Antigen-primed CD4+CD25+ Regulatory T Cells Prevent Skin Graft Rejection: *in vitro* and *in vivo* studies

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抗原刺激 CD4+CD25+ 調節性 T 細胞による皮膚拒絶反応の阻害：*in vitro* と *in vivo* における研究

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ABSTRACT

In the present studies, we examined the role of regulatory T cells in developing strategies to achieve skin graft-specific tolerance and explored the immune characteristic of Treg cells and the main mechanisms through which inducing transplantation tolerance. The 5 x 10^4 Treg could inhibit the MLR obviously, and the effect of the Treg to the MLR is dose dependent. The suppression rate of Treg to response T cell from the donor was higher than control group that came from the non-donor, indicating that the suppression of Treg to response T cell was antigen specific. SR of Treg in co-culture was greater than that in separate culture, inferring that CD4+CD25+ Treg cells exerted their suppressive effects on effector T cells through cell to cell contact mechanism and cytokines secretion mechanism. In the group 1 x 10^5 Treg injected, the mean survive time of skin grafts from C57BL/6 mice was obviously longer than the control group. These data suggest that antigen-primed CD4+CD25+Treg are effective therapeutic tool to prevent skin allograft rejection.

Key words : Regulatory T cells; Immune tolerance; Skin transplantation; Allotransplantation; Xenotransplantation

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INTRODUCTION

The mechanisms of immunologic tolerance are divided into 2 categories, central and peripheral. Central tolerance results from intrathymic deletion of T cells with high affinity for physically expressed antigen. The mechanisms that contribute to peripheral T-cell tolerance include deletion, the induction of anergy, and active immunoregulation. Regulatory T cells (Treg) that coexpress CD4 and CD25 has have been proposed to play an active immunoregulation role in the maintenance of peripheral tolerance. Thus $CD4^+CD25^+$ Treg could be a therapeutic tool to induce transplantation tolerance[1,2,3].

Skin is the most antigenic tissue in the body and is consistently refractory to tolerance induction. Immunosuppressant is not suitable for the prolongation of skin graft survival, especially in burn patients; furthermore, skin is the only organ that can be transplanted in different phases and parts. Thus skin transplantation is a very good model for research of Treg[4].

In these studies, we examined the role of regulatory T cells in developing strategies to achieve skin graft-specific tolerance and explored the immune characteristic of Treg cells and the main mechanisms through which inducing transplantation tolerance.

MATERIALS AND METHODS

$CD4^+CD25^+$ T cells were isolated from mouse spleen by means of magnetic cell sorting, The purity of $CD4^+CD25^+$ T cells was determined by flow cytometry and was more than 90%. Skin antigen extraction from C57BL/6 mouse was prepared through cutting, sonication followed by filtration with 0.44 μm membrane. BALB/c Treg was primed with syngeneic T-depleted splenocytes for 72 hours in the presence of C57BL/6 skin antigen extraction.

One-way mixed lymphocyte reactions were performed to explore the mechanisms of donor-specific hyporesponsiveness induced by antigen-primed Treg.In detail, 10^5BALB/c T cells were stimulated by 10^5 irradiated (60Gy) C57BL/6 splenocytes (irradiated splenocytes from Kunming mice as the third-party control) in the presence of different numbers of antigen-primed BALB/c Treg. For antibody blocking assays neutralizing anti–TGF-ß1 monoclonal antibodies, anti–IL-10 mAbs were added at the initiation of the culture at a concentration of 10 μg/mL. For transwell experiments, transwells of pore size 0.4 μm were used, 10^5 freshly isolated $CD4^+CD25^-$ cells from BALB/c mice were stimulated with10^5 irradiated (60Gy) C57BL/6 mice splenocytes in the lower wells and different numbers of antigen-primed BALB/c Treg were added to the upper wells. MTT technique was used to monitor the T-cell proliferation in cultures above. Stimulating Index(SI)=($OD_{experiments}$ – $OD_{negative\ control}$) x 100%/ ($OD_{positive\ control}$ – $OD_{negative\ control}$) ; Suppression Rate(SR)= ($OD_{positive\ control}$ – $OD_{experiments}$) x 100%/ ($OD_{positive\ control}$ – $OD_{negative\ control}$).

The following day after injection of 1 x 10^5 antigen-primed Treg via tail vena, BALB/c mice received full-thickness skin grafts from C57BL/6 mice donors with the counter-side of the same mouse received skin grafts from Kunming mice as the third-party. All grafts were evaluated daily for discoloration, adherence, pliability, necrosis, shrinkage, and hair growth. Rejection was noted when the graft exhibited 90 percent necrosis or when it sloughed off its bed.

