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# Feedback loop of immune regulation by $CD4^+CD25^+$ Treg

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## Abstract

Naturally occurring regulatory T cells (Tregs), residing in $CD4^+CD25^+$ fraction, are important in the maintenance of immune homeostasis. One of the functional characteristics of Tregs is close relationship between suppressive activity and anergy *in vitro*. Meanwhile, many *in vitro* assays have observed Treg proliferation and suppressive activities in different settings, i.e., in the absence and presence of $CD25^-$ responder cells. If the presence of responder cells affect the proliferation of Tregs, comparison between the two settings would be inappropriate. In the present study, we traced proliferation as well as suppressive activities of Tregs in the same setting of coculture in response to varying concentrations of anti-CD3 and anti-CD28. Quantitative analysis using two parameters, precursor frequency and CD25 mean fluorescence intensity, reflecting early and late proliferative responsiveness, respectively, showed that proliferation of Tregs was dependent on the responder cells and proliferating Tregs preserved suppressive activities. Transwell assay and neutralization assay showed that the enhancement of Treg proliferation by the responder cells was mediated through secreted IL-2. Quantitative analysis also showed distinct mode of suppression by Tregs according to the presence or absence of anti-CD28. In the absence of anti-CD28, Tregs suppressed the initial proliferation, whereas in the presence of anti-CD28, Tregs suppressed only the late expansion of the responder cells by lowering CD25 expression. Considering that Tregs cannot produce IL-2 by themselves while they constitutively express CD25 (IL-2R$\alpha$), dependency of Tregs on their target of suppression (responder cells) for proliferation supports the model for feedback loop of immune regulation by Tregs.


***Keywords:*** Regulatory T cell; Anergy; Suppression; Feedback

## Introduction

Spontaneously occurring population of T cells that regulate autoimmune reactions reside in $CD4^+CD25^+$ fraction, known as naturally occurring regulatory T cells (Tregs) (Sakaguchi et al., 1995). They constitute 5–10% of peripheral $CD4^+$ T cells, and are important in the maintenance of immunological self-tolerance in the periphery (Toda and Piccirillo, 2006). They also play important roles in the regulation of transplant rejection, graft versus host disease, infections, tumor immunity, etc. (Sakaguchi, 2004). Direct cell-to-cell contact is critical in the suppressive action of Tregs, although several inhibitory cytokines including IL-10 and TGF-$\beta$ are also implicated (Miyara and Sakaguchi, 2007; Thornton and Shevach, 2000).

One of the cardinal functional characteristics of Tregs is anergy in response to TCR engagement *in vitro* while

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they exert suppressive activities (Takahashi et al., 1998). This anergic state is closely linked with suppressive activity as abrogation of anergic state by TCR stimulation with high-dose IL-2 or CD28 ligation results in concomitant loss of suppressive activity (Oberg et al., 2006). Considering their low frequency in the periphery, it may be reasonable to raise the doubt how Tregs in anergic state can regulate the rapidly expanding effector cells properly in a contact-dependent manner when they are triggered. Furthermore, several *in vivo* studies have demonstrated that Tregs proliferate as well as $CD4^+CD25^-$ cells, suggesting different behavior of Tregs *in vitro* and *in vivo* (Klein et al., 2003; Walker et al., 2003; Yamazaki et al., 2003). To the contrary, it might be also reasonable to guess the *in vitro* assay may not reflect the *in vivo* situation properly. Because, many *in vitro* assays have observed Treg proliferation and suppressive activities in different settings, i.e. in the absence and presence of the responding cells. If the presence of responding cells may affect the proliferation of Tregs, comparison between the two settings would be inappropriate. In addition, there are several reports that human peripheral Tregs could be expanded *in vitro* with their suppressive activities preserved (Earle et al., 2005; Levings et al., 2001). Taken together, at the moment, it remains unclear if Tregs lose or preserve suppressive activities when they are proliferating *in vitro*. So, in the present study, we traced the proliferative response as well as the suppressive activities of Tregs in the same setting of coculture with the responding cells by selective labeling with CFSE.

