이학석사학위논문

동종이형 피부 이식 모델에서 C/EBPβ를 발현하는 유도 조절 T세포의 면역 반응 억제 효과

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2015 년 2 월

서울대학교 대학원

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지도교수 성노현

이 논문을 이학석사학위논문으로 제출함

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YEO HYUN KIM

Date Approved:

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Abstract

The immune suppressive effect of induced regulatory T cells expressing C/EBPβ in allogenic skin graft transplantation

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The selective immunosuppression by induced regulatory T (iT_{reg}) cells has the potential to protect transplanted tissue without sacrificing immunity against other infections, greatly enhancing the chances of successful engraftment. However, the relative instability of Foxp3 expression in iT_{reg} cells, and consequently their immunosuppressive function, deters the development of therapeutic iT_{reg} cells at present. Results from a recent study have identified C/EBPβ as a transcription factor that enables stable expression of Foxp3 even in the presence of inflammatory cytokines IL-4 and IFN-γ (Lee, 2014).

In the light of such findings, the present research investigated the immunosuppressive function of iT_{reg} cells expressing C/EBPβ in the context of allogenic skin transplantation. For this purpose, a

protocol for culturing retrovirally-transduced iT_{reg} cells in high purity was established by using the MIN retroviral expression vector and naïve $CD4^+$ T cells from $Foxp3^{eGFP}$-reporter mice. Highly purified populations of iT_{reg} cells were obtained by selecting for hNGFR and GFP double-positive cells.

Immunodeficient *Rag*$^{-/-}$ mice reconstituted with a 1:1 ratio mixture of naïve $CD4^+$ T cells ($CD4^+CD45RB^{high}$) and $CD8^+$ T cells were used as a model of T cell-mediated allogenic rejection response. Compared to control or transfer of control iT_{reg} cells, the transfer of iT_{reg} cells expressing C/EBPβ showed possibility of delay in the rejection of allogenic grafts.

Through macroscopic observation and analysis during the rejection response, the efficacy of iT_{reg} cells expressing C/EBPβ in suppressing the immune response against allogenic graft will continue to be evaluated. The future findings of this study will contribute to the development of iT_{reg} cells as a safe and reliable immunosuppressive therapy.

Keywords: regulatory T cell, allogenic tolerance, skin graft, transplantation

Student Number: 2011-20321

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Introduction

A. Immunological basis of allogenic transplant rejection

Transplantation, or the surgical implantation of a tissue or an organ from a donor to a recipient, requires careful follow-up to ensure success. As allogenic transplantation involves direct transfer of living tissue or organ between two non-identical individuals of the same species, it inevitably triggers responses from the recipient's immune system. The human immune system consists of an array of cells trained to recognize and destroy intruders in our body including viruses, bacteria and abnormal or foreign cells, such as transplanted tissue. A non-identical donor's cells are recognized as foreign due to mismatches in cell surface proteins called major histocompatibility complexes (MHC) classes I and II. Mismatched MHC proteins can serve as foreign antigens (alloantigens) that trigger inflammatory responses against the transplanted tissue. Thus, the number of MHC mismatches correlate with the rapidity and severity of transplant rejection.

Allogenic transplant rejection response can be divided into hyperacute, acute and chronic rejection responses according to the rapidity and underlying immunological mechanism. Hyperacute

rejection response occurs within minutes of transplantation, and is triggered by preexisting humoral response mediated by alloantigenic antibodies secreted from B cells. Acute rejection response occurs as early as one week after transplantation, and in most cases during the first three months. Acute rejection is related with the Th1 response and the production of pro-inflammatory cytokines IFNγ, IL-2, IL-12, TNFα and GM-CSF. It is mediated by cellular immunity exerted by activated cytotoxic T cells and mononuclear macrophages. Chronic rejection is related with the Th2 response, which culminates in the activation of antibody-secreting B cells (Wood & Goto, 2012).

The initiation of transplant rejection may occur through two distinct pathways of alloantigen recognition. Antigen presenting cells (APCs) such as dendritic cells of the donor may migrate from the transplant into nearby draining lymph nodes, where they come into contact and stimulate recipient alloantigen-specific T cells (direct recognition) (Lombardi, Sidhu, Batchelor, & Lechler, 1989). Alternatively, APCs of the recipient may uptake alloantigens released from the transplant and present them to recipient T cells (indirect recognition) (Benichou, Takizawa, Olson, McMillan, & Sercarz, 1992; Fangmann, Dalchau, & Fabre, 1992). The relative

importance of direct and indirect alloantigen recognition pathways in transplant rejection is under study, but direct alloantigen recognition seems to play a greater role in early acute rejection (Benichou, Valujskikh, & Heeger, 1999), when the transplanted tissue is impregnated with donor-derived passenger APCs. Indirect recognition pathway has been implicated to play a more significant role in long-term transplant rejection (Lechler & Batchelor, 1982).

Thus the activation of T cells, particularly those specific to alloantigens, propels allogenic transplant rejection response. Studies indicate that these alloreactive T cells are present at high frequencies in humans, accounting for as much as 10% of total T cells (Ford & Atkins, 1973; Matzinger & Bevan, 1977). The high frequency of alloreactive T cells is responsible for the extreme severity and rapidity at which allogenic transplant rejection occurs. When left untreated, these rejection responses ultimately lead to loss of normal function and necrosis of the donor tissue or organ.

