

Ex vivo activated OVA specific and non-specific CD4⁺CD25⁺ regulatory T cells exhibit comparable suppression to OVA mediated T cell responses

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Abstract

CD4⁺CD25⁺ regulatory T cells (Tr) are important in maintaining immune tolerance to self-antigen (Ag) and preventing autoimmunity. Reduced number and inadequate function of Tr are observed in chronic autoimmune diseases. Adoptively transferred Tr effectively suppress ongoing autoimmune disease in multiple animal models. Therefore, strategies to modulate Tr have become an attractive approach to control autoimmunity. Activation of Tr is necessary for their optimal immune regulatory function. However, due to the low ratio of Tr to any given antigen (Ag) and the unknown nature of Ag in many autoimmune diseases, specific activation is not practical for potential therapeutic intervention. It has been shown in animal models that once activated, Tr can exhibit immune suppression in a bystander Ag-non-specific fashion, suggesting the effector phase of Tr is Ag independent. To investigate whether the immune suppression by activated bystander Tr is as potent as that of the Ag specific Tr, Tr cells were isolated from BALB/c or ovalbumin (OVA) specific T cell receptor (TCR) transgenic mice (DO11.10) and their immune suppression of an OVA specific T cell response was compared. We found that once activated *ex vivo*, Tr from BALB/c and DO11.10 mice exhibited comparable inhibition on OVA specific T cell responses as determined by T cell proliferation and cytokine production. Furthermore, their immune suppression function was compared in a delayed type hypersensitivity (DTH) model induced by OVA specific T cells. Again, OVA specific and non-specific Tr exhibited similar inhibition of the DTH response. Taken together, the results indicate that *ex vivo* activated Ag-non-specific Tr are as efficient as Ag specific Tr in immune suppression, therefore our study provides additional evidence suggesting the possibility of applying *ex vivo* activated Tr therapy for the control of autoimmunity.

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1. Introduction

Naturally occurring CD4⁺CD25⁺ regulatory T cells (Tr) represent a subpopulation (5–10%) of peripheral CD4⁺ T lymphocytes and are crucial in maintaining immune tolerance to self-antigens [1]. It has been reported that either the number of Tr is reduced or their function is inadequate in subjects with chronic autoimmune diseases [1,2]. Adoptively

transferred Tr effectively suppress ongoing chronic disease in multiple animal models, such as type-1 diabetes [3,4], experimental autoimmune encephalomyelitis (EAE) [5], asthma [6], rheumatoid arthritis (RA) [7], and inflammatory bowel disease [8]. In addition, Tr also play an important role in maintaining organ acceptance in allograft transplantation [9]. All of these results strongly indicate that strategies to increase Tr number and to enhance their function can be an attractive therapeutic approach for restoring immune tolerance and controlling unwanted immune responses.

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Naturally occurring $CD4^+CD25^+$ regulatory T cells are generated in the thymus where they acquire antigen specificity [10]. Mature Tr need to be activated for full immune regulatory function [1]. It has been shown that antigen specific recognition was required for Tr to suppress autoimmune diseases [5], e.g. Tr from a myelin basic protein (MBP) specific TCR transgenic but not from non-transgenic mice achieved complete protection from the development of EAE when adoptively transferred [5]. Other studies demonstrated that once activated, Tr could inhibit the immune response in an antigen non-specific bystander fashion [13,14], e.g. Tr from Hemagglutinin TCR transgenic mice, when pre-activated, suppressed the proliferative response of effector T cells from mice expressing a different transgenic TCR. These observations suggest that antigen specificity is necessary for Tr generation and activation but may not be crucial for the effector phase of Tr.

The effectiveness of Tr in restoring immune tolerance in experimental systems suggests its therapeutic potential to control autoimmunity and other immune mediated inflammatory diseases. Due to the low ratio of Tr to any given Ag and the lack of clarification of the pathogenic autoantigens in many autoimmune diseases, polyclonal activation of Tr becomes an interesting and practical approach. However, it is not clear if the bystander Tr mediated immune suppression is as potent as the one through Ag specific Tr, and if antigen specific and bystander Tr function through similar mechanisms. To address these questions, we compared the effect of Tr isolated from the OVA specific TCR transgenic (DO11.10) and wild-type mice on an OVA specific T cell response *in vitro* and *in vivo*. Our results demonstrated that once activated, OVA non-specific Tr exhibited potent inhibition to OVA specific T cell response both *in vitro* and *in vivo* with equivalent potency to that of OVA specific Tr.

2. Material and methods

2.1. Mice

DO11.10 TCR transgenic mice specific for an ovalbumin (OVA) peptide were backcrossed to BALB/c mice for more than 10 generations. DO11.10 and BALB/c mice were purchased from Charles Rivers Laboratories (Raleigh, NC, USA). Mice were maintained in a pathogen-free animal barrier facility under a protocol approved by the Institutional Animal Care and Use Committee of Centocor.

2.2. Purification of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells

A single cell suspension of splenocytes was prepared from 8- to 10-week-old naive DO11.10 or BALB/c mice. $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were separated using a regulatory T cell isolation kit (Miltenyi Biotec, CA) according to the manufacturer's instructions. The purity of $CD4^+CD25^+$ cells and $CD4^+CD25^-$ was >92% and >95%, respectively, as shown by flow cytometry.

2.3. Cytokine production and T cell proliferation assays

$CD4^+CD25^-$ T cells (T_E) from DO11.10 mice and $CD4^+CD25^+$ T cells (Tr) from DO11.10 or BALB/c mice were plated at 1×10^5 cells/well of each in 200 μ l RPMI-1640 medium with 10% FBS. In the presence of immobilized anti-CD3 (10 μ g/ml, BD Biosciences, CA) and soluble anti-CD28 antibodies (2 μ g/ml, BD Biosciences, CA), the cells were cultured at 37 °C for 3 days. The cell culture supernatant was harvested and the level of cytokines was detected by using murine cytokine kit (LINCO Research, Inc., MO) with a Luminex instrument (Luminex Corporation, Texas). The T cell proliferation was measured on day four by using an ATPLite-M assay (Packard BioScience, CT).

