【研究報告】

Correlation of IL-10 with CD4+CD25+ T Regulatory Cells Acting on Effector T Cells

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ABSTRACT

Regulatory T (Treg) cells can suppress effector T cells, but the mechanism underlying its suppression function is not comprehended. Here we examine the inhibiting co-stimulating molecule CTLA4 and cytokines secreted by Treg cells, and explore the immunology mechanism of T regulatory cells acting on effector T cells in co-cultured system (CCS) and separating-cultured system (SCS). Compared with effector T cells, Treg cells expressed higher level CTLA4 and secreted much more IL-10 and TGF-β_1 ($P<0.01$). The inhibitory capacity of Treg cells co-cultured with effector T cells is much stronger than that in separating cultured group ($P<0.01$). Moreover, the inhibiting rate of Treg cells exerting on effector T cells through secreting IL-10 was more powerful than that through secreting TGF- β_1 ($P<0.01$). Both cell-to–cell contact and cytokines secretion mechanisms are involved in CD4+CD25+ Treg cells operating function. However, the former is more important. Interestingly, we for the first time point found that IL-10 make more powerful role than TGF- β_1 in the cytokines secretion mechanism.

Key words: regulatory T cells, effector T cells, cytokine

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INTRODUCTION

In such a short time, regulatory T (Treg) cells with their potent suppressive activity on normal effector T cells function have been the focus in the immunology although the phenotypic characteristics of Treg had not be identified and the cellular and molecules responsible for these suppressive phenomena were never characterized. The discovery of the Treg cells owes to the research on the treatment of autoimmunity diseases and tumor. The modern view of Treg cells begin with the observation that the adoptive transfer of T cells depleted of $CD25^+$ cells can induce multi-organ autoimmunity, while adoptive transfer of a well defined T cells subset from syngeneic healthy donors can prevent the development of autoimmunity in lymphopenic recipients [1-4]. They can inhibit the development of organ-specific autoimmunity by suppressing the auto-reactive T cells. So they were once named suppressor T cells (Ts). But it is not correct, as we now know; Treg cells can regulate the function of immune system by either secreting cytokines such as IL-10 and TGF- β_1 or inhibiting the mature of dendritic cells [5, 6]. Treg cells are one of the important mechanisms of immunologic homeostasis.

It is now identified that Treg cells can suppress effector T cells, but the mechanism underlying its suppression function is not comprehended. There are few researchers continued to keep interesting in it. So we put focus on the immune mechanisms of how Treg cells acting on effector T cells. In this study, we extend our *in vitro* studies of the mechanisms of Treg cells and demonstrate that $CD4^+CD25^+$ cells act through an antigen presenting cells (APC) independent mechanism *in vitro* and its suppressor effector function is antigen specific.

MATERIALS AND METHODS

Animals

8-10-week-old inbred BALB/c ($H\text{-}2^d$) mice, weighing 23-25g, 8-10-week-old C57bl/6 ($H\text{-}2^b$) mice weighing 20-23g. All animals were purchased from the laboratory animal center of the third military medical university. The animals were maintained under a standard condition according to the principle of laboratory animal care and the guide for the care and use of laboratory animals in our institution. The local ethical committee approved the animal studies.

Reagents and apparatus

Anti-Mouse IL-10, Anti-Mouse TGF-β_1, MTT (Sigma, USA), FITC-labeled Anti-Mouse CD4, FITC-labeled Anti-Mouse CD8, FITC-labeled Anti-Mouse CD25 (Pharmingen, USA). PE-labeled Anti-Mouse CTLA-4, PE-labeled Anti-Mouse CD28 (Pharmingen, USA). MiniMagnetic cell separator, (Meltiny, USA), TransWell Millicell-PCF (MilliPore, USA).

Analysis of the characteristics of Treg cells secreting cytokines IL-10, TGF-β_1

$CD4^+CD25^+$Treg cells were prepared as previously described [7]. Briefly BALB/c mice splenic T cells were purified by T cell enrichment column. Then $CD4^+CD25^+$ Treg cells were purified using a magnetic cell sorting system (MACS). The $CD4^+$ T cells were isolated by depletion magnetically labeled non-$CD4^+$ T cells. $CD4^+$ T cells labeled with PE-conjugated anti-CD25 were then magnetically labeled with anti-PE microbeads. The magnetically labeled cells were passed through a column placed in the magnetic field of a MACS separator. The purity of $CD4^+CD25^+$ cells ranged from 85% to 90% by flow cytometric

analysis. $CD4^+CD25^+$ cells ($2x10^5$) were cultured in 96-well plates for 48 hrs at 37°C/5%CO_2 and supernatant were collected after centrifugation and were stored at -20°C for detection. The supernatant of the T cells in the same condition were collected simultaneously as the control. The production of the cytokines was assayed by using ELISA kit.