B. Current immunosuppressive treatments and the advantages of regulatory T cell therapy

To prevent such loss of grafted tissue or organ, the immune system's natural inclination to attack non-self cells needs to be

curbed. In current clinical practices, high doses of immunosuppressive drugs are administered to patients post-surgery to weaken the immune system. For patients, this means a lifelong intake of immunosuppressive drugs to keep the transplanted organ alive.

Conventional immunosuppressive drugs include glucocorticoids, cytostatics, monoclonal or polyclonal antibodies, and calcineurin inhibitors. The majority of these drugs act by either directly (ex. via cell cycle inhibition) or indirectly (ex. via depletion of pro-inflammatory cytokines such as IL-2) inhibiting the proliferation and differentiation of T and B cells (Halloran, 2004).

The greatest drawback of these immunosuppressive drugs is the lack of antigen-specificity. Therefore, immunodeficiency is an inevitable result of continuous administration of these pan-immunosuppressive drugs. A dilemma arises at this point as continued administration of immunosuppressive drugs protects the transplanted tissue from attack, but the general suppression of the immune system leaves the patient virtually defenseless against other pathogenic threats. Without an active immune system, the patient may fall prey to the most trifling infections, and even the smallest

infections can turn lethal. Lack of a vigilant immune system also exposes the patient to increased risks of cancer as such malignantly transformed cells may proliferate undetected. Other side effects include hypertension, hyperglycemia, lipodystrophy, and liver and kidney injury (Halloran, 2004).

One of the ways immunologists have been exploring as a possible means to bypass this problem is to employ regulatory T cells. Most subtypes of T cells, which are educated in the thymus to distinguish self and non-self cells during development, engage in aggressive attack and elimination of intruders. Regulatory T cells do exactly the opposite – they repress other types of activated immune cells, preventing inflammatory responses from spinning out of control. The mechanisms by which regulatory T cells repress immune responses include direct cell-cell interactions, secreted immunosuppressive soluble factors and competitive absorption of cytokines (Josefowicz, Lu, & Rudensky, 2012; Pillai, Ortega, Wang, & Karandikar, 2007).

As the body's natural regulators of the immune system, the suppressive effect regulatory T cells have is not only effective, but also antigen-specific – their ability to distinguish self from non-self

and also among different pathogens sets them apart from the general immunosuppressive drugs currently in use. This property has important implications for the role of regulatory T cells in maintaining self-tolerance, and similarly in allogenic transplantation settings. Thus treating transplant recipients with regulatory T cells may allow both the preservation of engrafted tissue and the maintenance of sufficient immune responses against pathogens.

C. Natural vs. induced regulatory T cells and the challenges of regulatory T cell therapy

There are three major hurdles to harnessing regulatory T cells for immunosuppressive therapies: obtaining them in sufficient numbers, in high purity, and as stable populations. These challenges arise from the nature of the regulatory T cells, which include various subtypes. Most notable among them are natural regulatory T (nT_{reg}) cells and induced regulatory T cells (iT_{reg}), each of which will be addressed in turn.

Although there is no single established protocol for generating regulatory T cells for therapeutic use, most procedures involve harvesting these cells or their progenitors from the recipient or third-party donors and inducing their *ex vivo* expansion.

Regulatory T cells generated in this manner, multiplied several fold from the starting population, are then transferred to the patient before or at the time of transplantation to ensure maximum efficacy.

Regulatory T cells harvested directly from peripheral blood of donors are mostly nT_{reg} cells. Like other T cell subtypes, they are selected for their ability to recognize self-antigens during their development in the thymus. They are also distinguished by a relatively stable expression of forkhead box p3 (Foxp3), a key transcription factor that enables the differentiation and maintenance of regulatory T cell characteristics (Fontenot, Gavin, & Rudensky, 2003; Hori, Nomura, & Sakaguchi, 2003; Sakaguchi, Sakaguchi, Asano, Itoh, & Toda, 1995). Around 5~10% of mature T cells are estimated to be nT_{reg} cells, of which only a small portion are alloreactive regulatory T cells. Thus, relying solely on direct purification poses challenges for procuring regulatory T cells in large numbers. This is a serious concern especially for the purpose of treating transplant rejection, as studies in mice have implied high ratios of regulatory T cells are necessary to exert immunosuppressive effects (Wang, Lu, & Jiang, 2011). Therefore, many protocols involve *in vitro* stimulation of purified regulatory T cells to induce expansion.

An alternative approach is to generate regulatory T cells *de novo* from purified progenitor populations. Regulatory T cells generated by the cytokine TGF-β from progenitor naïve T cells are called iT_{reg} cells. These cells are naturally generated in the periphery, especially at sites of inflammation. The relative contribution of nT_{reg} versus iT_{reg} cells in immune suppression is unclear, but both are important in maintaining immune homeostasis. The feasibility of generating iT_{reg} cells from progenitor cells enables greater yields. Furthermore, iT_{reg} cells may be the only option for patients who are unable to generate functional and mature nT_{reg} cells. Therefore, iT_{reg} cells have a great advantage over nT_{reg} cells as therapeutic regulatory T cells.