2.4. Cytokine and proliferation inhibition assay

To test Tr suppressive activity, T_E (1×10^5) from DO11.10 mice were incubated with antigen presenting cells (APCs, 1×10^5 irradiated BALB/c mouse spleen cells) and the specific OVA peptide (1 μ g/ml) alone or co-culture with different numbers of activated Tr from naïve DO11.10 or BALB/c mice, which were previously stimulated with immobilized anti-CD3 (10 μ g/ml) and 10 ng/ml of mIL-2 (R&D Systems, MN) in 96-well plates for 3 days. The cell culture supernatant was collected for cytokine analysis on day three. Cell proliferation was measured by ATPLite-M assay on day four.

2.5. Flow cytometric analysis

To identify co-stimulatory molecule expression on T_E after co-cultures with Tr, T_E from DO11.10 mice were labeled with CFSE (Molecular Probes, Eugene, OR) by incubation of the cells with 0.5 μ M CFSE in PBS for 8 min at 37 °C. The cells were then blocked with 50% FBS for 3 min at 37 °C and washed twice with cold PBS. The labeled cells were co-cultured with APCs and activated Tr (at 1:1:1 ratio) in the presence of OVA peptide. On day four, the cells were treated with FcR blocker (BD PharMingen, CA) at 2 μ g/ml on ice for 30 min and then stained with FITC- or PE-conjugated antibodies recognizing CTLA-4 (BD PharMingen), GITR (R&D Systems, MN), or PD-1 (eBioscience, CA). Antibodies were used at 2 μ g/ml and staining was performed in 0.1% BSA/PBS buffer on ice for 30 min. Foxp3 intracellular staining was conducted by using Foxp3 staining kit following the instruction of the manufacture (e-Bioscience, CA). Flow cytometric analyses were performed on a FACS Calibur equipped with CellQuest software (Becton Dickinson).

2.6. IL-10 and TGF β neutralization assay

To test the role of IL-10 and TGF β on Tr mediated immune suppression of the OVA-induced immune response, T_E (1×10^5) were incubated with APCs (1×10^5 /well) and OVA peptide (1 μ g/ml), alone or with pre-activated

Tr from BALB/c or DO11.10 mice (1×10^5 /well) and in the presence or absence of anti-IL-10 and anti-TGF β (TGF β 1, β 2, and β 3) neutralization antibodies (10 μ g/ml, R&D Systems, MN, the biological activities of the antibodies have been confirmed in biological assays). On day three, part of the cell culture supernatant was collected for cytokine analysis and on day four, the cell proliferation was measured by ATP Lite-M assay.

2.7. CFSE labeling and transwell experiments

Purified CD4⁺CD25⁻ cells from DO11.10 mice were labeled with CFSE (Molecular Probes, OR) by incubation with 1 μ M CFSE in PBS containing 5% fetal bovine serum (FBS) for 5 min at 37°C. Cells were then washed twice with PBS. To assess whether CD4⁺CD25⁺ cells mediated immune suppression through direct cell–cell contact, transwell studies were conducted. CFSE labeled CD4⁺CD25⁻ cells were mixed with APCs (irradiated BALB/spleen cells) at 1:1 ratio at 6×10^5 /well in the lower chamber of a 96 well HTS transwell plate (Corning, NY) with 1 μ g/ml of the specific OVA peptide. Same number of the CD4⁺CD25⁺ cells were either cultured in the lower chambers directly in contact with the CFSE labeled CD4⁺CD25⁻ cells or in the upper chambers separated from the CFSE labeled CD4⁺CD25⁻ cells by a semi-permeable membrane, which allows diffusion of cytokines but not of cells. Cells are cultured for 4 days in RPMI 1640, supplemented with 1% penicillin and streptomycin and 10% inactivated FBS at 37°C and 5% CO₂. CFSE labeled CD4⁺CD25⁻ cells proliferation was detected by flow cytometry.

2.8. DTH response

Th1-like TE: CD4⁺CD25⁻ T cells from naïve DO11.10 mice (2×10^6 /ml) were cultured with APCs (2×10^6 /ml), 1 μ g/ml of the OVA peptide, 20 μ g/ml of anti-mouse IL-4 neutralization Ab (R&D Systems) and 1 ng/ml of recombinant mIL-12 (R&D Systems) in 24 well tissue culture plate. Fifty percentage of culture media (RPMI medium with 10% FBS) was replaced on days 3 and 5. The cells were harvested on day 7. Those cells produced a high level of IFN γ after re-stimulation with anti-CD3 and CD28 mAbs *in vitro* (data not shown). After washing with PBS, the cells were ready for adoptive transfer.

Tr: CD4⁺CD25⁺ T cells isolated from naïve DO11.10 or BALB/c mice were stimulated with immobilized CD3 mAb (10 μ g/ml) and recombinant mouse IL-2 (10 ng/ml, R&D System) in 10% FBS RPMI medium for 3 days. After washing, the cells were ready for adoptive transfer.