RESULTS

It was found that the 5 x 10^4 Treg could inhibit the MLR obviously, and the effect of the Treg to the MLR is dose dependent. (as Tab 1. showed)

Tab 1. The role of Treg number on immune response intensity in model of MLR.

Treg Number	SR	SI
Control	0.00%	100.0%
5 x 10^5	81.5%	18.5%
2 x 10^5	79.6%	20.4%
1 x 10^5	79.6%	20.4%
5 x 10^4	77.1%	22.9%
1 x 10^4	41.4%	58.6%

The suppression rate of Treg to response T cell from the donor was higher than control group that came from the non-donor, indicating that the suppression of Treg to response T cell was antigen specific (as Tab 2. showed).

Tab 2. The antigen-specificity of Treg acting on T effector cells

Group	SR	SI
I (responsor:BALB/C; stimulator:C57BL/6)	79.6% a	20.4% c
II (responsor:BALB/C; stimulator:Kunming)	45.8% b	54.2% d

X^2 $P_{a/b} < 0.01$ $P_{c/d} < 0.01$
#: The number of antigen-primed Treg is 10^5

SR of Treg in co-culture was greater than that in separate culture inferring that CD4+CD25+ Treg cells exerted their suppressive effects on effector T cells through cell to cell contact mechanism and cytokines secretion mechanism, but the former was more intensive (as Tab 3. showed).

Tab 3. The suppressive mechanism of Treg.

groups	SR	SI
control	0.00%	100.0%
Co-culture (No Transwell)	74.5% **	25.5% **
Co-culture + Anti-TGF-ß1	72.8% **	27.2% **
Co-culture + Anti-IL-10	61.8% **	39.2% **
Separate culture (using Transwell)	31.5% *	68.5% *
Separate culture + Anti-TGF-ß1	19.6%	80.4%
Separate culture + Anti-IL-10	4.30%	95.7%

**: $P < 0.01$, *: $P < 0.05$ *VS* control

In the control group no cells injected, the mean survive time of grafts was 8.5 ± 1.0 (n=11) days with no skin graft was viable beyond 10 days. In the group 1 x 10^5 Treg injected, the mean survive time of skin grafts from C57BL/6 mice was 17.0 ± 2.1days (n=14) whereas that of the third-party grafts was 10.2 ± 1.5days (as Fig 1. showed).

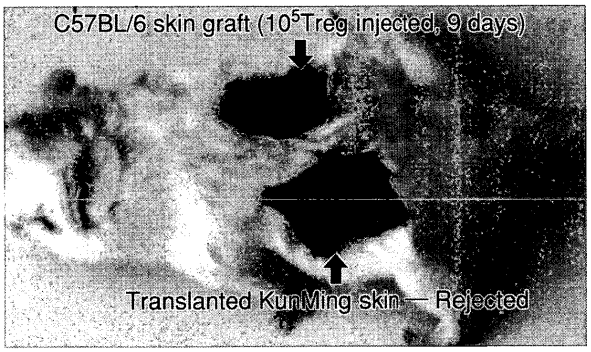

Fig 1. A gross photograph of skin allografts

After the injection intra tail vein, the T cells were separated, purified from the receipt mice and stimulated with ConA, the results revealed that from the 7 days post operation to the injection of the skin graft, the proliferation has the trend to be very strong, but no significance has been found during the stimulating group and control statistically.

DISCUSSION

T cell plays the crucial role in regulating the immune system, and it was acting as the main responding cell in acute graft rejection, inducing the recepient T cell specific tolerance is the most principal and most effective way to solve the acute rejection in organ transplantation. Sakaguchi et al found that nude mice rejected allogeneic skin grafts faster if transferred lymphocytes were first depleted of $CD25^+$ cells[5]. Taylor et al found that $CD4^+CD25^+$ cells were an essential requirement for the ex vivo induction of tolerance to alloantigen via costimulatory blockade[6]. Accumulated evidence show that $CD4^+CD25^+$Treg can maintain the anergy of the allo-reactive T cell; which is an active mechanism of the immunologic homeostasis.

On the other hand, antigen specificity of $CD4^+CD25^+$Treg remains a matter of debate and Treg is a scarce cell subset difficult to be isolate enough for clinical aim. So, antigen-specific expansions of Treg become an alluring goal. Trenado[7] et al performed regulatory T cell expansion ex vivo by stimulation with allogenic APCs, which has the additional effect of producing alloantigen-specific regulatory T cells, and used expanded Treg to control GVHD successfully.

In these studies, we design the experiments to induce the antigen-specific Treg to suppress the response T cell, and to restrain the antigen specific acute rejection. Recipient mice become tolerant to skin allografts whose antigen Treg experienced while retaining partial immunity to third-party alloantigens. These data suggest antigen-primed $CD4^+CD25^+$Treg are effective therapeutic tool to prevent skin allografts rejection.

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