Another difficulty in employing regulatory T cells for immunosuppressive therapy is obtaining them in high purity. Studies have identified several cell surface markers found on regulatory T cells, but often these markers are not exclusive to regulatory T cells. For instance, though $CD4^+CD25^+$ T cells are enriched in regulatory T cells, CD25 is also found on activated T cells (Sakaguchi, 2004). At present, the only definitive marker that distinguishes regulatory T cells in mice is the transcription factor Foxp3. In humans, the problem becomes even more complicated by the fact that activated

T cells also upregulate FOXP3 expression (Pillai et al., 2007). Developing ways to increase the purity of regulatory T cells not only improves the ease and efficacy of treatment (i.e. smaller number of cells required to achieve the same immunosuppressive effect), but also decreases the possible risks of introducing non-suppressive T cells to the recipient.

A concern closely associated with purity in using regulatory T cells for therapy is their stability and plasticity. Natural regulatory T cells have been reported to lose their expression of Foxp3 upon repeated rounds of activation *in vitro*, which can render efforts at expansion pointless (Hoffmann et al., 2009). Likewise, iT_{reg} cells generated *in vitro* have been found to lose their expression of *Foxp3* over time. Not only are iT_{reg} cells prone to losing Foxp3 expression (Floess et al., 2007; Wieczorek et al., 2009), but their plasticity allows them to differentiate into effector T cells such as T_H17 cells (L. Zhou, Chong, & Littman, 2009; X. Zhou, Bailey-Bucktrout, Jeker, & Bluestone, 2009). Thus unstable iT_{reg} cells infused to a transplant recipient not only have the potential to lose their regulatory function, but also to aggravate the rejection response. The unstable iT_{reg} cells may participate in the aggressive immune response against the transplanted tissue, ultimately accelerating the

destruction of the graft.

Thus, the issue of maintaining Foxp3 expression and thereby regulatory characteristics of *in vitro* generated iT_{reg} cells must be addressed before they can be safely implemented in clinical settings. This issue of safety is precisely what limits their use at present to merely a subsidiary treatment to conventional immunosuppressive drugs. Even with such limited usage, results from clinical trials have demonstrated the efficacy of iT_{reg} cells as immune suppressors in transplant recipients, greatly increasing the chances of successful engraftment (Kang, Tang, & Bluestone, 2007; Sagoo, Lombardi, & Lechler, 2008; Waldmann, Adams, Fairchild, & Cobbold, 2008; Walsh, Taylor, & Turka, 2004; Wood & Sakaguchi, 2003). Experiments in mouse models have shown regulatory T cells capable of repressing both acute (Bushell & Wood, 2007) and chronic (Nadig et al., 2010; Warnecke, Bushell, Nadig, & Wood, 2007) allogenic transplant rejection.

The powerful immune suppressive ability of regulatory T cells has excited interest with their potential for use in clinical settings since their identification in 1995 (Sakaguchi et al., 1995). Without a reliable means to prevent the loss of Foxp3, however, the dangers

are far too great to consider *in vitro* generated iT_{reg} cells as a primary immunosuppressive therapy.

D. CCAAT/enhancer-binding protein beta (C/EBPβ) as an inducer and stabilizer of Foxp3

CCAAT/enhancer-binding protein beta (C/EBPβ) is a bZIP transcription factor with DNA binding ability as homodimers (Stein, Cogswell, & Baldwin, 1993). Studies have identified its importance in the immune system via regulation of genes encoding cytokines such as IL-6, IL-4, IL-5 and TNF-α (Akira et al., 1990; Delphin & Stavnezer, 1995; Li-Weber, Giaisi, & Krammer, 2001; Wedel, Sulski, & Ziegler-Heitbrock, 1996), and by enabling macrophages to retain normal functions (Screpanti et al., 1995; Zhang et al., 1996).

A recent study has identified a function of C/EBPβ in regulatory T cells (Lee, 2014). While the presence of inflammatory cytokines such as IL-4 and IFN-γ normally inhibit the expression of Foxp3 in regulatory T cells (Bettelli et al., 2006; Dardalhon et al., 2008), the presence of C/EBPβ overrides their effects. Thus, the expression of C/EBPβ in iT_{reg} enables both the attainment and maintenance of regulatory function with sustained Foxp3 expression (Lee, 2014).

Therefore, the expression of C/EBPβ may be able to increase the stability of iT_{reg} at the site of transplant, an inflammatory environment where the cytokines IL-4 and IFN-γ are found in abundance.

Based on results from the aforementioned study demonstrating the ability of C/EBPβ to induce Foxp3 in inflammatory environments (Lee, 2014), the present research investigates the efficacy of iT_{reg} cells expressing C/EBPβ in a solid tissue transplantation setting. To this end, iT_{reg} cells expressing C/EBPβ have been generated *in vitro* and have been transferred into mice receiving allogenic skin grafts. Through macroscopic observation and analysis of the graft post-transplantation, the efficacy of iT_{reg} cells expressing C/EBPβ in suppressing the immune response against allogenic graft has been evaluated.

Methods

Mice. All mice were bred and maintained in specific pathogen-free barrier facilities at Seoul National University and were used according to protocols approved by the Institutional Animal Care and Use Committees (IACUC) of Seoul National University. C57BL/6 ($H-2^b$) and BALB/c ($H-2^d$) mice were purchased from The Jackson Laboratory. $Foxp3^{eGFP}$ reporter mice were kindly provided by T. A. Chatila (University of California at Los Angeles).