Adoptive transfer. Naïve BALB/c mice were divided into three groups (Table 1). In the DTH control group, equal numbers of T_E and APCs (1.6×10^6) were mixed and injected s.c. in a volume of 30 μ l (in PBS) with 10 μ g of OVA peptide in the left hind footpad. In the two groups testing the *in vivo* function of Tr, equal numbers of T_E, APCs and Tr (1.6×10^6) were mixed and injected s.c. in a volume of 30 μ l (in PBS)

Table 1

DTH response study groups and treatments

Group	Left footpad	Right footpad
1	T _E + APC + OVA peptide	T _E + APC
2	T _E + APC + OVA peptide + Tr/BALB/c	T _E + APC
3	T _E + APC + OVA peptide + Tr/DO11.10	T _E + APC

with 10 μ g of OVA peptide in the left hind footpad. In each mouse, equal numbers of T_E and APCs (1.6×10^6) were mixed without OVA peptide and injected s.c. in a volume of 30 μ l (in PBS) in the right hind footpad as a background control. Initial footpad measurements were taken before injection as a baseline. Footpad swelling is measured 24 h post cell injection and expressed as the percentage increase of the left footpad over the control right footpad.

Mice were sacrificed at 24 h post injection, and their footpads were removed. The footpad tissues were homogenized for cytokines measurement.

2.9. Measurement of footpad tissue cytokine

Mice were terminated 24 h post cell transfer and their footpads were removed. The footpads were homogenized and the supernatants were collected. The level of cytokines in the supernatants was detected using murine cytokine kit (LINCO Research, Inc., MO) with a Luminex instrument.

2.10. Statistics

Results are expressed as mean $\pm$ SD. Data were analyzed using a 2-sided Students' *t* test and ANOVA one-way test. All analyzes were performed using Prism software (Graph-Pad Software, CA). Differences were considered significant at a *p* level less than 0.05.

3. Results

3.1. Activated OVA-specific and bystander T regulatory cells exhibited equivalent inhibition on OVA-specific T cell responses *in vitro*

To determine the relative potency of the immune suppressive effects provided by Ag specific vs. non-specific Tr, we compared the inhibition of OVA-specific T cell responses by OVA-specific and OVA non-specific Tr in an *in vitro* system. CD4⁺CD25⁺ Tr were isolated from spleens of either the DO11.10 OVA specific TCR (recognizing an OVA peptide in the context of I-A^d MHC) or naïve BALB/c mice using the regulatory T cell isolation kit. As expected, CD4⁺CD25⁺ Tr from BALB/c mice did not express detectable levels of OVA transgenic TCR (data not shown), but both CD4⁺CD25⁺ Tr showed poor proliferation and cytokine production to anti-CD3 and CD28 antibodies stimulation indicated by (data not shown). In addition, intracellular staining results showed that those cells expressed high level of Foxp3 (Fig. 1).

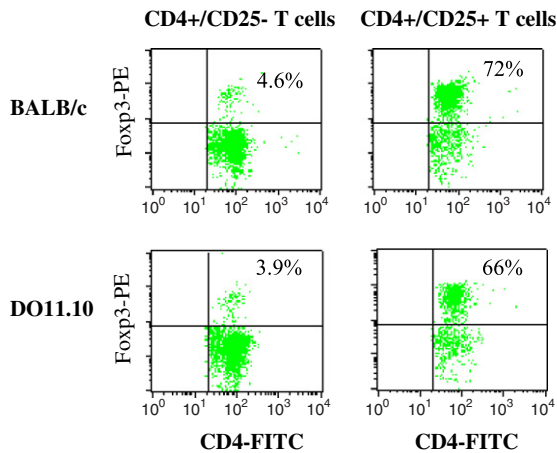


Fig. 1. $CD4^+CD25^+$ cells from both DO11.10 and BALB/c mice express high level of intracellular Foxp3: purified $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from BALB/c and DO11.10 mice were stained with CD4-FITC, CD25-PE antibodies. After fixation and permeabilization, Foxp3s-APC antibody was used. The expression of Foxp3 was evaluated using flow cytometry.

OVA-specific $CD4^+CD25^-$ T effector cells (T_E) from DO11.10 mice were activated with APCs and the specific OVA peptide. Freshly isolated or pre-activated (with anti-CD3 mAb and IL-2) Tr were added to the OVA specific T_E cell culture, and the immune suppression was measured by cell proliferation and cytokine production. Consistent with previously reported observations that pre-activated Tr exhibited much more potent immune suppression than that of freshly isolated Tr, confirming that activation is needed for optimal inhibitory functions of Tr. Although freshly isolated Tr from DO11.10 mice was slightly more potent than those from wild-type BALB/c mice, once activated, Tr from both strains of mice exhibited comparable (statistically non-significant) inhibition on the proliferation of OVA-specific T_E cells (data not shown). This result clearly demonstrated that polyclonal activation enhanced the immune suppressive function of antigen specific and non-specific Tr to a similar extent.

To compare the relative potency of the inhibition of OVA specific T cells by OVA-specific and OVA non-specific Tr, the numbers of Tr were titrated and the inhibition was measured by T cell proliferation and cytokine production. Constant numbers of OVA-specific T_E were activated with APCs plus the OVA peptide and cultured with different ratio of pre-activated Tr from DO11.10 or naïve BALB/c mice. As shown in Fig. 2A, activated Tr from DO11.10 and BALB/c mice exhibited similar cell number-dependent inhibition (statistically non-significant) of OVA-specific T cell proliferation, suggesting that once activated, OVA-specific and OVA non-specific Tr were equally potent in inhibiting OVA-specific T cell responses. This result was further confirmed by the inhibition of cytokine production. Activated Tr from DO11.10 and BALB/c mice exhibited similar cell number-dependent

inhibition of OVA-induced production of Th1 (IL-2 and $IFN\gamma$), Th2 (IL-4, IL-5, and IL-10), as well as pro-inflammatory cytokines (IL-6) (Fig. 2B). Taken together, our results demonstrate that activated OVA specific and non-specific Tr exhibit similar immune suppressive potency to OVA specific T cell responses. Therefore, the effector phase of Tr mediated inhibition does not require Ag specific TCR recognition.

