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A Soluble Antigen-Specific Factor from $CD4^+CD25^+$ T Cells of OVA Tolerant Mice Inducing OVA-Specific Peripheral Tolerance Independently *in vivo*

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Abstract: $CD4^+CD25^+$ T cells played a critical role in the establishment and maintenance of peripheral tolerance via adoptive transfer. However, whether one or more molecules in $CD4^+CD25^+$ T cells that could independently mediate peripheral tolerance was disputed by worldwide researchers. In the present study, one soluble antigen-specific factor was extracted from splenic lymphocytes lysates of OVA-tolerant mice (named OVA Immune Tolerance Factor, OVA-ITF) with molecular mass <3 ku which could establish OVA-specific immune tolerance in recipient mice via transfer treated and induce the same effect of peripheral tolerance as those of splenic lymphocytes from OVA-tolerant mice. Treated with OVA-ITF to naive BALB/c mice resulted in significant suppression of DTH reaction and T cell proliferation in an antigen-specific manner as well as a significant increase in the percentage of $CD4^+CD25^+$ T cells within the $CD4^+$ T cell subset in peripheral blood. Present study showed that OVA-TIF was produced by $CD4^+CD25^+$ T cell subset and could induce OVA-specific peripheral tolerance independently *in vivo* with TGF-β_1 as its main suppressive cytokine in recipient mice. These results suggested that OVA-TIF is a novel, low MW factor and totally different from other suppressive components reported previously which have important implications for expanding new potential therapeutic routes of prevention and control of graft rejection, autoimmune and related diseases.

Key words: OVA-TIF, $CD4^+CD25^+$ T cells, peripheral tolerance, graft rejection, potential therapeutic, China

INTRODUCTION

Establishment and breakdown of peripheral immune tolerance has close relation with autoimmune diseases (Kukreja *et al*., 2002; Van Amelsfort *et al*., 2004; Viglietta *et al*., 2004; Lindley *et al*., 2005; Atfy *et al*., 2009; La Cava, 2008), graft-rejection (Hall *et al*., 2007; Fu *et al*., 2010), cancers (Onizuka *et al*., 1999; Shimizu *et al*., 1999; Mougiakakos *et al*., 2010; Nishikawa and Sakaguchi, 2010) and some of parasitic and virus diseases (Long *et al*., 2003; Vahlenkamp *et al*., 2005; Bourreau *et al*., 2009; Rodrigues *et al*., 2009; Wongyanin *et al*., 2010; Langhans *et al*., 2010; Weiss *et al*., 2010). At present, several mechanisms for the induction of tolerance to self-antigens have been reported in cluding clonal deletion of autoreactive T cells in duction of T cell anergy, active suppression by regulatory T cells and present of pathogenic auto-reactive T cells (Kukreja *et al*., 2002; Viglietta *et al*., 2004; Kappler *et al*., 1987; Ota *et al*., 1990; Sakaguchi, 2004; Shevach *et al*., 2001). There is growing evidence that $CD4^+CD25^+$ regulatory T (Treg) cells play a crucial role in the establishment and maintenance of peripheral tolerance (Cobbold *et al*., 2003; Kohm *et al*., 2002; Sakaguchi, 2004; Cohen *et al*., 2002) which was first demonstrated by the induction of body-wide autoimmune diseases in athymic nude mice after injection with $CD4^+$ T cells depleted of $CD25^+$ T cells (Sakaguchi *et al*., 1995; Takahashi *et al*., 1998). Therefore, Tregs cells would be the target to determine the clinical effectiveness of novel therapy to modulate Tregs *in vivo* besides the conventional treatments (Atfy *et al*., 2009).

Recently, many specific cell therapies based on Treg cells and other T cell subsets were developed (Grupp and June, 2010; Lee *et al*., 2010; Turtle and Riddell, 2010) which have been presented to be promising approaches for cancer immunotherapy. Adoptive Cell Therapy (ACT)

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has been proved to be an effective treatment for viral infections which also could induce regression of cancer in early stage clinical trials and would be a particularly important and efficacious modality in the period following Hematopoietic Stem Cell Transplantation (HSCT) (Grupp and June, 2010). However, the cell therapies have lots of shortcomings (Miyara *et al*., 2009; Schumacher and Restifo, 2009) and the major limitation is the rapid decline in viability and function of the transplanted cells. Consequently, new therapeutic routes of these diseases should be reconsidered.

