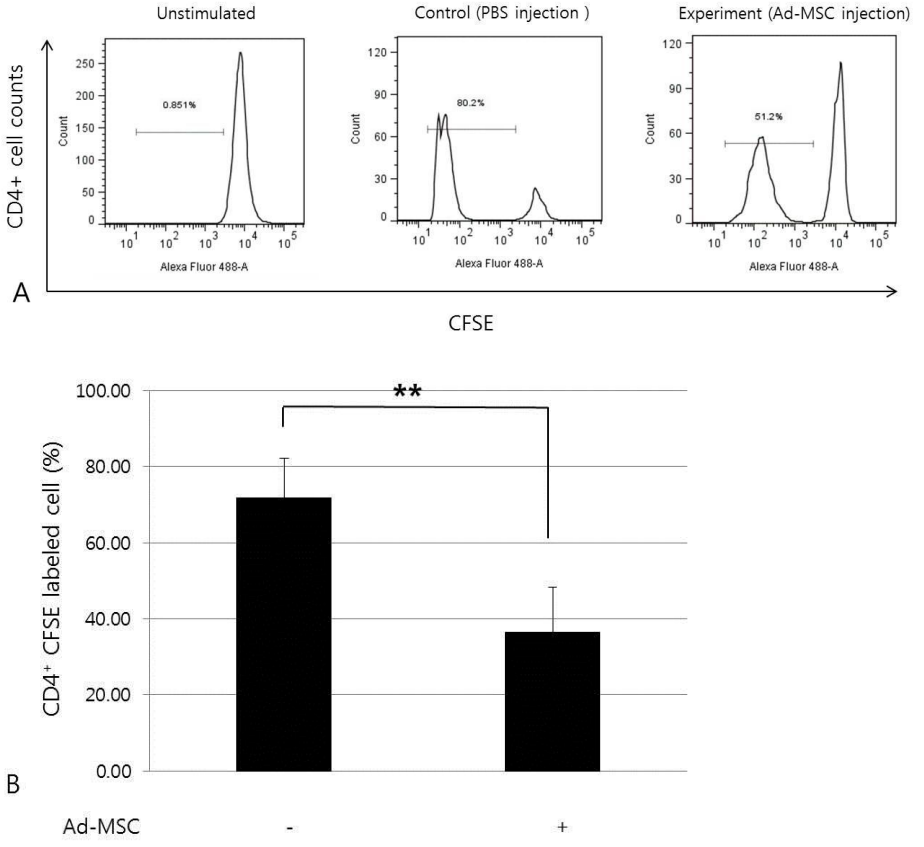


Figure 14. Mixed lymphocyte reaction analysis of splenocytes from BALB/C mice that received skin allografts, either with or without injection of Ad-MSCs. (a) CSFE-stained $CD4^+$ responder cells were co-incubated with stimulator cells and Ad-MSC. Histograms show the proliferation of $CD4^+$ T cells after four days. (b) Quantification of the MLR results show that the percentage of $CD4^+$ responder cells in the Ad-MSC-injection group was reduced compared to the control group. (n=7, p=0.002, Mann-Whitney test). **p<0.01

연세대학교
YONSEI UNIVERSITY

IV. Discussion

Initial research on MSCs primarily used BM-MSCs.[9,12] However, more recently, research has extended to other sources of MSC's, such as cord blood and adipose tissue.[16,33,34] Ad-MSCs share many of the same features as other MSCs, but the characteristics of MSCs have been show to vary depending on their origin.[15,33,35-37] Here, it was examined whether Ad-MSCs promote immune tolerance through a similar mechanism as other MSCs. Specifically, MSCs have been shown to modulate immune tolerance by inducing Tregs,[16,38] and here it was sought to determine whether Ad-MSCs can induce Tregs through direct contact, or through the release of soluble factors. To this end, the induction of Tregs from $CD4^+$ T cells in conditions were investigated that allowed for direct contact with Ad-MSCs, and conditions that did not, *in vitro*. In addition, the survival of skin allografts *in vivo* according to the method were investigated by which Ad-MSCs were delivered.