DNA constructs. Full-length mouse *C/ebpb* gene was amplified from genomic DNA. PCR products were sequenced and inserted into the EcoRI restriction site of the MSCV-IRES-hNGFR (MIN) retroviral vector. MIN vector was kindly provided by U. N. Pear (University of Pensylvania).

***In vitro* iT_{reg} cell differentiation.** All T cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 10 μg/ml streptomycin, and 55 μM 2-mercaptoethanol. iT_{reg} cells expressing C/EBPβ were generated *in vitro* from a starting population of highly purified naïve $CD4^+$ T cell progenitors ($CD4^+CD25^-CD44^{low}$). The purified progenitors were first stimulated with plate-bound anti-CD3 (3 μg/ml) and soluble anti-

CD28 (1 μg/ml), in the presence of soluble anti-IFN-γ (1 μg/ml), anti-IL-4 (1 μg/ml), and anti-TGF-β (10 ng/ml) for 24 hours. The activated cells were transfected with retroviruses by spin infection, after which they were allowed to rest overnight in the presence of soluble anti-IFN-γ, anti-IL-4, and anti-TGF-β. The transfected cells were stimulated with plate-bound anti-CD3 and anti-CD28 once more with the addition of the cytokines TGF-β (5 ng/ml) and IL-2 (10 U/ml) for 48 hours to induce iT_{reg} differentiation.

Retroviral transduction. Phoenix packaging cells were calcium phosphate trasfected with retroviral expression vectors 48 hours prior to retroviral supernatant harvest. Activated T cells were spin-infected for 1.5 hours with retroviral supernatant containing 8 μg/ml polybrene. Viral supernatants were then replaced with T cell culture medium containing appropriate antibodies and cytokines.

Intracellular staining. Cultured T cells were stained with anti-CD4-PE-cy7, fixed and permeabilized with cytofix/permeabilization solution (BD Pharmingen), and stained with anti-Foxp3-APC mAb. Stained cells were analyzed by FACS Canto II (BD Biosciences).

Cell purification. Peripheral $CD4^+$ and $CD8^+$ T cells were obtained from spleen and mesenteric lymph nodes of 8- to 12-week-old mice. Naïve $CD4^+$ T cell progenitors ($CD4^+CD25^-CD44^{low}$) from

Foxp3^{eGFP} reporter mice, and naïve CD4^{+} T cells (CD4^{+}CD45RB^{high}) and CD8^{+} T cells from wild-type C57BL/6 mice were purified using FACS Aria II (BD). Differentiated T cells were stained with anti-CD271-PE Ab (BD Pharmingen) and sorted for GFP and PE double-positive cells to obtain cells that have been transduced and have successfully differentiated into iT_{reg} cells.

Adoptive transfer. A 1:1 ratio mixture of naïve CD4^{+} T cells (CD4^{+}CD45RB^{high}) and CD8^{+} T cells and purified iT_{reg} cells were adoptively transferred to recipient mice by intravenous injection, with or without the addition of $1x10^{5}$ retrovirally transduced iT_{reg} cells.

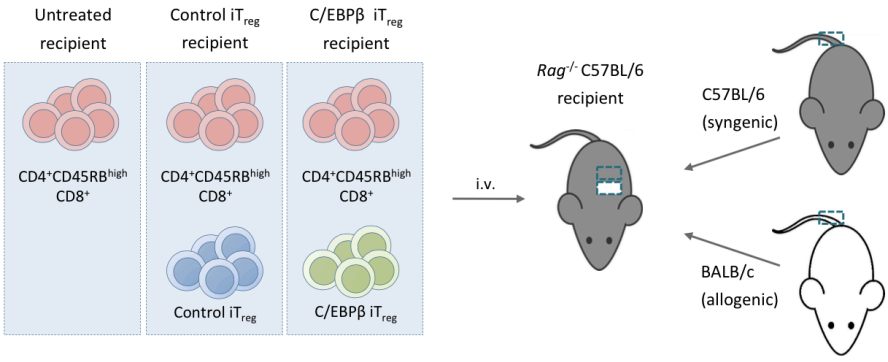
Skin transplantation. One day after adoptive transfer, each of the *Rag*$^{-/-}$ C57BL/6 recipient mice were grafted with full thickness skin obtained from the tails of syngenic and allogenic donors. The graft sites were dressed and bandaged for one week. After one week, the dressing was removed and the graft sites were observed daily for macroscopic signs of inflammation. Observation continued until complete rejection of the allogenic graft, defined as loss of greater than 90% of the original graft.

Results

A. Allogenic skin graft transplantation as an *in vivo* mouse model for evaluating the immunosuppressive effect of iT_{reg} cells expressing C/EBPβ

To study the immunosuppressive effect of generated iT_{reg} cells *in vivo*, an allogenic skin transplantation model was designed (Figure 1). Immunodeficient *Rag* knockout mice ($Rag^{-/-}$) were used as recipients to allow better control of the naïve $CD4^+$ ($CD4^+CD45RB^{high}$) and $CD8^+$ T cells to regulatory T cell ratio. By consulting previous studies (Schliesser et al., 2013), a 1:1 ratio mixture of naïve $CD4^+$ T cells and $CD8^+$ T cells was adoptively transferred to initiate an allogenic transplant rejection response. These cells were transferred with or without the addition of iT_{reg} cells generated *in vitro* to test their efficacy. Cultured iT_{reg} cells had been retrovirally transduced with either control expression vector (abbreviated control iT_{reg}) or vector encoding C/EBPβ (abbreviated C/EBPβ iT_{reg}). Reconstituted mice received both syngenic and allogenic skin grafts the next day. The efficacy of iT_{reg} cells was evaluated by macroscopic observation of inflammation at graft sites.