Previous studies showed several cell-surface markers are associated with Treg-cell phenotype and function and the most prominent molecules are cytotoxic T lymphocyteassociated protein 4 (CTLA-4) and Glucocorticoid-Induced TNFR-Related protein (GITR) (McHugh *et al*., 2002; Shimizu *et al*., 2002). Additionally, IL-10 and TGF-β although, rarely expressed *in vitro* might have functional importance for Treg cells *in vivo*, particularly in the context of disease (Hara *et al*., 2001; Nakamura *et al*., 2001). Although, these molecules are related to the regulation of suppressor activity by $CD4^+CD25^+$ T cells, they are not candidate molecules for the independent establishment of peripheral tolerance. Transfer treated of spleen cell lysates from tolerant mice could help to establish peripheral tolerance to certain antigens (HGG or HIgG) in naive mice (Jones and Kaplan, 1977; Taniguchi and Miller, 1978). This lysate-induced tolerance was acted in an antigen-specific manner that the immune response to related antigens was significantly suppressed while to other antigens was not affected. However, most of these cell lysates are >10 ku in molecular weight which would induce immune reaction of receptor to these exotic antigens. The origin and chemical nature were also unknown.

In the present study, a novel and smaller (<3 ku) soluble antigen-specific factor named OVA Immune Tolerant Factor (OVA-ITF) was identified which was generated by $CD4^+CD25^+$ T cells in OVA tolerant mice and could mediate OVA specific tolerance in naive mice. The findings would provide direct molecular evidence on the mechanism of Treg-mediated peripheral tolerance and expand new routes for clinical therapy.

MATERIALS AND METHODS

Mice: Female BALB/c mice (4 weeks old) were originally purchased from the Animal Center at the Fourth Military Medical University, Xi'an, P.R. China and housed under specific pathogen-free conditions in the facility with free access to food and water.

Induction of OVA-tolerant mice, preparation of Tolerant Splenic Lymphocytes (TSLC) and different tolerant T cell subsets and OVA Immune Tolerance Factor (OVA-ITF) purification: BALB/c mice received i.v. 3 times (5 day intervals) with 5 μg of OVA (Sigma) in 0.1 mL of PBS. Control mice received an equivalent volume of PBS. Then each mouse were injected s.c with 250 μg OVA in 0.1 mL PBS at 3 and 12 days after the last injection.

Lymphocytes were prepared from OVA-tolerant mice at 6 days after the last injection. Spleens were removed and pooled in D-Hank's solution with 5% heat-inactivated FCS (HyClone, USA). Then the tissue was dispersed by passing through a stainless steel mesh. Lymphocytes were isolated by Ficoll-Hypaque density gradient centrifugation, rinsed twice with D-Hank's solution and then resuspended in RPMI 1640 medium (HyClone, USA). The final solutions were split into two equal portions. The lymphocyte concentration in one portion was adjusted to 1×10^8 cells mL^{-1} for cell adoptive transfer. The other one was adjusted to 1.5×10^8 cells mL^{-1} for sorting different tolerant T cell subsets.

Different tolerant T cell subsets were sorted using flow cytometry. Single lymphocyte suspension of OVA-tolerant mice was prepared by negative selection using MACS microbeads labeled with various mAbs (StemCell Biotec) according to the manufacturer's suggested protocol. The $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were obtained using anti-PE coated microbeads labeled with PE-conjugated anti-CD25 (StemCell Biotec). The purity was consistently >95% or 90% for $CD4^+$ or $CD4^+CD25^+$T cell preparations, respectively. The purified $CD4^+CD25^+$ T cells were adjusted to a concentration of 6×10^6 cells mL^{-1} in PBS for cell adoptive transfer and preparation of OVA-ITF.