3.2. Activated OVA-specific and bystander T regulatory cells inhibited effector T cells through similar mechanisms

To investigate the mechanisms of the inhibition of T_E activation by OVA-specific and bystander Tr, we first looked at if immune suppressive cytokine were involved, since one possible mechanism of Tr-mediated suppression is through $TGF\beta$ and IL-10 [17,18]. We found that although OVA stimulation induced IL-10 production in T_E from DO11.10 mice, Tr from either DO11.10 or BALB/c mice did not produce detectable IL-10 after activation (data not shown). Furthermore, when IL-10 or $TGF\beta$ neutralization antibody was added, neither IL-10 nor $TGF\beta$ antibody reversed Tr mediated inhibition, demonstrated by cell proliferation (Fig. 3A) and cytokine production results (Fig. 3B).

To assess whether OVA-specific and bystander Tr mediated immune suppression was through direct cell–cell contact, we conducted transwell studies. OVA peptide induced proliferation of CFSE labeled T_E from the DO11.10 mice (Fig. 4A) and the proliferation was inhibited to the same extent by Tr from either DO11.10 or BALB/c mice when T_E and Tr were mixed (Fig. 4B). However, the inhibition was abrogated when Tr and T_E were separated using transwell culture (Fig. 4C). This result indicated that both the Ag-specific and the bystander Tr inhibit T_E through cell–cell contact rather than via immune suppressive cytokines.

It has been reported that a Tr like phenotype and function could be induced in T_E when they were co-cultured with Tr [17]. Several cell surface molecules, cytotoxic T-lymphocyte antigen 4 (CTLA-4), glucocorticoid-induced TNF receptor family-related protein (GITR), and programmed death-1 (PD-1) are highly expressed on Tr [21–24]. In this study, we examined if OVA-specific and OVA non-specific Tr could induce increased expression of these cell surface proteins on OVA specific T_E . CFSE labeled OVA-specific $CD4^+CD25^-$ T_E were activated with APC plus OVA peptide with or without co-culturing with Tr, and the expression of CTLA-4, GITR, and PD-1 was detected on CFSE positive cells. We found that OVA activation increased expression of GITR (untreated 2.5% vs. OVA stimulated 22.3%); CTLA-4 (2.65% vs. 5.91%); and PD-1 (9.96% vs. 23.2%) in T_E . While co-culture T_E with Tr from either BALB/c or DO11.10 mice, the expression of CTLA-4, GITR, or PD-1 on OVA specific T_E cells was further increased (Fig. 5), and there was no significantly difference between OVA-specific and bystander Tr in terms of the induction of these markers.