While designing this study, it was heavily considered the likelihood that using an Ad-MSC injection as the only treatment might not be enough to maintain immune tolerance and inhibit the rejection of the skin allografts. Previous research studies performed in clinical practices have reported differential outcomes that likely vary according to conditions such as pre-radiation treatment of the patients.[6,39-41] Moreover, reproducibility of previous work focusing on immune tolerance and survival has been difficult to achieve even in experiments performed under the same conditions.[6,42-44] Despite of this, only an Ad-MSC injection was chose to observe their singular effect on the allografts.

In this study, it was confirmed that Ad-MSCs can induce Treg

연세대학교
YONSEI UNIVERSITY

differentiation from $CD4^+$ T cells. Induction of Tregs in both the co-culture and transwell culture models was high, and no statistical were observed difference between the two: the Ad-MSCs exerted the same effects on Treg induction regardless of cell-cell contact. These results are in contrast with previous work by English and colleagues performed on BM-MSCs, which found that transwell cultures were less effective than co-cultures.[10]

TGF-β and IL-10 have been reported as the soluble factors responsible for the paracrine effects of MSCs.[34,45] In this study, these soluble factors were evaluated by blocking them with antibodies, instead of by quantifying their respective levels to examine their roles more conclusively. TGF-β and/or IL-10 were blocked in both culture conditions (transwell and co-culture), whereas previously, most studies that have performed this type of experiment have investigated only a co-culture model. It was our hope that by blocking the signaling of soluble factors in the transwell culture, wherein direct cell-cell contact did not occur, it could be isolated the effects of these cytokines.

Even in conditions in which cytokine signaling was blocked, under either transwell or co-culture, it was found that the induction of Tregs was still significantly higher, compared to $CD4^+$ T cells cultured alone. This indicates that there might be additional cytokines that underlie the effects of Ad-MSCs.

When cultured individually, it was found that both cytokines were much more highly expressed in Ad-MSCs than in $CD4^+$ T cells alone. However, when $CD4^+$ T cells were cultured in the presence of Ad-MSCs, the expression of both TGF-β and IL-10 significantly increased. This indicates that Ad-MSCs can cause $CD4^+$ T cells to exert autocrine effects.

연세대학교
YONSEI UNIVERSITY

It was thought that the Ad-MSC can exert mutual effects to $CD4^+$ T cells on cytokine induction.

Following our experiments *in vitro*, I hoped confirm these findings regarding mechanisms of immune tolerance in an *in vivo* study. For this study, skin allografts were performed. Under normal conditions, these skin allografts should be rejected by the recipient without treatment,. Using this animal model, two methods of Ad-MSC delivery were tested: systemic injections and local injections around the graft site. For our systemic delivery, IP injections were performed rather than IV injections in an attempt to block direct contact of Ad-MSCs with immune cells, and block potential homing effects. An IV injection might have allowed for more direct contact between MSCs and PBMCs in the blood stream. Ad-MSCs had a chance to reach the skin graft site by the homing effect.

In our other method of delivery, MSCs were injected directly into the area surrounding the graft. These local injections are thought to lead to cell-cell contact with immune cells since they are spread via the plasma in the early phases, rather than through direct blood flow to the grafted skin.

For an analysis of survival, rejection as 100% and 50% rejection were classified, and an analysis of the area of survival were also performed, which is usually used in wound healing models. IP injection (Group 1) trended toward a higher survival than controls, but this was not statistically significant. Conversely, the local injection group (Group 2) showed a statistically significant improvement at 50% rejection. Neither Group 1 nor Group 2 was significant at 100% rejection.

For our assessment of inflammatory cells, total inflammatory cell number were counted, but also checked for differences in distribution

연세대학교
YONSEI UNIVERSITY

between the dermis and the subcutaneous layer, including the panniculus muscle, in grafted skin. Therefore, inflammatory cells were counted in both the dermis and the subcutaneous layer. It was found that the number of inflammatory cells in the subcutaneous layer was significantly lower in the experimental group than in the PBS control group for both injection methods (Group 1 and 2) on postoperative day 3. Both IP and local injection of Ad-MSCs showed some effects toward delayed rejection of the skin allograft; however, local injections were more effective in inducing immune tolerance than IP injections.