Analysis of the expression of the costimulatory molecules receptor CTLA4, CD28 on the Treg cells

$CD4^+CD25^+$Treg cells were purified using a magnetic cell sorting (MACS) kit as shown above. The expression of the costimulatory molecules receptor CTLA4, CD28 were assayed on the $CD4_+CD25^+$Treg cells and the effector T cells separatively by using FACS.

Analysis of the mechanisms of Treg cells acting on the effector T cells by cell-cell contact and cytokines secreted.

The mixed lymphocyte reaction (MLR) system consists of the splenic T cells of BALB/c ($T_{BALB/c}$) mice as the responder and the splenic cells of C57bl/6 mice ($T_{C57BL/6}$) as the stimulator. Then Treg cells was added to the system in CCS and Trans Well Millicell-PCF SCS, at the same time, adding or not adding anti-IL-10 or TGF-β_1 to the reacting systems. $T_{BALB/C}$ +$T_{C57BL/6}$ works as a positive control while $T_{BALB/C}$ as a negative control.

Experiment groups were divided as following:

Group 1: (negative control) $T_{BALB/C}$;

Group 2: (positive control group) $T_{BALB/C}$ + $T_{C57BL/6}$;

Group 3: Treg + $T_{BALB/C}$ + $T_{C57BL/6}$; (ccs)

Group 4: Treg + $T_{BALB/C}$ + $T_{C57BL/6}$; (scs)

Group 5: Treg + $T_{BALB/C}$ + $T_{C57BL/6}$ + Anti-IL- 10; (ccs)

Group 6 : Treg + $T_{BALB/C}$ + $T_{C57BL/6}$ + Anti- TGF-β_1; (ccs)

Group 7: Treg + $T_{BALB/C}$ + $T_{C57BL/6}$ + Anti-IL- 10; (scs)

Group 8 : Treg + $T_{BALB/C}$ + $T_{C57BL/6}$ + Anti- TGF-β_1; (scs)

*Treg: $CD4^+CD25^+$Treg cells induced by the complex antigen of C57BL/6 mice skin.

Assay of the inhibition of the Treg cells

Splenic T cells of the C57BL/6 mice and BALB/c mice were purified by T cell enrichment column. 0.5ml $T_{BALB/C}$. $T_{C57BL/6}$ ($1*10^6$/ml) were cultured in 24-well plates according to the experiment groups. Treg cells, Anti-IL-10 (0.5mg) and Anti-TGF-β_1 (1.0mg) were added according to the experiment groups. In the separating-cultured groups, Treg cells were put in the Well-Millicell-PCF before put in the reaction system. After cultured for 48 hrs at 37°C/5%CO_2, the cells were transport to 96-well plates.(Treg cells in the Well-Millicell-PCF were not transported in the separating-cultured groups. Then assay the *OD* in the 595nm according to the MLR.

Analysis of antigen-specific of Treg cells acting on the effector T cells

Experiment design

Treg cells of BALB/c mice were induced by the complex antigen of C57BL/6 mice skin. Then the Treg cells ($1x10^5$) were added to the MLR system which were composed of $T_{BALB/C}$ ($5x10^5$) + $T_{C57BL/6}$($5x10^5$) and $T_{BALB/C}$ ($5x10^5$) + T_{KM} ($5x10^5$) according to the experiment groups. After 96 hrs cultured, the *OD* in the 595nm were assayed according to MTT method. It was designed to divide into following groups:

Group 1: (negative control group) $T_{BALB/C}$;

Group 2: (positive control group I) $T_{BALB/C}$ + $T_{C57BL/6}$;

Group 3: $T_{BALB/C}$ + $T_{C57BL/6}$ + Treg (induced by C57BL/6);

Group 4: positive control group II: $T_{BALB/C}$ + T_{KM};

Group 5: $T_{BALB/C}$ + T_{KM} + Treg (induced by C57BL/6)

Calculation of the results

Simulating Index (SI):

$$SI=\frac{\overline{OD}_{Exp}-\overline{OD}_{negifiveControl}}{\overline{OD}_{positiveControl}+\overline{OD}_{negifiveControl}}\times 100\%$$

Suppression Rate (SR)

$$SR=\frac{\overline{OD}_{PositiveControl}-\overline{OD}_{Exp}}{\overline{OD}_{positiveControl}-\overline{OD}_{negifiveControl}}\times 100\%$$

Statistical analysis

Pooled data was computed as mean ± SEM using the Microsoft Excel 97 statistical program and the SPMR program box programmed by the department of mathematics of the Third Military Medical University. P<0.05 means significant difference; P<0.01 means significant difference.