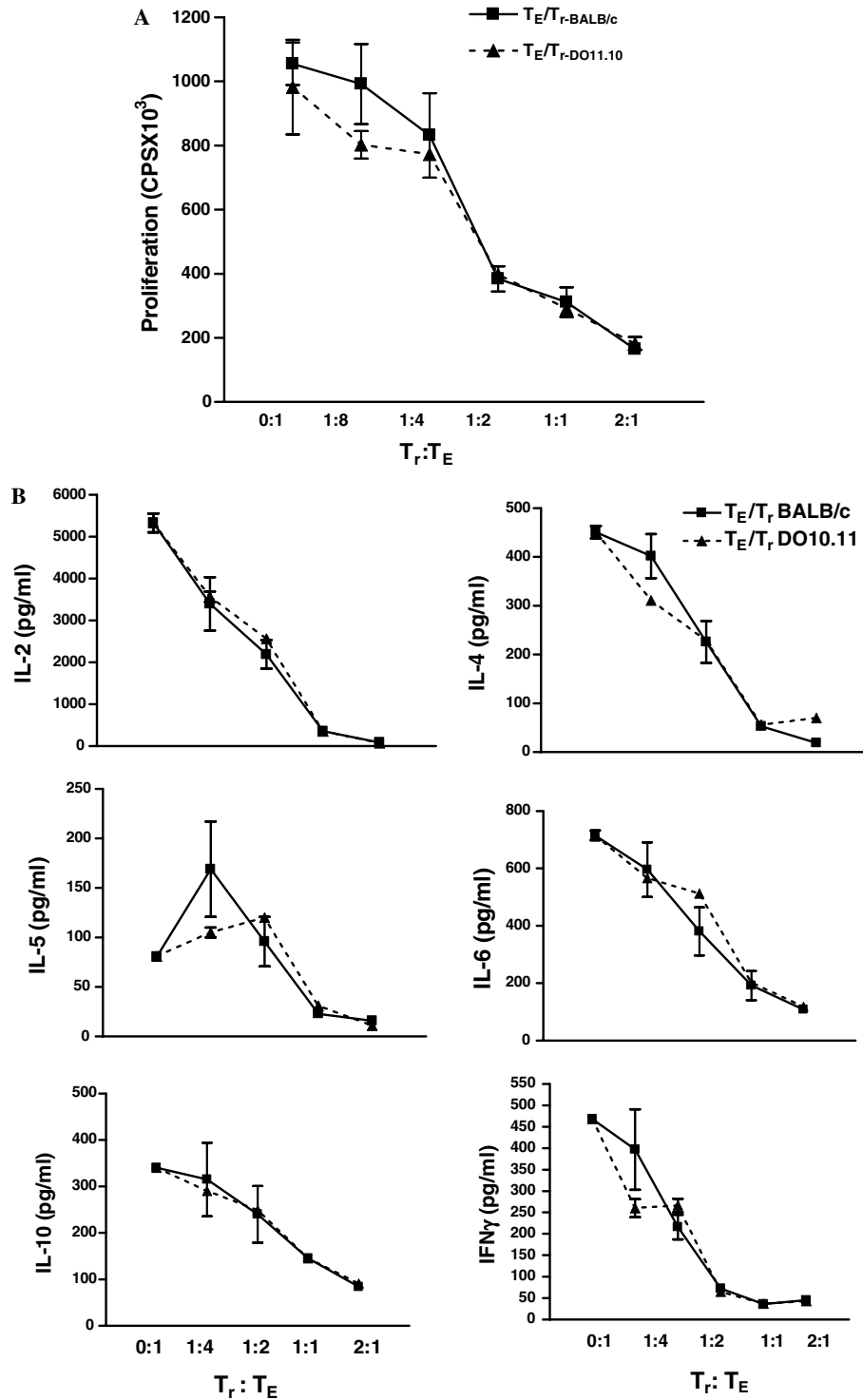


Fig. 2. Tr from BALB/c and DO11.10 mice showed equal potency in the inhibition of OVA-induced T cell response: a constant number of T_E from DO11.10 mice was cultured with APCs and OVA peptide in the presence or absence of different ratios of pre-activated Tr from BALB/c or DO11.10 mice. Proliferation was measured on day 4 (A) and cytokines were detected from the culture supernatants on day 3 (B). Each point represents the means $\pm$ SD of three wells. Data are representative of two independent experiments. There is no statistic significance between inhibition by Tr from DO11.10 and BALB/c mice.

3.3. OVA specific and OVA non-specific Tr exhibited similar inhibition on an OVA specific DTH response *in vivo*

To investigate whether *ex vivo* activated OVA specific and non-specific Tr exhibit similar immune suppression in

an *in vivo* system, a T cell adoptive transfer DTH model was used. OVA-specific T_E were pre-skewed into a Th1-like phenotype, and adoptively transferred into the left hind footpads of BALB/c mice along with APCs and OVA peptide. The right footpad of the same mouse was injected

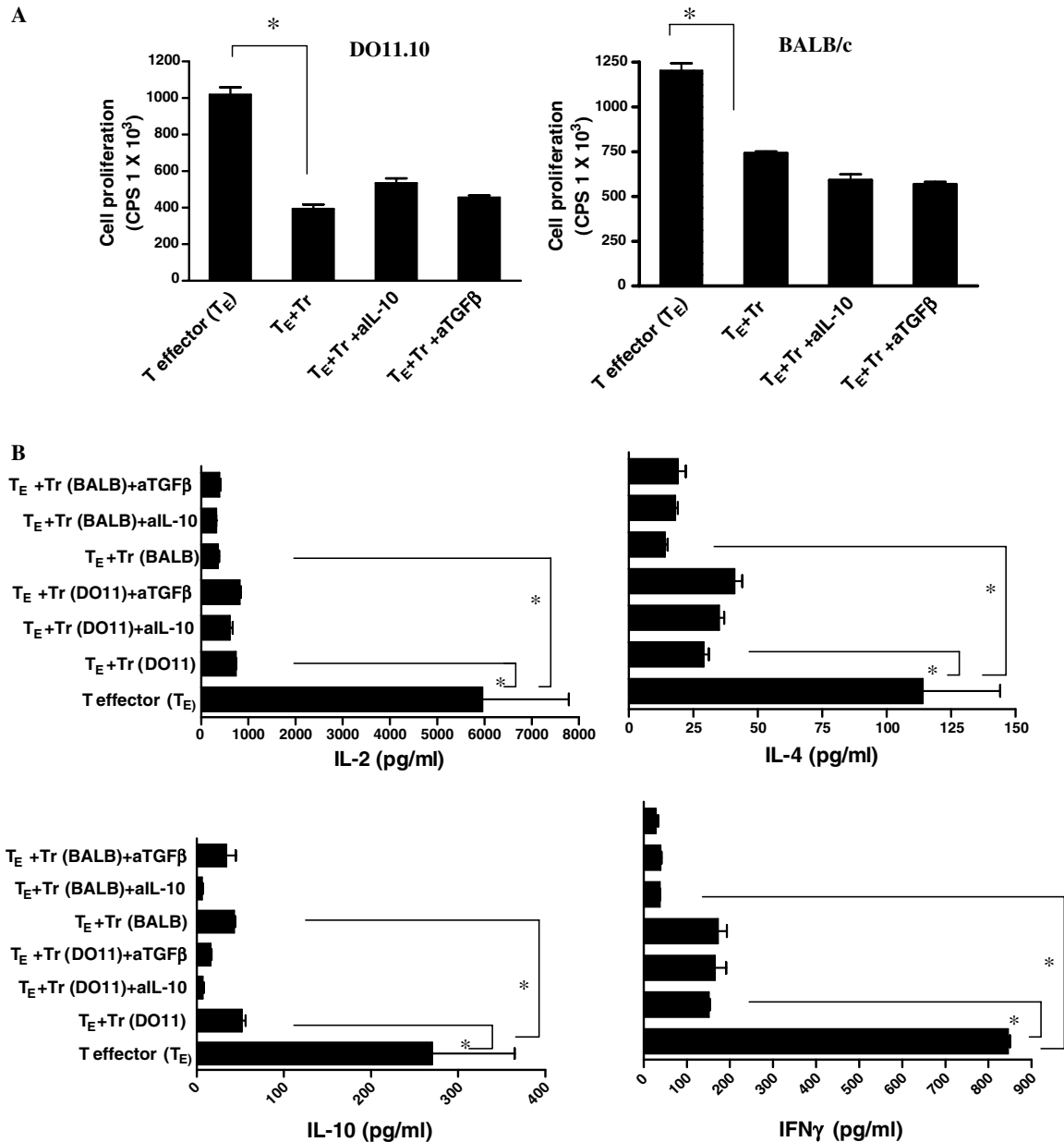


Fig. 3. The effects of IL-10 and TGFβ neutralization antibody on Tr mediated inhibition. T_E from DO11.10 mice were cultured with APCs, OVA peptide and equal numbers of Tr in the presence or absence of anti-IL-10 or anti-TGFβ neutralization antibody. Proliferation was measured on day 4 (A) and cytokines were detected from the culture supernatant on day 3 after the culture (B). Each bar represents the mean ± SD of three wells. Data are representative of two independent experiments. **p* < 0.05 compared to T_E control mice.

with OVA specific T_E plus APCs but without OVA peptide as an internal control. DTH was evaluated by the measurement of footpad swelling 24 h post cell injection. OVA activated T_E induced significant footpad swelling 24 h post transfer compared to that of the control foot, indicating an OVA-specific DTH response (Fig. 6). Co-transferring either the pre-activated OVA-specific or OVA non-specific Tr significantly inhibited the DTH response to a similar extent (Fig. 6). This result demonstrated that pre-activated, OVA specific and non-specific Tr mediated similar immune suppression *in vivo*.