The final $CD4^+CD25^+$ T cells were frozen-thawed at -20 and 37°C for 3 times and then centrifuged at 12,000×g for 15 min to remove cell debris. The supernatant was filtered through ultrafiltration tubes by centrifugation at 7000×g for 35-40 min at room temperature according to suggested protocol (Vivaspin2, Vivascience, Sartorius Group, Germany) and separated into three fractions that contained molecules of <3 ku, 3~5 ku and >5 ku in molecular weight.

Each fraction was transferred to BALB/c mice and the tolerated effect was evaluated using the lymphocyte proliferation assay. The results showed that the optima effect was molecules of <3 ku fraction (data requested from researchers) which was named as OVA-ITF. As a control, splenic lymphocyte suspensions of naive mice were also prepared and the fraction with the molecules of <3 ku was harvested (named as naive ITF) and transferred to naive mouse.

To address the origin of OVA-ITF, purified $CD4^-$, $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were resuspended in RPMI 1640 medium, respectively and the final concentrations were adjusted to 9×10^6 cells mL^{-1}. Fractions with the same molecular weight as OVA-ITF were harvested according to the protocols above. These fractions were referred to as ITF_{CD4-}, $ITF_{CD4+CD25+}$ and $ITF_{CD4+CD25-}$.

Transfer treated and immunization: BALB/c mice were randomly assigned to eight groups. The first seven groups received i.v. with TSLC, OVA-ITF, naive ITF, isolated $CD4^+CD25^+$ T cells, $ITF_{CD4+CD25+}$, ITF_{CD4-}, $ITF_{CD4+CD25-}$ in a volume of 0.4 mL (day 0) and the last group was injected with PBS as blank control. The blood were collected at 1, 3, 7 and 10 days later to determine the percentage of $CD4^+CD25^+$ T cells in peripheral blood after adoptive transfer and Peripheral Blood Mononuclear Cells (PBMCs) were also analyzed using flow cytometry.

The cell-transferred mice were immunized s.c. with 250 μg of OVA on day 1 and sacrificed on day 5 to test for lymphocyte proliferation and cytokine secretion. ITF-treated mice (transferred with OVA-ITF, naive ITF, $ITF_{CD4+CD25+}$, ITF_{CD4-}, $ITF_{CD4+CD25-}$) as well as PBS control mice were also immunized s.c. with the same dose of OVA on day 3 and sacrificed on day 9. Then spleens of these mice were harvested and to prepare lymphocytes cultured *in vitro*.

For DTH reactions response, cell-transferred mice were immunized s.c. on day 1 with 100 μg of OVA in 50 μL of PBS and an equivalent volume of complete Freund's adjuvant (CFA, Sigma). ITF-treated mice and PBS control mice were also immunized with the same dose of OVA and 50 μL of CFA on day 3.

Flow cytometry analysis: The whole blood aliquots (100 μL) with heparin were incubated with a mixture of FITC-labeled rat anti-mouse CD4 and PE-labeled rat anti-mouse CD25 (Biolegend, USA; 2 μL/100 μL blood) or their isotype control mAbs for 30 min at room temperature. A 2 mL aliquot of FACS lysing solution (BD Pharmingen) was added to each mixture and incubated for 10 min at room temperature. Then the solution were centrifuged at 800×g for 5 min and the labeled PBMCs were resuspended in 500 μL PBS for analysis, analyzed on a FACScan flow cytometer (BD Biosciences) and quantified with WinMDI 2.9 (WinRAR) software.

Proliferation assay: Lymphocyte proliferation was determined by MTT assay as previous studies with some modification (Keller *et al*., 2005; Peng *et al*., 2009; Liu *et al*., 2010). Briefly, Lymphocytes (1×10^6 cells $well^{-1}$) were cultured alone or with OVA (500 μg mL^{-1}, Sigma), BSA (750 μg mL^{-1}, Roche, USA) for 68 h at 37°C in 96 well flat-bottom plates (Costar, NY, USA) in RPMI 1640 cell culture medium supplemented with 10% heat-inactivated FCS, 5×10^{-5} M 2 ME (Biotech) and antibiotics (100 U mL^{-1} penicillin and 100 U mL^{-1} streptomycin), 10 mM Hepes buffer (Sigma), 2 mM L-glutamine (Amresco) and 1 mM sodium pyruvate (Amresco). Cultures were added MTT (5 mg mL^{-1}, Amresco) with 10 μL per well at 64 h later. Before harvest, DMSO (Amresco) was added with 100 μL per well and the mixtures were oscillated for 10 min. The absorbance was measured at 630 nm using a Model 680 Microplate Reader (BIO-RAD). The proliferation values were presented as a stimulation index value (SI value).