Figure 1. Experimental scheme of adoptive transfer and allogenic skin transplantation in mice. *Rag*$^{-/-}$ C57BL/6 mice were adoptively transferred with 1x10^5 CD4$^+$CD45RB^{high} and 1x10^5 CD8$^+$ T cells, with or without the addition of 1x10^5 iT$_{reg}$ cells. Recipient mice were grafted with BALB/c (allogenic) and wild-type C57BL/6 (syngenic) tail skin grafts 1 day later.


Untreated recipient
Control iTreg recipient
C/EBPβ iTreg recipient
CD4+CD45RBhigh CD8+
CD4+CD45RBhigh CD8+
CD4+CD45RBhigh CD8+
Control iTreg
C/EBPβ iTreg
i.v.
Rag-/- C57BL/6 recipient
C57BL/6 (syngenic)
BALB/c (allogenic)

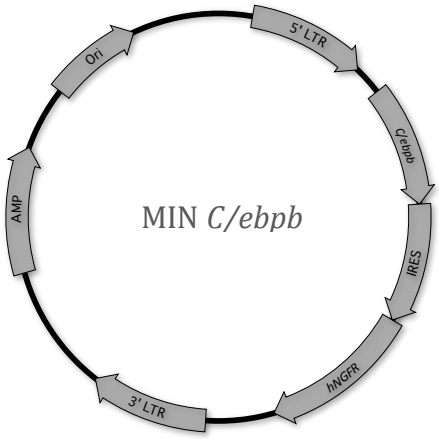
B. Generation of retroviral DNA constructs for ectopic C/EBPβ expression

First, to generate highly purified populations of iT_{reg} cells expressing C/EBPβ for *in vivo* study, retroviral vectors that allow easy detection of virally transduced cells were constructed. To this end, the mouse *C/ebpb* gene was cloned into the multiple cloning site of the MSCV-IRES-hNGFR (MIN) retroviral vector (Figure 2a). The MIN vector has an IRES-hNGFR sequence following its multiple cloning site. This feature enables transduced cells to express a truncated form of the human nerve growth factor receptor (NGFR) molecule, which can be cell surface stained and identified. The starting population of naïve $CD4^+$ T cells was obtained from $Foxp3^{eGFP}$-reporter mice, in which Foxp3 expressing cells are also GFP^+. With this donor mice and viral vector combination, virally transduced iT_{reg} cells with Foxp3 expression could be purified (Figure 2b).

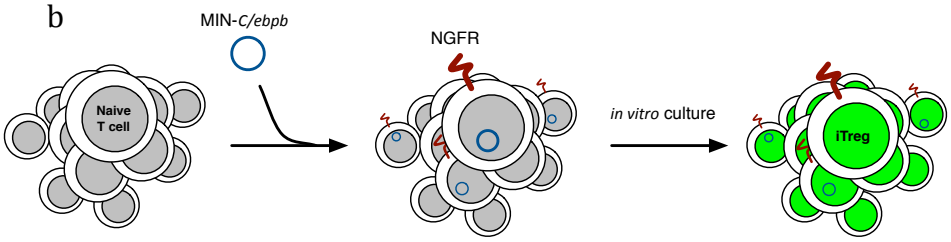
The protein expression of C/EBPβ in virally transduced cells was confirmed by western blotting (data not shown).

Figure 2. Strategy for obtaining retrovirally transduced iT_{reg} cells expressing C/EBPβ. (a) Map of MSCV-IRES-hNGFR (MIN) retroviral expression vector constructed for the expression of mouse *C/ebpb* gene. (b) Cells double-positive for hNGFR and GFP were purified to obtain retrovirally transduced $Foxp3^+$ iT_{reg} cells.

a



b



From **Foxp3 - eGFP** Knock-in mice

C. Retroviral transduction and *in vitro* differentiation of iT_{reg} cells

To verify the differentiation of iT_{reg} cells *in vitro*, a starting population of naïve $CD4^+$ T cells ($CD4^+CD25^-CD44^{low}$) was obtained from mice, retrovirally transduced, and cultured in iT_{reg} cell polarizing conditions. The protocol for iT_{reg} cell differentiation has largely followed that outlined in the preceding study (Lee, 2014), but the recovery time after viral transduction was reduced by half to ensure maximum survival of cells. Intracellular staining of cultured T cells confirmed Foxp3 expression after 2 days of culture in iT_{reg} cell polarizing conditions (Figure 3). In some instances, the retrovirally-transduced expression of C/EBPβ increased the proportion of cells expressing Foxp3.