To further compare *in vivo* immune suppression induced by Ag specific vs. non-specific Tr, the local cyto-

kine production in the DTH footpads was measured. Twenty-four hours after cell transfer, the footpads were removed, homogenized and multiple cytokines were measured. In the control footpads where T_E were injected with APC without OVA peptide, the levels of most of the cytokines tested was undetectable (Fig. 7). In contrast, elevated levels of Th1 (IL-2, IFNγ), Th2 (IL-4 and IL-10) and pro-inflammatory cytokines (IL-6 and TNFα) were detected in the DTH footpad, where OVA specific T_E were injected with APCs and OVA peptide (Fig. 7). Both OVA-specific and non-specific Tr exhibited similar (statistically non-significant) but significantly inhibited local production of IL-2, IL-4, IL-6, IFNβ, and TNFα (Fig. 7).

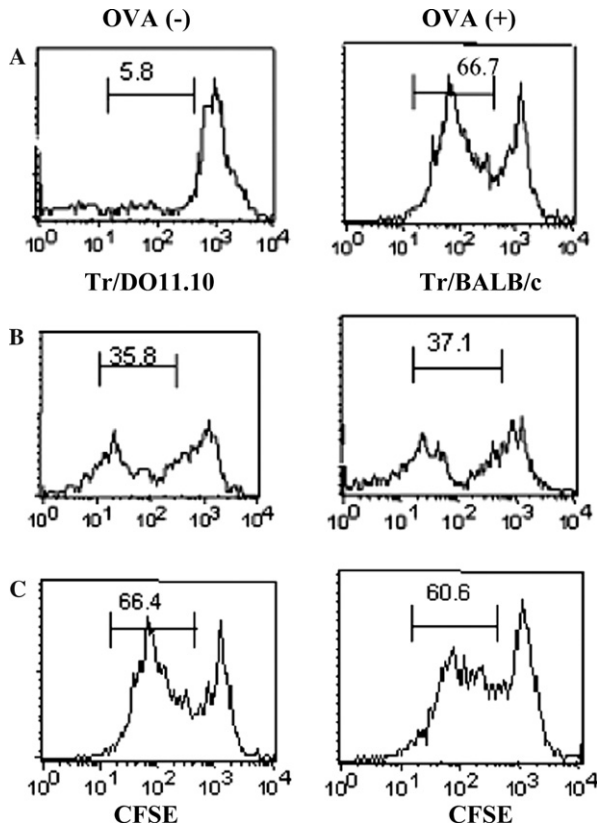


Fig. 4. Tr from both DO11.10 and BALB/c mice inhibit T_E proliferation through cell–cell contact: (A) $CD4^+CD25^-$ cells from DO11.10 mice were labeled with CFSE and cultured with or without OVA peptide. After 4 days culture the cell proliferation was detected by FACS. Data represent CFSE positive proliferating cells. (B) CFSE labeled $CD4^+CD25^-$ cells from DO11.10 mice were cultured with Tr from either DO11.10 or BALB/c mice at 1:1 ratio in a mixed culture with OVA peptide (B) or CFSE labeled $CD4^+CD25^-$ cells were cultured in the lower chambers with OVA peptide and $CD4^+CD25^+$ cells were in the upper chambers separated from the CFSE labeled $CD4^+CD25^-$ cells (C). Cells were cultured for 4 days, and T_E proliferation was measured using flow cytometry.

This result indicates that pre-activated Ag-specific and non-specific Tr demonstrated similar immune suppression *in vivo*.

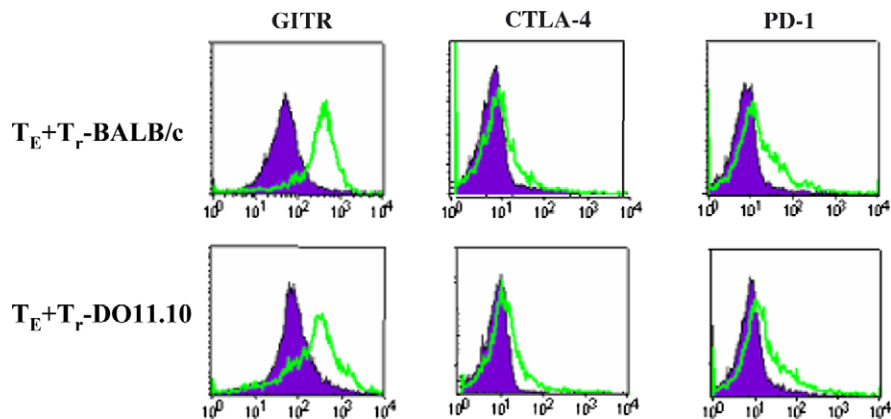


Fig. 5. Tr from BALB/c or DO11.10 up-regulated the expression of CTLA-4, GITR and PD-1 on T_E . T_E from DO11.10 mice were labeled with CFSE and activated with APCs and OVA peptide alone or in the presence of Tr from BALB/c or DO11.10 mice. Four days after the culture, the expression of GITR, CTLA-4, and PD-1 on T_E were analyzed by flow cytometry. Closed: T_E alone, open: T_E cultured in the presence of Tr. Data are representative of three independent experiments.