Several questions remain regarding the effects of Ad-MSCs on immune tolerance. For example, it was found that inflammatory cell counts were significantly altered by Ad-MSC treatment on postoperative day 3, and on day 14 the Ad-MSC groups exhibited a lessened MLR response and FoxP3 expression was observed in group 1. Therefore it is still unclear how effective immunotolerance will be on day 3 or 14. While most preclinical and clinical studies of the effects of MSCs on immune tolerance have shown good results,[40,41,46] several studies reported that MSC treatment alone was not effective, and only achieved good results when used in combination with an immunosuppressant.[47-49] Similarly, in our previous experiments with dental pulp-derived MSCs, changes in MLRs were observed *in vitro*, but no significant effect on the survival of grafts.[50]

In organ transplantation and VCA, the recipient's blood and immune cells are circulated to the donor tissue through the vessel anastomosis. Therefore, delivery via IV may have been a more effective method to achieve immune tolerance. A comparative study of IV and IP may be needed in the future. In addition, while IP injections were used in an

연세대학교
YONSEI UNIVERSITY

attempt to prevent cell-cell contact and isolate any paracrine effects, potential paracrine effects cannot be ruled out in the local injections. Moreover, it is likely that a higher concentration of Ad-MSCs elicited effects on the grafted skin in the local injection than the IP injection. Future studies should explore other mechanisms for increasing cell contact through the delivery method, and examine how effectively soluble factor are delivered in MSC treatments. It is also critical that the effects of Ad-MSC dosing, timing, and injection intervals be examined in future work. Unlike skin allograft, VCA is directly connected to blood flow. That means that the location of immune response may be different between skin allograft and VCA. Therefore, further research is needed to investigate delivery method in VCA procedures, as well as the effects of varying levels of antigens.

연세대학교
YONSEI UNIVERSITY

V. Conclusion

In conclusion, it was found that Ad-MSCs in culture can induce T cell differentiation into $CD4^{+}CD25^{+}FoxP3^{+}$ Tregs. Treg was induced regardless of cell contact. TGF-β and IL-10 secreted from adipose stem cells affected regulatory T cell expression. TGF-β and IL-10 mRNA expressions were elevated in $CD4^{+}$ T cells cultured with adipose stem cells compared to $CD4^{+}$ T cells alone. It was also found that local injection of Ad-MSCs increased skin allograft survival and was more effective than IP injections. Finally, it was observed that Ad-MSC injections can be effective on immune tolerance for 14 days in the aspect of MLR and FoxP3 in histology. But clinically meaningful effects regarding graft rejection were only seen in the early days after skin allograft.

연세대학교
YONSEI UNIVERSITY

REFERENCES

1. Bloom RD, Crutchlow MF. New-onset diabetes mellitus in the kidney recipient: diagnosis and management strategies. Clin J Am Soc Nephrol 2008;3 Suppl 2:S38-48.
2. Khalifian S, Brazio PS, Mohan R, Shaffer C, Brandacher G, Barth RN, et al. Facial transplantation: the first 9 years. Lancet 2014;384:2153-63.
3. Sarhane KA, Tuffaha SH, Broyles JM, Ibrahim AE, Khalifian S, Baltodano P, et al. A critical analysis of rejection in vascularized composite allotransplantation: clinical, cellular and molecular aspects, current challenges, and novel concepts. Front Immunol 2013;4:406.
4. Baccarani A, Follmar KE, Baumeister SP, Marcus JR, Erdmann D, Levin LS. Technical and anatomical considerations of face harvest in face transplantation. Ann Plast Surg 2006;57:483-8.
5. Brown CS, Gander B, Cunningham M, Furr A, Vasilic D, Wiggins O, et al. Ethical considerations in face transplantation. Int J Surg 2007;5:353-64.
6. Heyes R, Iarocci A, Tchoukalova Y, Lott DG. Immunomodulatory Role of Mesenchymal Stem Cell Therapy in Vascularized Composite Allotransplantation. J Transplant 2016;2016:6951693.
7. Schneeberger S, Gorantla VS, Brandacher G, Zeevi A, Demetris AJ, Lunz JG, et al. Upper-extremity transplantation using a cell-based protocol to minimize immunosuppression. Ann Surg 2013;257:345-51.
8. Del Bene M, Di Caprio AP, Melzi ML, Pioltelli PE, Bonomi S. Autologous mesenchymal stem cells as a new strategy in immunosuppressant therapy in double hand allotransplantation. Plast Reconstr Surg 2013;131:305e-7e.
9. Le Blanc K. Immunomodulatory effects of fetal and adult mesenchymal stem cells. Cytotherapy 2003;5:485-9.
10. English K, Ryan JM, Tobin L, Murphy MJ, Barry FP, Mahon BP. Cell contact, prostaglandin E(2) and transforming growth factor beta 1 play non-redundant roles in human mesenchymal stem cell induction of CD4+CD25(High) forkhead box P3+ regulatory T cells. Clin Exp