IL-2 is critical in the development of Tregs in thymus and expansion in the periphery (de la Rosa et al., 2004; Nelson, 2004). Although Tregs constitutively express high level of IL-2Rα, they do not produce IL-2 by themselves, probably because FoxP-3 turn off the expression of effector cytokines, including IL-2 (Levings et al., 2002; Thornton and Shevach, 1998). Accordingly, Tregs cannot expand in an autocrine fashion, and they must depend on exogenous source of IL-2 for their expansion (de la Rosa et al., 2004). In the present study, we also demonstrate that $CD4^+CD25^+$ Tregs can expand in the coculture with the responding $CD4^+CD25^-$ cells, depending on IL-2 secreted by the latter. Here we also demonstrate that the proliferating Tregs in the coculture preserve their suppressive activities. Based on these results, we propose a conceptual model for the feedback loop of immune regulation by Tregs.

## Materials and methods

### Preparation of cells

Male BALB/c mice were purchased from Koatech (Pyungteck, South Korea) and were maintained in SPF condition in the animal facility of the Ewha Womans University School of Medicine (Seoul, South Korea), and were used in the experiments when they were 8–12 weeks old, in accordance with the institutional guideline for animal welfare. $CD4^+CD25^+$ fraction was separated from the spleens for Tregs by immunomagnetic selection using the regulatory T-cell isolation kit from Miltenyi Biotech (Auburn, CA, USA). For intranuclear staining for FoxP3, cells were fixed and permeabilized using the mouse regulatory T-cell staining kit (eBiosciences, San Diego, CA, USA) and were stained with FJK-16s-PE-Cy5. The purity of $CD4^+CD25^+$ cells purified from splenocytes was $95.6+3.1\%$, and $86.7+5.1\%$ of the purified fraction expressed FoxP3 ($n=6$). $CD4^+CD25^-$ fraction was also separated for the responder cells (Teff), while the $CD4^-$ fraction was used for antigen-presenting cells (APCs) after irradiation. Parts of Tregs and $CD25^-$ responder cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen, Carlsbad, CA, USA) as described elsewhere, to trace the proliferation of each fraction separately (Venken et al., 2007).

### In vitro proliferation assay

Ten thousand CFSE-labeled Teffs were cocultured with $10^5$ APCs in the presence or absence of $10^4$ Tregs to investigate if the presence of Tregs inhibits the proliferation of Teffs. Reversely, $10^4$ CFSE-labeled Tregs were cocultured with $10^5$ APCs in the presence or absence of $10^4$ Teffs to investigate if the presence of Teffs influences the proliferation of Tregs. Sometimes, varying numbers of Teffs were added in the cocultures to investigate dose responsiveness. The cells were cultured in DMEM supplemented with 10% FCS (Hyclone, Logan, UT, USA) in round-bottomed 96-well plates by stimulating with various concentrations of anti-CD3e (e-Biosciences) alone or in combination with anti-CD28 (e-Biosciences). To investigate the effects of exogenous IL-2 on Treg or Teff proliferation, varying concentrations of recombinant mouse IL-2 (Chemicon, Temecula, CA, USA) were added to the cultures while the cells were stimulated with a low concentration (20 ng/mL) of anti-CD3.

### Flow cytometry

On the 3rd and 5th day of culture, the cells were harvested for staining with anti-CD25-PE (BD Biosciences, San Jose, CA, USA), anti-CD4-APC-Cy7 (BD Biosciences) and 7-aminoactinomycin D (7-AAD, BD Biosciences). Whole cells were acquired for analysis by using Winlist software (Verity, Topsham, ME, USA). Precursor frequency (Pf) was estimated for the exclusively gated $7\text{-AAD}^-CD4^+$ cells, using the proliferation