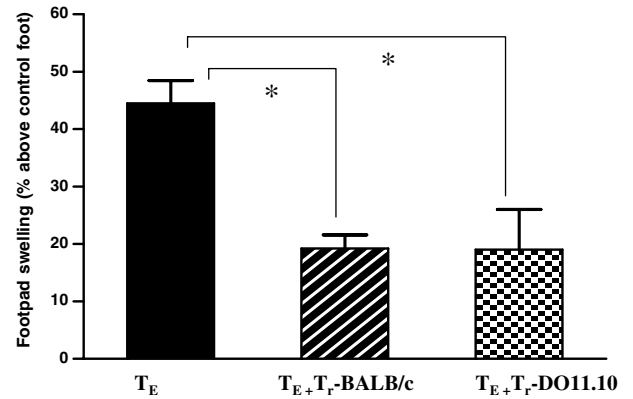


Fig. 6. Tr from BALB/c and DO11.10 mice showed equal inhibition of OVA-induced DTH response. Th1-like T_E from DO11.10 mice were mixed with APCs and OVA peptide in the presence or absence of pre-activated Tr from either DO11.10 or BALB/c mice and were injected s.c. in the left hind footpads of naïve BALB/c mice. The right hind footpad of the same mouse received the same number of Th1-like T_E mixed with APCs in the absence of OVA peptide as an internal control. Footpad swelling was measured 24 h post cell injection and expressed as the percentage increase of the left foot over the right control foot. Each bar represents the mean $\pm$ SD of four mice. This graph is a representative of three independent experiments. * $p < 0.05$ compared to T_E control mice.

4. Discussion

Tr require activation through their TCR to be fully functional for maintaining immune homeostasis and preventing autoimmune diseases *in vivo* [1,2]. $CD4^+CD25^+$ Tr are developed from the thymus and migrate to the periphery where they recognize various self-antigens [10,31]. Several studies suggested that Tr are activated by tissue-specific self-antigens [11,12,15,16]. $CD4^+CD25^+$ Tr from pancreatic lymph nodes and the periphery of pre-diabetic mice transferred protection against diabetes in NOD mice [4,16,30]. In an adoptive transfer study, MBP-specific Tr are more potent in preventing the development of MBP-induced EAE *in vivo* than non-transgenic Tr, which suggests that