연세대학교
YONSEI UNIVERSITY

Immunol 2009;156:149-60.

11. Selmani Z, Naji A, Zidi I, Favier B, Gaiffe E, Obert L, et al. Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4+CD25highFOXP3+ regulatory T cells. Stem Cells 2008;26:212-22.
12. Di Ianni M, Del Papa B, De Ioanni M, Moretti L, Bonifacio E, Cecchini D, et al. Mesenchymal cells recruit and regulate T regulatory cells. Exp Hematol 2008;36:309-18.
13. Salibian AA, Widgerow AD, Abrouk M, Evans GR. Stem cells in plastic surgery: a review of current clinical and translational applications. Arch Plast Surg 2013;40:666-75.
14. Tobita M, Orbay H, Mizuno H. Adipose-derived stem cells: current findings and future perspectives. Discov Med 2011;11:160-70.
15. Ikegame Y, Yamashita K, Hayashi S, Mizuno H, Tawada M, You F, et al. Comparison of mesenchymal stem cells from adipose tissue and bone marrow for ischemic stroke therapy. Cytotherapy 2011;13:675-85.
16. Burr SP, Dazzi F, Garden OA. Mesenchymal stromal cells and regulatory T cells: the Yin and Yang of peripheral tolerance? Immunol Cell Biol 2013;91:12-8.
17. Tsuji W, Schnider JT, McLaughlin MM, Schweizer R, Zhang W, Solari MG, et al. Effects of immunosuppressive drugs on viability and susceptibility of adipose- and bone marrow-derived mesenchymal stem cells. Front Immunol 2015;6:131.
18. Melief SM, Zwaginga JJ, Fibbe WE, Roelofs H. Adipose tissue-derived multipotent stromal cells have a higher immunomodulatory capacity than their bone marrow-derived counterparts. Stem Cells Transl Med 2013;2:455-63.
19. Plock JA, Schnider JT, Zhang W, Schweizer R, Tsuji W, Kostereva N, et al. Adipose- and Bone Marrow-Derived Mesenchymal Stem Cells Prolong Graft Survival in Vascularized Composite Allotransplantation. Transplantation 2015;99:1765-73.