Cytokine ELISA: For cytokine assays, lymphocytes (5×10^6 cells $well^{-1}$) were cultured with OVA (500 μg mL^{-1}, Sigma) in 24 well flat-bottom plates (Costar, NY, USA) in RPMI 1640 cell culture medium at 37°C. Supernatants were harvested 48 and 72 h later for detection of IL-10 and TGF-β_1, respectively.

The levels of IL-10 and TGF-β_1 were measured using commercial ELISA kits (IL-10, Jingmei Biotech, China, detection limit, 7 pg mL^{-1}; TGF-β_1, Uscnlife, USA, detection limit, 7.8 pg mL^{-1}) according to the manufacturer's instructions.

Delayed-Type Hypersensitivity (DTH) reaction: About 6 days after immunization, mice were challenged s.c. with 50 μg OVA in 20 μL PBS in the left ear. Ear thickness was measured 24 h after challenge with a micrometer caliper (Mitutoyo, Japan). Results are shown as the difference between left and right ear thickness.

Statistical analysis: Statistical analyses were performed with the Student's t-test between experimental and control groups. Results are expressed as mean±Standard Deviation (SD) and p-values <0.05 or <0.01 represented significant or highly significant differences between groups, respectively.

RESULTS AND DISCUSSION

The OVA-specific peripheral tolerant model in BALB/c mice was induced using normal mice which were firstly received i.v. 3 times with OVA solution or PBS and then immunized twice with OVA. The OVA-specific lymphocyte proliferation and the percentage of splenic $CD4^+CD25^+$ T cells were analyzed. The proliferation of OVA-specific T cells was significant lower in OVA-induced mice compared with control mice ($p<0.01$)