D. Immunosuppressive effect of iT_{reg} cells expressing C/EBPβ in allogenic skin transplantation mouse model

To verify the effectiveness of the previously described allogenic skin graft transplantation model, the dynamics of the rejection response was studied. Adoptive transfer of a 1:1 ratio mixture of naïve $CD4^+$ T cells ($CD4^+CD45RB^{high}$) and $CD8^+$ T cells to *Rag*$^{-/-}$ recipient mice induced shrinkage, redness, scabbing, and eventually

Figure 3. *In vitro* differentiation of retrovirally transduced iT_{reg} cells. Naïve $CD4^+$ T cells ($CD4^+CD25^-CD44^{low}$) were obtained from wild-type C57BL/6 mice, retrovirally transduced with either control MIN vector or vector encoding C/EBPβ, and cultured in iT_{reg} cell polarizing conditions for 2 days. Foxp3 expression in transduced cells was confirmed by intracellular staining.

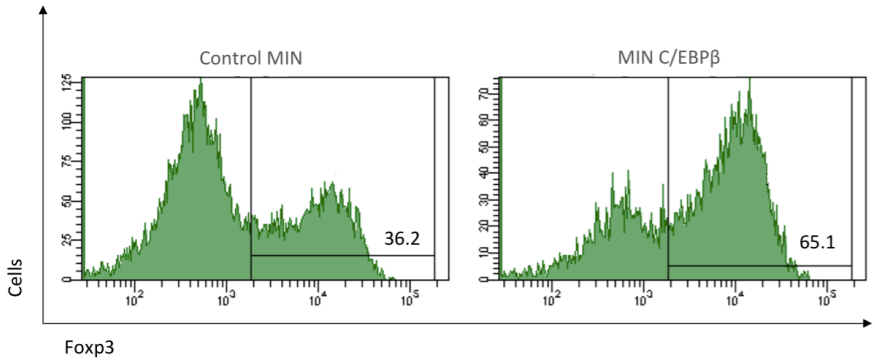
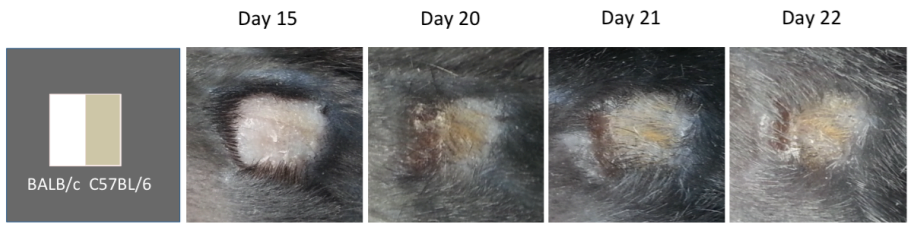

Control MIN
MIN C/EBPβ
36.2
65.1
Cells
Foxp3

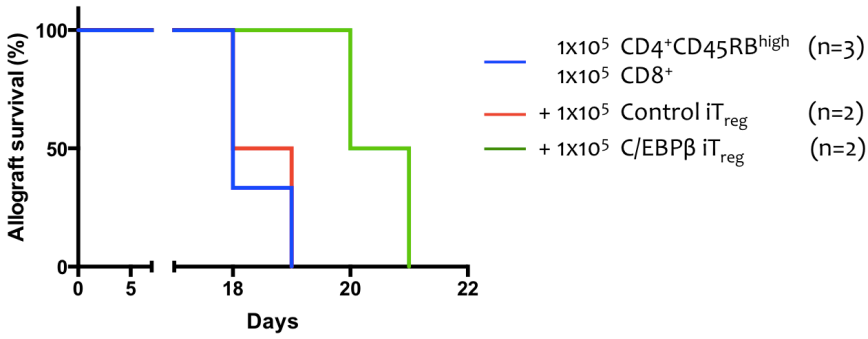
Figure 4. Allogenic skin graft rejection in *Rag*$^{-/-}$ mice adoptively transferred with T cells. *Rag*$^{-/-}$ C57BL/6 mice were adoptively transferred with 1x10^5 CD4$^+$CD45RB^{high} and 1x10^5 CD8$^+$ T cells. Recipient mice were grafted with, from left to right, BALB/c (allogenic) and wild-type C57BL/6 (syngenic) tail skin grafts 1 day later. Macroscopic observation showed that the allogenic graft is lost by day 20.


Day 15
Day 20
Day 21
Day 22
BALB/c C57BL/6

complete rejection and loss of allogenic grafts within 20 days (Figure 4). In comparison, the control syngenic grafts survived indefinitely on the same recipient mice, and showed signs of successful engraftment such as wound healing and hair growth.

The efficacy of *in vitro* differentiated iT_{reg} cells expressing C/EBPβ was tested in the established *Rag*$^{-/-}$ allogenic skin transplantation model. Differentiated iT_{reg} cells, transduced with either control MIN vector (abbreviated control iT_{reg}) or vector encoding C/EBPβ (abbreviated C/EBPβ iT_{reg}) were adoptively transferred with naïve $CD4^+$ T cells and $CD8^+$ T cells at 2:1 ratio. Macroscopic observation of grafted skin showed that untreated recipient adoptively transferred with only naïve $CD4^+$ T cells and $CD8^+$ T cells completely rejected the allogenic skin graft in 18.3 days, as was demonstrated in preliminary trials. Mice receiving control iT_{reg} cells or C/EBPβ iT_{reg} cells rejected the allogenic skin graft in 18.5 and 20.5 days, respectively, on average (Figure 5). All control syngenic grafts survived indefinitely on the same recipient mice.