연세대학교
YONSEI UNIVERSITY

20. Shi M, Liu ZW, Wang FS. Immunomodulatory properties and therapeutic application of mesenchymal stem cells. Clinical and Experimental Immunology 2011;164:1-8.
21. Fudaba Y, Spitzer TR, Shaffer J, Kawai T, Fehr T, Delmonico F, et al. Myeloma responses and tolerance following combined kidney and nonmyeloablative marrow transplantation: in vivo and in vitro analyses. Am J Transplant 2006;6:2121-33.
22. Yi T, Song SU. Immunomodulatory properties of mesenchymal stem cells and their therapeutic applications. Arch Pharm Res 2012;35:213-21.
23. Baharlou R, Ahmadi-Vasmehjani A, Faraji F, Atashzar MR, Khoubyari M, Ahi S, et al. Human adipose tissue-derived mesenchymal stem cells in rheumatoid arthritis: Regulatory effects on peripheral blood mononuclear cells activation. Int Immunopharmacol 2017;47:59-69.
24. Gonzalez-Rey E, Gonzalez MA, Varela N, O'Valle F, Hernandez-Cortes P, Rico L, et al. Human adipose-derived mesenchymal stem cells reduce inflammatory and T cell responses and induce regulatory T cells in vitro in rheumatoid arthritis. Ann Rheum Dis 2010;69:241-8.
25. Ma OK, Chan KH. Immunomodulation by mesenchymal stem cells: Interplay between mesenchymal stem cells and regulatory lymphocytes. World J Stem Cells 2016;8:268-78.
26. Eom YW, Lee JE, Yang MS, Jang IK, Kim HE, Lee DH, et al. Rapid isolation of adipose tissue-derived stem cells by the storage of lipoaspirates. Yonsei medical journal 2011;52:999-1007.
27. Yanez R, Lamana ML, Garcia-Castro J, Colmenero I, Ramirez M, Bueren JA. Adipose tissue-derived mesenchymal stem cells have in vivo immunosuppressive properties applicable for the control of the graft-versus-host disease. Stem Cells 2006;24:2582-91.
28. Bourin P, Bunnell BA, Casteilla L, Dominici M, Katz AJ, March KL, et al. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and

연세대학교
YONSEI UNIVERSITY

Science (IFATS) and the International Society for Cellular Therapy (ISCT). Cytotherapy 2013;15:641-8.

29. Uder C, Bruckner S, Winkler S, Tautenhahn HM, Christ B. Mammalian MSC from selected species: Features and applications. Cytometry A 2017; doi:10.1002/cyto.a.23239.
30. Cendales LC, Kanitakis J, Schneeberger S, Burns C, Ruiz P, Landin L, et al. The Banff 2007 working classification of skin-containing composite tissue allograft pathology. Am J Transplant 2008;8:1396-400.
31. Siemionow MZ. The Know-How of Face Transplantation. New York: Springer; 2011.
32. Woo YL, Sterling J, Crawford R, van der Burg SH, Coleman N, Stanley M. FOXP3 immunohistochemistry on formalin-fixed paraffin-embedded tissue: poor correlation between different antibodies. J Clin Pathol 2008;61:969-71.
33. Kern S, Eichler H, Stoeve J, Kluter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 2006;24:1294-301.
34. Ichim TE, Harman RJ, Min WP, Minev B, Solano F, Rodriguez JP, et al. Autologous stromal vascular fraction cells: a tool for facilitating tolerance in rheumatic disease. Cell Immunol 2010;264:7-17.
35. Ock SA, Baregundi Subbarao R, Lee YM, Lee JH, Jeon RH, Lee SL, et al. Comparison of Immunomodulation Properties of Porcine Mesenchymal Stromal/Stem Cells Derived from the Bone Marrow, Adipose Tissue, and Dermal Skin Tissue. Stem Cells Int 2016;2016:9581350.
36. Ribeiro A, Laranjeira P, Mendes S, Velada I, Leite C, Andrade P, et al. Mesenchymal stem cells from umbilical cord matrix, adipose tissue and bone marrow exhibit different capability to suppress peripheral blood B, natural killer and T cells. Stem Cell Res Ther 2013;4:125.
37. Jin HJ, Bae YK, Kim M, Kwon SJ, Jeon HB, Choi SJ, et al. Comparative analysis of human mesenchymal stem cells from bone marrow, adipose tissue, and umbilical cord blood as sources of cell therapy. Int J Mol Sci 2013;14:17986-8001.