RESULTS

Contrast analyses of the cytokines secreted by Treg cells and effector T cells

The cytokines in the supernatant of the $CD4^+CD25^+$Treg cells and the effector T cells cultured ex vivo examined by ELISA shows that Treg cells secret more cytokines (IL-10 and TGF-β_1) than effect T cells. IL-10 and TGF-β_1 are 380±36.3 pg/ml and 790±56.8pg/ml in the supernatant of the $CD4^+CD25^+$Treg cells respectively; while only 150±27.3pg/ml and 130±18.6pg/ml of the effector T cells respectively. Both of them have significant difference (Fig. 1).

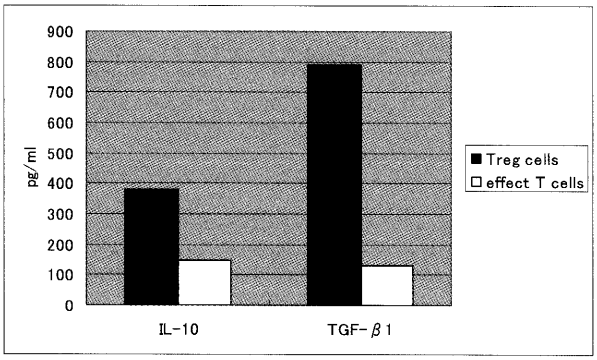

Fig 1. Cytokines secreted by Treg cells and T effector cells. Treg cells secret more cytokines (IL-10 and TGF-β_1) than effect T cells. IL-10 and TGF-β_1 are 380±36.3 pg/ml and 790±56.8pg/ml in the supernatant of the $CD4^+CD25^+$Treg cells respectively; while only 150±27.3pg/ml and 130±18.6pg/ml of the effector T cells respectively. Both of them have significant difference ($p<0.01$).

Contrast analyses of the costimulatory molecules expressed by Treg cells and effector T cells

There is significant difference in the expression of costimulatory molecules CTLA4, CD28 on the $CD4^+CD25^+$Treg cells and the effector T cells. $CD4^+CD25^+$Treg cells express CTLA4 mainly which is about 5 fold than CD28. While there are about 42.03% of the effector T cells express CD28 but they do not express CTLA4. The results are as follows:

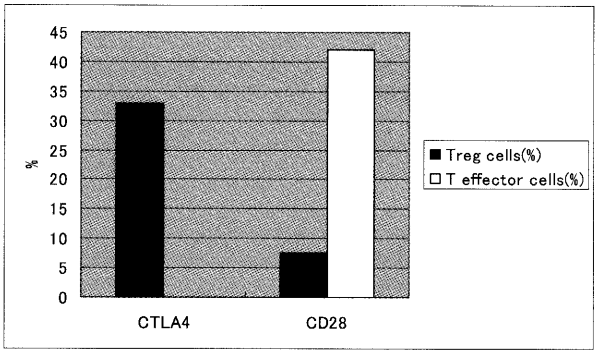

Fig 2. Costimulatory molecules (CM) expressed by Treg cells and effector T cells. $CD4^+CD25^+$Treg cells express CTLA4 mainly which is about 5 fold than CD28. While there are about 42.03% of the effector T cells express CD28 but they do not express CTLA4.

Antigen-specific of Treg cells acting on the effector T cells

There are significant difference in the suppression rate (SR) and stimulating index (SI) between the induced groups and uninduced groups. The result shows that the function of Treg cells is antigen-specific (Fig. 3).

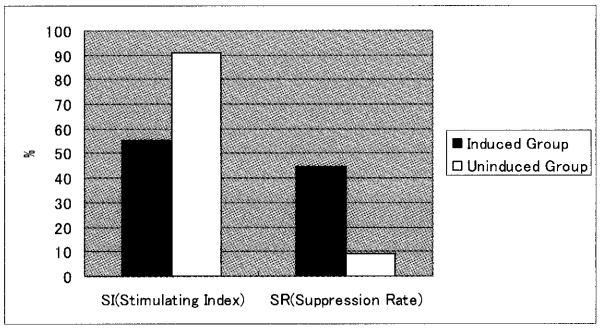

Fig 3. The antigen-specific mechanism of Treg acting on T effector cells. There are significant difference in the suppression rate (SR) and stimulating index (SI) between the induced groups and uninduced groups ($p<0.01$).