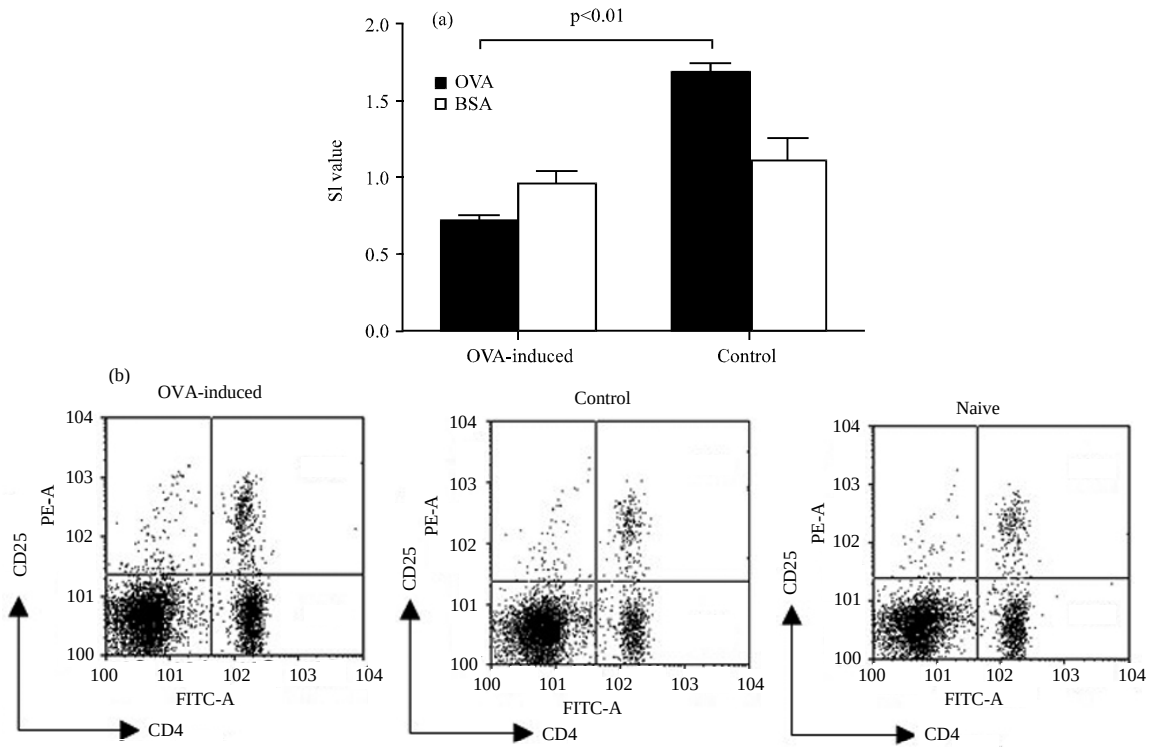

Fig. 1: Establishment of OVA-specific tolerance in BALB/c mice. (a) Lymphocytes were cultured with or without OVA (500 μg mL^{-1}) or BSA (750 μg mL^{-1}) for 72 h. The results are shown using the mean±SD of two independent experiments with six mice. (b) Flow cytometry analyses of splenic lymphocytes stained with CD4 and CD25. One representive experiment of six mice from two independent experiments was shown

whereas no significant suppression of BSA-specific T cell proliferation was observed in OVA-induced mice (Fig. 1a). The $CD4^+CD25^+$ T cell subset was significantly increased in groups that received i.v. with antigens. Compared with naive mice (9.97±1.38%), the percentage of splenic $CD4^+CD25^+$ T cells was significantly increased in OVA-induced mice (15.37±0.22%, $p<0.01$) while that was slightly increased in control mice (11.23±0.41%) (Fig. 1b). These results suggested that OVA-specific tolerance had been induced in BALB/c mice.

Previous studies indicated that the splenocytes, $CD4^+$ T cells and $CD4^+CD25^+$ T cells could transfer the antigen-specific tolerance which would be used to the prevention and control of autoimmune diseases and transplantation rejection (Graca *et al*., 2002; Becker *et al*., 2003; Morgan *et al*., 2005). To determine whether OVA-ITF could transfer OVA-specific tolerance to normal BALB/c mice, the OVA-ITF, splenic lymphocytes, naive ITF or an equivalent volume of PBS were injected into recipient mice. Then mice were tested for DTH reaction and sacrificed to determine OVA-specific T cell proliferation. The DTH reactions to OVA of mice that received OVA-ITF and lymphocytes were significantly reduced to that of naive ITF and PBS control mice ($p<0.01$) (Fig. 2a) which prompted that OVA-ITF could transfer peripheral tolerance in an OVA-specific manner. The proliferation of OVA-specific T cells was significantly suppressed by OVA-ITF and TSLC from OVA tolerant mice ($p<0.01$). In contrast, the transfer of naive ITF from naive mice had little effect on the proliferation of OVA-specific T cells. BSA stimulation also had no significant effect on splenic T cell proliferation in any experimental group, though the response to BSA was partly suppressed by both OVA-ITF and TSLC as a result of antigen-driven bystander suppression (Fig. 2b). These results suggested that tolerance induced by OVA-ITF was antigen-specific.

Preliminary experiments indicated that maximum suppression of lymphocyte proliferation occurred at different time in mice that received OVA-ITF compared with that received TSLC (data not shown) which revealed that the time of OVA-specific tolerance establishment was present to be diverse.

Considering the importance of $CD4^+CD25^+$ T cells in the induction and maintenance of peripheral tolerance, kinetic analysis of $CD4^+CD25^+$ T cell subset in peripheral blood due to peripheral tolerance established by OVA-ITF were performed. The percentage of $CD4^+CD25^+$ T cells