연세대학교
YONSEI UNIVERSITY

38. English K. Mechanisms of mesenchymal stromal cell immunomodulation. Immunol Cell Biol 2013;91:19-26.
39. Aksu AE, Horibe E, Sacks J, Ikeguchi R, Breitinger J, Scozio M, et al. Co-infusion of donor bone marrow with host mesenchymal stem cells treats GVHD and promotes vascularized skin allograft survival in rats. Clin Immunol 2008;127:348-58.
40. Kuo YR, Chen CC, Goto S, Huang YT, Wang CT, Tsai CC, et al. Immunomodulatory effects of bone marrow-derived mesenchymal stem cells in a swine hemi-facial allotransplantation model. PLoS One 2012;7:e35459.
41. Jeong SH, Ji YH, Yoon ES. Immunosuppressive activity of adipose tissue-derived mesenchymal stem cells in a rat model of hind limb allotransplantation. Transplant Proc 2014;46:1606-14.
42. Pan H, Zhao K, Wang L, Zheng Y, Zhang G, Mai H, et al. Mesenchymal stem cells enhance the induction of mixed chimerism and tolerance to rat hind-limb allografts after bone marrow transplantation. J Surg Res 2010;160:315-24.
43. Kuo YR, Chen CC, Goto S, Lee IT, Huang CW, Tsai CC, et al. Modulation of immune response and T-cell regulation by donor adipose-derived stem cells in a rodent hind-limb allotransplant model. Plast Reconstr Surg 2011;128:661e-72e.
44. Casiraghi F, Perico N, Remuzzi G. Mesenchymal stromal cells to promote solid organ transplantation tolerance. Curr Opin Organ Transplant 2013;18:51-8.
45. Chien CM, Chen YW, Chen CC, Wu YC, Huang SH, Lee SS, et al. Adipose-Derived Stem Cell Modulation of T-Cell Regulation Correlates with Heme Oxgenase-1 Pathway Changes. Plast Reconstr Surg 2016;138:1015-23.
46. Lefaucheur C, Loupy A, Vernerey D, Duong-Van-Huyen JP, Suberbielle C, Anglicheau D, et al. Antibody-mediated vascular rejection of kidney allografts: a population-based study. Lancet 2013;381:313-9.
47. Popp FC, Eggenhofer E, Renner P, Slowik P, Lang SA, Kaspar H, et al.

연세대학교
YONSEI UNIVERSITY

Mesenchymal stem cells can induce long-term acceptance of solid organ allografts in synergy with low-dose mycophenolate. Transpl Immunol 2008;20:55-60.

48. Eggenhofer E, Renner P, Soeder Y, Popp FC, Hoogduijn MJ, Geissler EK, et al. Features of synergism between mesenchymal stem cells and immunosuppressive drugs in a murine heart transplantation model. Transpl Immunol 2011;25:141-7.

49. Inoue S, Popp FC, Koehl GE, Piso P, Schlitt HJ, Geissler EK, et al. Immunomodulatory effects of mesenchymal stem cells in a rat organ transplant model. Transplantation 2006;81:1589-95.

50. Hong JW, Lim JH, Chung CJ, Kang TJ, Kim TY, Kim YS, et al. Immune Tolerance of Human Dental Pulp-Derived Mesenchymal Stem Cells Mediated by CD4(+)CD25(+)FoxP3(+) Regulatory T-Cells and Induced by TGF-beta1 and IL-10. Yonsei Med J 2017;58:1031-9.

연세대학교
YONSEI UNIVERSITY

ABSTRACT(IN KOREAN)

지방유래 중간엽줄기세포가 조절 T세포 유도와 동종피부이식에 미치는 영향

<지도교수 유 대 현>

연세대학교 대학원 의학과

홍 종 원

목적: 동종이식에서 이식편에 대한 면역거부반응을 예방하기 위하여 면역억제제가 반드시 사용되어야 하지만 장단기적으로 많은 부작용을 유발하기에 이식 후 면역관용의 유도는 면역억제의 부작용을 줄이며 동시에 이식편의 장기생존을 위한 대안으로 제시되어 왔다. 중간엽줄기세포 중 지방에서 유래한 줄기세포는 세포간 접촉과 몇몇 사이토카인의 작용이 면역관용 유도의 기전으로 알려져 있으나, 줄기세포를 주입하는 방법에 따른 연구는 거의 없는 실정이다. 본 연구자는 지방에서 유래한 지방줄기세포를 사용하여 세포접촉에 따라 TGF-β1, IL-10가 CD4+ CD25+ FoxP3+ 조절T세포 발현에 미치는 영향을 분석하였으며, 동종피부이식에서 지방줄기세포 주입방법에 따른 면역관용의 차이를 보고자 하였다.