Figure 5. Survival of allogenic skin grafts on adoptively transferred *Rag*$^{-/-}$ recipient mice. *Rag*$^{-/-}$ C57BL/6 mice were adoptively transferred with 1x10^5 CD4$^+$CD45RB^{high} and 1x10^5 CD8$^+$ T cells with or without 1x10^5 indicated populations of iT$_{reg}$ cells. (n=3 for untreated and n=2 control iT$_{reg}$ and C/EBPβ iT$_{reg}$)


Allograft survival (%)
100
50
0
0
5
18
20
22
Days
1x10⁵ CD4⁺CD45RB^high (n=3)
1x10⁵ CD8⁺
+ 1x10⁵ Control iT_reg (n=2)
+ 1x10⁵ C/EBPβ iT_reg (n=2)

Discussion

The founding study of the present paper (Lee, 2014) demonstrated the importance of C/EBPβ as an inducer of Foxp3 and therefore iT_{reg} cells in inflammatory environments. However, this founding study was performed largely *in vitro* with *in vivo* experiments designed to compare the generation of iT_{reg} cells depending on the expression of C/EBPβ. The present study examined whether these findings hold significance in physiological contexts by employing the allogenic skin transplantation model in mice. This model is an excellent example of T cell-mediated inflammation, with the transplanted graft acting as a source of donor APCs and alloantigens. These alloantigens stimulate the release of inflammatory cytokines that inhibit the induction of iT_{reg} cells. The expression of C/EBPβ allows the generation and maintenance of iT_{reg} cells where they are needed the most.

The present study is still in its early stages and experiments thus far have focused on developing a protocol for culturing large volumes of retrovirally-transduced iT_{reg} cells and testing their immunosuppressive effect in mice receiving skin transplants. Graft acceptance and tolerance between two distinct strains of

laboratory-bred mice was examined to determine the suppressive function of control iT_{reg} cells and iT_{reg} cells expressing C/EBPβ. This mouse model can be classified as allogenic transplantation, which is comparable to transplantation between non-identical donors and recipients in clinical settings.

By using the MIN retroviral expression vector and $Foxp3^{eGFP}$-reporter mice as naïve $CD4^+$ T cell donors, selecting for hNGFR and GFP double-positive cells enabled collection of highly purified populations of iT_{reg} cells. These measures were crucial to ensure high purity, as intracellular staining analysis showed that only about 35~65% of transduced cells expressed Foxp3 despite iT_{reg} cell differentiation conditions, and therefore could be called true iT_{reg} cells. The $Foxp3^-$ cells among differentiated cells may introduce other complications as they may mediate rather than suppress immune response against the transplanted tissue. Thus, these measures were taken due to the lack of definitive cell surface markers for iT_{reg} cells, and may not be necessary if other markers are identified.

Immunodeficient *Rag*$^{-/-}$ mice reconstituted with a 1:1 ratio mixture of naïve $CD4^+$ T cells ($CD4^+CD45RB^{high}$) and $CD8^+$ T cells

were used as recipients to allow control of the naïve $CD4^+$ T cell and $CD8^+$ T cell to regulatory T cell ratio. Previous studies have implicated that the ratio must be greater than 2:1 for regulatory T cells to have an effect. Such high ratios of regulatory T cells may be required due to their slower homing to inflamed sites compared to effector T cells. Effector T cells are known to quickly infiltrate the graft, and the iT_{reg} cells enter the site later on (Fan et al., 2010). If the effector T cells that reach the graft site first manage to mount an acute response, the graft may be damaged beyond repair and lost.

In the aforementioned *Rag*$^{-/-}$ allogenic skin transplantation model, adoptive transfer of naïve $CD4^+$ T cells and $CD8^+$ T cells with either control iT_{reg} cells or C/EBPβ iT_{reg} cells at 2:1 ratio showed possibility of slight delay in allogenic graft rejection (18.5 and 20.5 days, respectively, compared to 18.3 days in untreated control recipients). Reproducibility in future trials will enable evaluation of whether C/EBPβ enables iT_{reg} cells to maintain their regulatory phenotype and function in inflammatory environments.

Although such preliminary data may make the immunosuppressive effect of iT_{reg} cells appear to fall short of expectations, even limited success is enough to make the case for

continued development of therapeutic iT_{reg} cells. The skin is a highly sensitive organ to transplant rejection, as the transplanted graft carries many donor APC that can initiate a severe acute rejection response. Therefore, even a delay of a few days may be indicative of significant immune suppression by the transferred iT_{reg} cells. Also, the allogenic donor and recipient mice that have been chosen for study have completely mismatched MHC, molecules which enable immune cells to differentiate self from non-self. The genetic disparity is greater in this case than most clinical cases of transplantation, where every effort is made to obtain matching donors and recipients. In addition, it is unlikely that cultured iT_{reg} cells will be used as stand-alone therapy any time soon and is most likely to be coupled with other known immunosuppressive drugs to act synergistically.

Based on these findings and considerations, future work of this study will focus on examining the reproducibility of the immunosuppressive effect of iT_{reg} cells expressing C/EBPβ. Variables such as the naïve $CD4^+$ T cell and $CD8^+$ T cell to iT_{reg} cell ratio, allospecificity of iT_{reg} cells, and degree of MHC mismatch should be explored to confirm the immunosuppressive function of C/EBPβ iT_{reg} cells. In particular, using allospecific iT_{reg} cells may make the

effect of C/EBPβ overexpression more evident by increasing the overall immunosuppressive effect of cultured iT_{reg} cells. Previous studies have shown that T_{reg} cells specific for the alloantigen are more effective at inducing tolerance towards the grafted tissue than polyclonal T_{reg} cells (Sagoo et al., 2011).