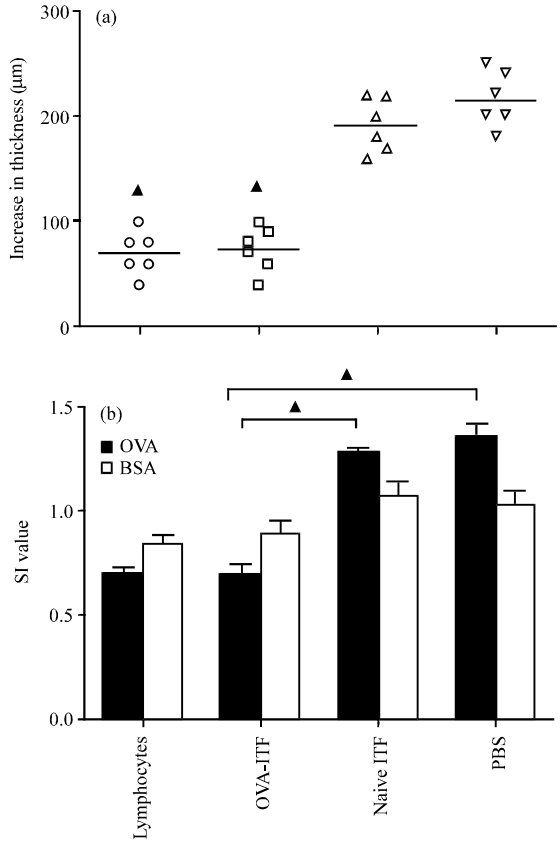

Fig. 2: The regulatory functions of OVA-ITF from splenic lymphocytes in DTH reaction and lymphocyte proliferation assays. a) OVA-ITF or lymphocytes from OVA-tolerant mice, naive ITF or PBS were transferred into naive BALB/c mice and then the mice were immunized s.c. with 100 μg OVA/CFA. Ear challenge was performed 6 days after the immunization with 50 μg OVA in 20 μL PBS. The DTH reaction was measured by the increase of ear thickness 24 h later. The results are shown using the mean±SD from two independent experiments with six mice per group. ▲, $p<0.01$ vs naive ITF or PBS control. b) OVA-ITF, lymphocytes, naive ITF or PBS solution were transferred to mice followed by immunization with 250 μg OVA. Splenic lymphocytes were isolated and cultured with or without OVA (500 μg mL^{-1}) or BSA (750 μg mL^{-1}) for 72 h. The results are shown using the mean±SD of six mice from two independent experiments. ▲, $p<0.01$

in $CD4^+$ T cells from mice received OVA-ITF was significantly increased ($p<0.01$) after injection with peaks of 10.106±0.108% on day 7 in peripheral blood. While the percentage of $CD4^+CD25^+$ T cells in mice received TSLC

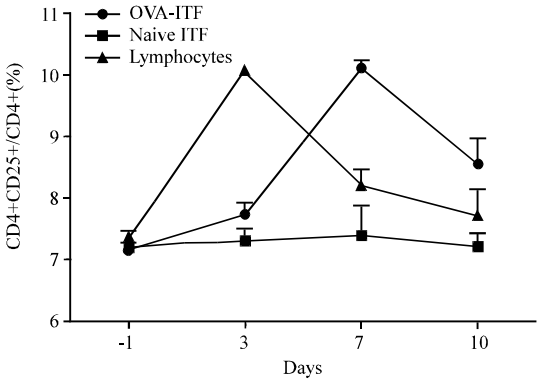

Fig. 3: Kinetic analysis of $CD4^+CD25^+$ T cells in $CD4^+$ T cells from peripheral blood before and after adoptive transfer. The results are shown using the mean±SD from two independent experiments with six mice per group

(positive control) also significantly increased but no change was detected in mice received naive ITF. Interestingly, a peak of $CD4^+CD25^+$ T cells in peripheral blood could reach only 3 days after injection of TSLC (Fig. 3).