방법 및 재료: 지방줄기세포와 $CD4^+$ T 세포를 혼합 배양한 그룹과 트렌스웰 배양한 두 실험군과 대조군인 조절 T 세포 단독 배양군에서 세포접촉 유무에 따른 $CD4^+CD25^+FoxP3^+$ 조절 T 세포 발현을 FACS 로 비교하였으며, TGF-β, IL-10 항체를 사용한 후

연세대학교
YONSEI UNIVERSITY

조절 T 세포의 변화를 관찰하였다. 지방줄기세포와 혼합 배양군에서는 CD25, FoxP3, TGF-β1, IL-10 mRNA 변화를 실시간 중합효소연쇄반응으로 확인하였다. 지방줄기세포 주입방법에 따른 면역관용 유도 효과를 알아보고자 마우스를 이용한 2×2 cm 동종피부이식 모델을 사용하였다. 지방줄기세포 2.0×10^6을 복강 내 주입한 군과 국소 주입한 군으로 나누어 이식피부편의 생존 및 면적을 계산하고 혼합림프구반응을 관찰하여 그 효과를 비교하였다.

결과: 혼합배양, 트렌스웰배양 모두에서 지방줄기세포와 함께 배양한 $CD4^+$ T 세포에서 조절 T 세포 발현이 증가하였다. 혼합배양에서 트렌스웰배양 조건에 비하여 조절 T세포 발현이 증가한 경향이 관찰되었으나 통계학적 의미는 없었다. 혼합배양군, 트렌스웰배양군 모두에서 TGF-β, IL-10 신호를 억제한 경우 조절 T세포 발현이 감소하였으나, 대조군 보다는 조절 T세포의 발현이 증가하였다. 지방줄기세포와 함께 배양한 두 군에서 CD25, FoxP3 mRNA가 증가하는 것이 관찰되었다. 지방줄기세포에서 TGF-β1, IL-10 mRNA가 높았으며, $CD4^+$ T 세포의 TGF-β1, IL-10 mRNA도 증가시켰다. 마우스를 이용한 동종피부이식 실험에서 지방줄기세포를 국소주입한 실험군에서 50% 생존율이 의미있게 증가하였으나 복강내 주입한 실험군에서는 생존율의 차이를 보이지 않았다. 그러나 이식 후 생존면적을 관찰한 결과, 양군간의 통계학적인 차이는 없었으나 국소주입 실험군에서 조금 더 생존면적이 넓은 경향을 보였다. 그럼에도 불구하고 지방줄기세포를 주입한 개체에서 대조군과 비교했을 때 혼합림프구반응, 염증세포 숫자에서 의미 있게 감소하였다.

결론: 지방줄기세포는 $CD4^+$ T 세포와 접촉 여부와 상관없이 $CD4^+CD25^+FoxP3^+$ 조절 T 세포 발현을 증가시켰다.

연세대학교
YONSEI UNIVERSITY

지방줄기세포에서 분비된 TGF-β, IL-10은 조절 T 세포 발현에 영향을 주었다. 지방줄기세포와 함께 배양했던 CD4+ T 세포에서 TGF-β, IL-10 mRNA 발현이 증가되어 자가분비되는 것으로 생각되었다. 동종피부이식 시 지방줄기세포 주입은 14일간 혼합림프구반응을 감소시키는 효과가 있었으며, 지방줄기세포를 국소 주입한 경우 복강 내 주입했을 때 보다 생존율은 높았으나, 그 효과는 이식 초기에만 국한되었다.

핵심되는 말 : 지방줄기세포, 면역관용, 조절 T 세포, 동종피부이식

연세대학교
YONSEI UNIVERSITY

PUBLICATION LIST

Hong JW, Lim JH, Chung CJ, Kang TJ, Kim TY, Kim YS, et al. Immune Tolerance of Human Dental Pulp-Derived Mesenchymal Stem Cells Mediated by CD4(+)CD25(+)FoxP3(+) Regulatory T cell and Induced by TGF-βeta1 and IL-10. Yonsei Med J 2017;58:1031-9.