A more comprehensive analysis of the graft recipients will also be needed to evaluate the stability and function of C/EBPβ iT_{reg} cells *in vivo* and their effect on transplanted tissue. The spleen and the draining lymph nodes of the recipient mice will be harvested and analyzed. These secondary lymphoid organs will be analyzed for the presence of the transferred iT_{reg} cells, in particular for regulatory T cell markers and for the expression of Foxp3 to evaluate their stability. The effector T cells present in these organs will also be examined to determine the severity of inflammation.

The grafted tail skin will also be harvested and sections will be analyzed with hematoxylin and eosin stain. The stain will visualize the distribution of leucocyte infiltration to the area and enable detailed assessment of the immune responses found at the site of the graft. For instance, the stain will differentiate between lessened inflammation due to absence of leucocyte homing versus immune

suppression of leucocytes at the graft site.

The greatest roadblock to introducing *in vitro* expanded regulatory T cells into the patient has not been the lack of evidence of their immunosuppressive effect but the uncertainty of their safety. Due to their tendency to lose FoxP3 expression during repeated rounds of activation *in vitro* (Hoffmann et al., 2009) and their ability to revert to alternative effector lineages such as T_H17 (L. Zhou et al., 2009; X. Zhou et al., 2009), the risks associated with regulatory T cells were far too great to push for development of therapeutic applications.

Although results acquired from mouse model systems require extensive rounds of clinical trials before they can be considered for therapy, the findings of this study may contribute to the development of iT_{reg} cell therapy. In particular, the protocol for mass culturing highly stable iT_{reg} cells expressing C/EBPβ for therapy is applicable not only for skin graft transplantation but numerous other clinical conditions that will benefit from suppression of excessive immune response. These may include other types of solid organ transplantations as well as autoimmune diseases such as rheumatoid arthritis, diabetes mellitus type 1, Crohn's

disease, eczema, and other chronic inflammations. With experimental evidence of the possibility of generating iT_{reg} cells with stable Foxp3 expression, regulatory T cell therapy will no longer be merely an idea that works well only in theory.

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국문초록

동종이형 피부 이식 모델에서 C/EBPβ를 발현하는 유도 조절 T세포의 면역 반응 억제 효과

유도 조절 T 세포는 생체외에서 다량 분화시킬 수 있고 항원 특이적인 면역 억제 기능을 가지고 있어 각종 염증 질환에 대한 차세대 치료제로 떠오르고 있다. 그러나 유도 조절 T 세포는 핵심 조절자 전사 인자인 Foxp3 의 발현이 불안정하고, Foxp3 의 발현을 잃게 되면 면역 반응 억제 기능을 잃을 뿐만 아니라 염증 반응을 증가시키는 T_H17 으로 분화할 수 있는 가소성을 가지고 있어 아직까지는 치료제로 도입되지 못하고 있다.

이 연구는 전사 인자 C/EBPβ 가 IL-4 와 IFN-γ 와 같은 염증성 사이토카인이 있는 환경에서도 유도 조절 T 세포의 Foxp3 발현을 안정적으로 유지해 준다는 선행연구의 결과를 바탕으로, 생체내에서 C/EBPβ 를 발현하는 유도 조절 T 세포의 면역 반응 억제 효과와 안정성을 평가할 수 있는 시스템을 수립하였다. 이를 위해 T 세포에 의한 염증 반응에 대한 면역 반응 억제 효과를 쉽게 관찰할 수 있는 동종이형 피부 이식 모델에 생체외에서 분화한 유도 조절 T 세포를 높은 순도로 얻어 넣어 줄 수 있는 실험 방법을 정립하였다.

이를 위해 $Foxp3^{eGFP}$ 마우스에서 얻은 세포를 MIN 레트로바이러스 벡터로 형질전환을 시켜서 바이러스 벡터를 가지며 동시에 Foxp3 를 발현하는 유도 조절 T 세포를 높은 순도로로 얻었다. 면역결핍 *Rag* 유전자제거 마우스에 $CD4^{+}CD45RB^{high}$ 와 $CD8^{+}$ T 세포를 입양전달한 모델로 실험을 진행한 결과, 대조군 마우스에 비해 C/EBPβ 를 발현하는 유도 조절 T 세포를 추가로 받은 마우스는 이식한 동종이형 피부에 대한 이식 거부 반응이 다소 늦추어지는 것을 실험을 통하여 확인하였다.

따라서 이 연구의 결과는 C/EBPβ 를 발현하는 유도 조절 T 세포가 동종이형 피부 이식 거부 반응을 늦출 수 있다는 가능성을 보여주어 치료제로서 개발 가능성을 확인하였다. 또한 이 연구에서 수립한 생체내 모델은 미래에 안전하고 효과적인 유도 조절 T 세포 치료법을 개발하는데 기여할 것이다. 동종이형 이식 거부 반응에 대한 관찰과 분석을 통하여 향후에도 C/EBPβ 를 발현하는 유도 조절 T 세포의 치료제로서 개발 가능성이 탐구될 것이다.

주요어: 조절 T 세포, 동종이형, 피부 이식

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