To address the origin of OVA-ITF, $ITF_{CD4+CD25+}$, ITF_{CD4-}, $ITF_{CD4+CD25-}$ as well as $CD4^+CD25^+$ T cells from OVA-tolerant mice were transferred into recipient mice. Then DTH reaction and OVA-specific T cell proliferation were determined. The DTH reaction and OVA-specific T cell proliferation were significantly ($p<0.01$) reduced in mice received $ITF_{CD4+CD25+}$ or $CD4^+CD25^+$ T cells (Fig. 4a, b) which hinted that OVA specific tolerance could be induced by $ITF_{CD4+CD25+}$ or $CD4^+CD25^+$ T cells. However, no significant effect was observed in the mice injected with ITF_{CD4-} and $ITF_{CD4+CD25-}$. The proliferative responses of splenic lymphocytes from mice received $ITF_{CD4+CD25+}$, ITF_{CD4-}, $ITF_{CD4+CD25-}$ and $CD4^+CD25^+$ T cell exhibited no significantly different with those received BSA (control) (Fig. 4b). But the DTH reaction and OVA-specific T cell proliferation of splenic lymphocytes in the $ITF_{CD4+CD25+}$ injected mice were nearly the same as those of OVA-ITF treated. These results suggested that the OVA-ITF was only produced by $CD4^+CD25^+$ T cell.

Generally, high levels of TGF-β and/or IL-10 would be observed in peripheral tolerant mice (Strid *et al*., 2004; Ostroukhova *et al*., 2004). In the present study, the supernatants of OVA-stimulated splenic lymphocytes were harvested from each group to measure the level of IL-10 and TGF-β_1. Significant high level of TGF-β_1 was detected in lymphocyte supernatants from mice transferred with OVA-ITF, TSLC, $ITF_{CD4+CD25+}$ and $CD4^+CD25^+$ T cells compared with naive ITF or PBS control mice ($p<0.01$). However, IL-10 was not detectable

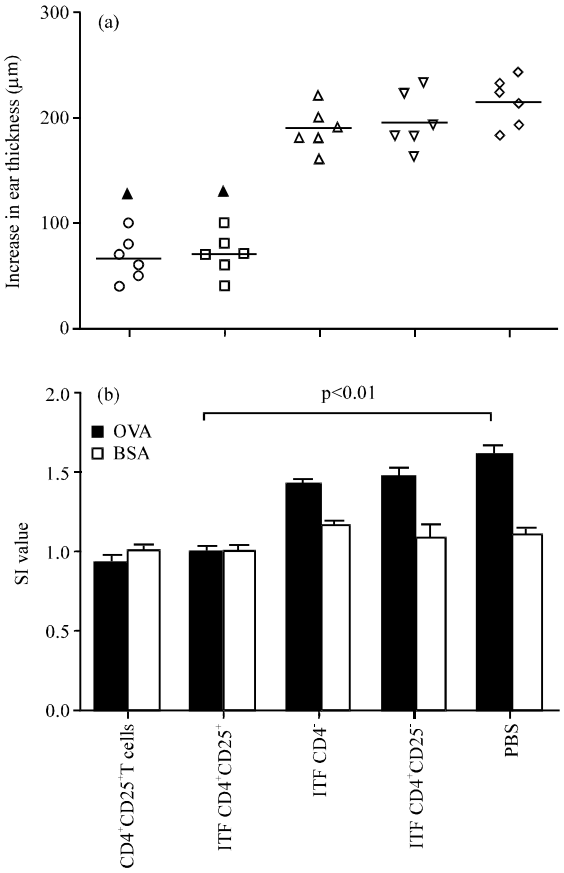

Fig. 4: The regulatory functions of OVA-ITF from $CD4^+CD25^+$ T cells are shown in DTH reaction and lymphocyte proliferation assays. a) Mice received $ITF_{CD4+CD25+}$, ITF_{CD4-}, $ITF_{CD4+CD25-}$, $CD4^+CD25^+$ T cells or PBS and then immunization s.c. with OVA. Ear challenge was performed with 50 μg OVA in 20 μL of PBS 6 days after immunization. The DTH reaction was measured by the increase in ear thickness 24 h later. The results are shown using the mean±SD from two independent experiments with six mice per group. ▲, $p<0.01$ vs PBS control. b) Mice received $ITF_{CD4+CD25+}$, ITF_{CD4-}, $ITF_{CD4+CD25-}$, $CD4^+CD25^+$ T cells or PBS and then followed immunized s.c. with OVA. Splenic lymphocytes were isolated and cultured with or without OVA (500 μg mL^{-1}) or BSA (750 μg mL^{-1}) for 72 h. The results are shown using the mean±SD from two independent experiments