Treg cells acting on the effector T cells mainly by cell-to-cell contact ex vivo

According to possible mechanisms that Treg cells acting on the effector T cells, we devise a simple experiment working on it. And the result shows that cell-to-cell contact is the main way that Treg cells acting on the effector T cells. While in the cytokines mechanisms, we show that IL-10 plays a more important role than TGF-β_1. the results are as follows:

DISCUSSION

It is now no doubt that Treg cells exist as a functional subset of T cells [8, 9]. In such a short time, Treg cells have generate so much interest in immunology because of their recognized importance in tolerance, autoimmune disease, transplantation, and so on. Nev-

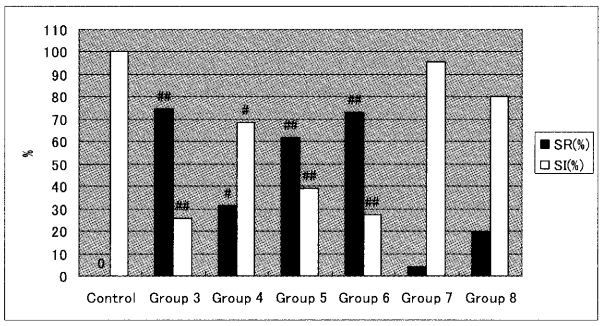

Fig 4. The Mechanism of Treg Acting on effector T cells. Cell-to-cell contact is the main way that Treg cells acting on the effector T cells. While in the cytokines mechanisms, IL-10 plays a more important role than TGF-β_1.
##: $p<0.01$, #: $p<0.05$, *versus* control

ertheless, the mechanisms underlying their ability to suppress immunity remain ill defined and hostly contested. The interest in Treg cells has brought with it several new observations and in some cases, these developments have led to controversies. New studies have supposed that CTLA4 plays a role in the suppression of effect T cells mediated by Treg cells. Takahashi et al [10] demonstrated that anti-CTLA4 antibody (or Fab of CTLA4) can reverse the suppression by $CD4^+CD25^+$Treg cells acting on the effector T cells. So it is supposed that CTLA4 may be the suppressor effector molecule by activating reverse signaling through B7 [11]. Our data show that CTLA4 has different expression on the surface of $CD4^+CD25^+$Treg cells and effector T cells. There are about 32.9% of the $CD4^+CD25^+$Treg cells express CTLA4 while none of the effector T cells express it.

It is now known that Treg cells can secrete two types of suppression cytokine: IL-10 and TGF-β_1(also said can secrete IL-4); and there exist CTLA4 which can transmit suppression signal [12]. There are possible two kinds of manners in which Treg cells regulate immune: one is by cell contact, the other is via cytokine and/or other soluble factor. So it arise two questions on which

persons are quarreling on:

Is the suppressor effector function of $CD4^+CD25^+$Treg cells antigen-specific or not? In which manner does $CD4^+CD25^+$Treg cells play their role in regulating immune reaction?

In order to clarify these questions, we devise a new, simple way by co-culture or cultured separately by TransWell-PCF of Treg cells and the effector T cells. Our data shows that both cell contact dependent manner and cytokine dependent manner play role in the suppressive function of $CD4^+CD25^+$Treg cells. But cell-to-cell contact is central to it. Cell contact manner is much more important than the cytokine dependent way. While in the latter manner, IL-10 plays an important role than TGF-β_1.

As to the problem of whether the suppressor effector function of $CD4^+CD25^+$Treg cells is antigen-specific or not. Treg cells suppression requires T cell receptor (TCR) engagement and cell-to-cell contact with the target cells [13]. It suggests that it is antigen-specific. Oluwole et al also found that adoptive transfer of enriched $CD4^+CD25^+$host thymic T cells combined with in vivo P5 primed syngeneic peripheral T cells restored permanent graft survival. But do not lead to survive of third-party allografts [14]. we can see from table 3 that T reg cells induced by the skin complex of the donor have a high suppression rate than that uninduced (44.7% vs 9.20%) *in vitro* experiment. The results of our *in vivo* experiment also confirm this point (data not shown). Grafts survive significantly longer in the host when the host accepts the Treg cells induced by the donor strain's antigen. In contrast, Treg cells uninduced by the donor strain's antigen results in no significant prolongation of the graft survival. Therefore, we considered that $CD4^+CD25^+$Treg cells regulating suppression is an antigen-specific. The result is according with the assumption of Bluestone et al [15].

Another important question on the mechanisms of the suppression function mediated by Treg cells is whether antigen presenting cells (APS) is involved in or not [16]. Treg cells can suppress the expression of B7 on the APC, inhibit the mature of dendritic cells (DC), so some scholar conjecture that Treg cells act on the effector T cells by inhibiting the DCs or binding to B7 on the DC competing with the effector T cells [11]. An alterative possibility is that the function of Treg cells do not need the help of APCs, because $CD4^+CD25^+$Treg cells activated by TCR can suppress effector T cells directly. Some cellular interactions such as TRANCE/RANK, GITR/GITR ligand are involved in the suppressive effects [17, 18]. And blockade of CD152 also can abrogated suppression [19]. These new discover has proved that there exist the cell-to-cell contact mechanisms indirectly. In our experiment, both of the effector T cells and Treg cells are purified without the attention of APC. Our results show that Treg cells act on the effector T cells directly by cell-to-cell contact independent of APC. So we now confirm that Treg cell can suppress effector T cells directly. Because our experiment is ex vivo, so we don't exclude the manner that APCs mediate, especially *in vivo*.

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