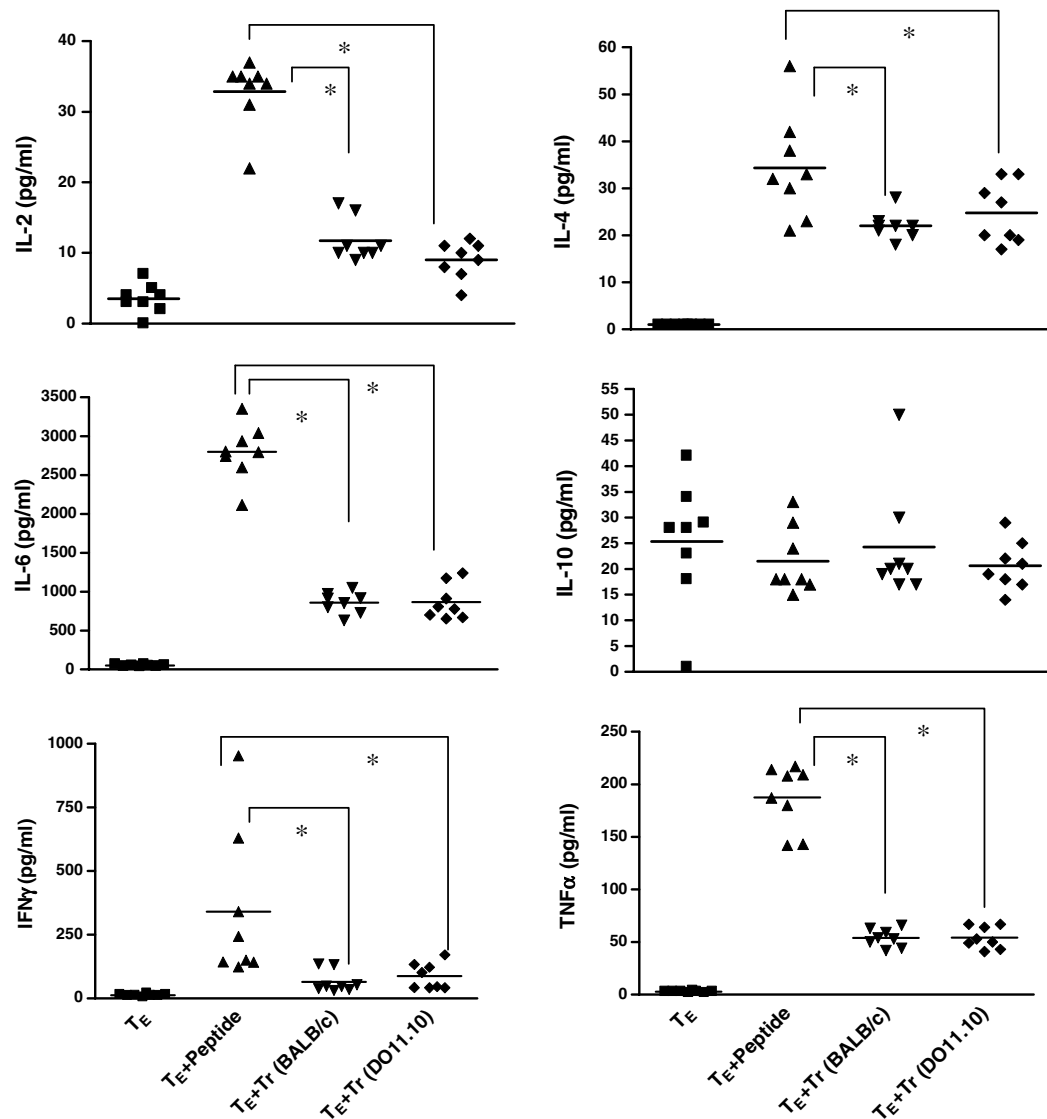


Fig. 7. Tr from BALB/c and DO11.10 mice showed equal inhibition of *in vivo* cytokine production in OVA specific DTH response. Th1-like T_E from DO11.10 mice were mixed with APCs and OVA peptide in the presence or absence of pre-activated Tr from either DO11.10 or BALB/c mice and injected s.c. in the left hind footpads of naïve BALB/c mice. The right hind footpad of the same mouse received the same number of Th1-like T_E mixed with APCs in the absence of OVA peptide as an internal control. Twenty-four hours after cell transfer, the footpads were removed and homogenized. Cytokine was measured from the tissue homogenizes. Each point represents an individual mouse. Data represents two independent experiments.

antigen specific activation of Tr would enhance their function [5]. However, due to the low numbers of antigen specific Tr *in vivo*, it is difficult to specifically expand the antigen specific Tr for therapeutic purposes. Although Tr require antigen-specific activation for their function, they are generally able to suppress bystander T cell responses once activated [13,14]. It was reported that CD4⁺ CD25⁺ T cells from HA TCR transgenic mice, when activated with HA peptide and IL-2, suppressed the proliferation of T effector cells from mice that express a different transgenic TCR *in vitro* [13,14]. The observation of bystander regulation by Tr provided an alternate potential cell therapy to stimulate the functional capability of polyclonal regulatory T cells [17–20]. However, it was not clear if the effector inhibitory activity is similar between Ag-specific and bystander Tr.

To compare the *in vitro* inhibitory activities between Ag-specific and bystander Tr, Tr from OVA specific TCR transgenic mice (DO11.10) and wild-type BALB/c mice were evaluated on OVA-induced T cell responses. Tr from both strains of mice expressed similar levels of CTLA-4, GITR, PD-1, and Foxp3. Those cells were poor responders to anti-CD3 and OVA peptide stimulation (data not shown). This indicated that they were functionally anergic. Pre-activated OVA specific and bystander Tr exhibited similar potency in inhibiting OVA specific T effector cell proliferation and cytokine production *in vitro*. In addition, immune inhibition through both Tr seemed to be mediated through similar mechanisms. The inhibition was cell contact-dependent and IL-10 and TGFβ may not play a role in the inhibition. Furthermore, both OVA-specific and bystander Tr induced Tr-like phenotype (increased expression

of CTLA-4, PD-1, and GITR) on T effector cells, which could be associated with Tr function as indicated by other studies [21–28]. However, although Tr up-regulated the expression of CTLA-4, PD-1, and GITR on T_E, we did not see the increase of Foxp3 in those cells (data not shown). We do not know if we have picked the right time point of the co-culture for the potential upregulation of Foxp3. Using purified T_E from co-cultured system for functional analysis could help us to define the role of those T effectors.

By co-transfer of OVA-specific T_E with pre-activated OVA specific or bystander Tr in a footpad DTH model, we demonstrated that pre-activated OVA-specific and bystander Tr significantly inhibited the OVA-induced DTH response and local inflammatory cytokine production with similar potencies. Inhibition of the DTH response by Tr was most likely through the inhibition of OVA-specific T_E activation *in vivo*. The limitation of this adoptively transferred model is that we can not use this model to evaluate the impacts of antigen specific and non-specific Tr on antigen specific T_E migration in local inflammation site. The potential impact of Ag-specific and bystander activation on Tr migration will be evaluated in other mouse disease models.

One main obstacle for potential use of Tr for therapeutic purpose in clinical is how can we generate enough number of Tr with inhibition potency? Due to the low frequency of antigen-specific Tr *in vivo* and unknown pathogenic auto antigens, it is difficult to generate and expand antigen specific Tr for therapeutic use. One alternative approach is to expand polyclonal Tr *in vitro* for adoptive transfer therapy *in vivo*. In this study, our results demonstrated that after polyclonal activation, Tr inhibit antigen specific immune response with similar potency to that of antigen specific Tr. Therefore, Tr may be effective in suppressing autoimmune or other inflammatory diseases through polyclonal stimulation.

In addition to anti-CD3 polyclonal activation, non-TCR signaling could also activate Tr, and therefore could enhance bystander immune regulation *in vivo*. It was reported that stimulation of CD4⁺CD25⁺ T cells with a high concentration of lipopolysaccharide through TLR4 elicited Tr proliferation, prolongation of survival, and augmentation of their suppressive activity *in vitro* suggesting a non-TCR dependent mechanism for Tr activation [29]. Additional approaches to activate Tr *in vivo* need to be explored.

In summary, we have investigated the impact of activated antigen specific and non-specific Tr on OVA-induced immune response. We found that after anti-CD3 mAb treatment, naturally occurring Tr from BALB/c (antigen non-specific) and DO11.10 (antigen specific) mice inhibit OVA-induced CD25⁺ T cell proliferation, cytokine production *in vitro* and footpad DTH response *in vivo* with similar inhibition potency and similar mechanisms. The results further indicate that anti-CD3 activation *in vitro* renders antigen specific and non-specific Tr with similar immune suppression potency *in vitro* and *in vivo*. These results pro-

vide evidence to support the potential use of *ex vivo* activated CD4⁺CD25⁺ T regulatory cells for controlling autoimmune diseases.

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