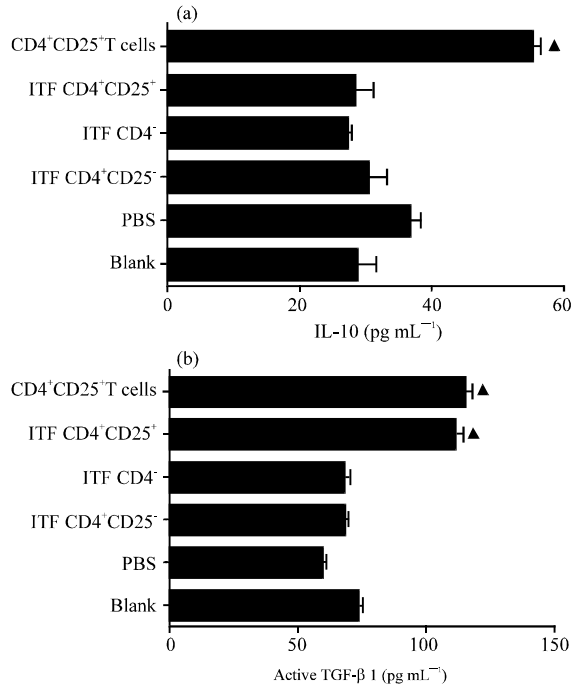

Fig. 5: Cytokine production after adoptive transfer. Naive mice were injected i.v. with $ITF_{CD4+CD25+}$, ITF_{CD4-}, $ITF_{CD4+CD25-}$, $CD4^+CD25^+$ T cells or PBS, followed by immunization with 250 μg of OVA. Naive mice served as an additional control. Splenic lymphocytes were cultured with OVA (500 μg mL^{-1}). The supernatants were harvested after 48 h for IL-10 determination (Fig. 5a) and 72 h for TGF-β_1 determination (Fig. 5b) using ELISA. The results are shown using the mean±SD from two independent experiments with six mice in each group. ▲, $p<0.01$ vs PBS control

in lymphocyte supernatants from mice transferred with OVA-ITF or TSLC (Fig. 5a, b). The level of IL-10 in mice received $ITF_{CD4+CD25+}$ was nearly equal to normal mice and that in mice transferred with $CD4^+CD25^+$ T cells was increased with nearly 2 fold. But no significant difference was observed in the level of IL-10 and TGF-β_1 secreted by lymphocytes in mice injected ITF_{CD4-} or $ITF_{CD4+CD25-}$ (Fig. 5). Thus, OVA-ITF from splenic lymphocytes and $CD4^+CD25^+$ T cells displayed a high level of TGF-β1 and low of IL-10 profile in recipients which indicated that TGF-β_1 was the main suppressive cytokine in recipient mice transferred with OVA-ITF. In the present study, we firstly demonstrated that adoptive transfer of splenic lymphocytes and $CD4^+CD25^+$ T cells from OVA-tolerant mice was able to establish OVA-specific tolerance in recipient mice. A smaller (<3 ku) soluble antigen-specific factor (OVA-TIF) was also extracted from splenic lymphocyte lysates of OVA-tolerant mice which could establish OVA-specific immune tolerance in recipient mice via transfer treated and induce the same effect of peripheral tolerance as those of splenic lymphocytes from OVA-tolerant mice.

The percentage of $CD4^+CD25^+$ T cells could reflect the maintenance of peripheral tolerance. Usually, there is a significant higher percentage of $CD4^+CD25^+$ T cells in tolerant mice than normal mice (Zhang *et al*., 2001). In this study, $CD4^+CD25^+$ T cells in peripheral blood reached a peak 7 days after the treated with OVA-ITF which was 4 days delayed to lymphocyte transfer mice but the percentage of $CD4^+CD25^+$ T cells was not significantly different between the two groups. These results suggested that OVA-ITF could induce peripheral immune tolerance and there must be additional means of suppression enacted by the $CD4^+CD25^+$ T cells other than through production of OVA-TIF. The suppressive function of $CD4^+CD25^+$ T cells depended on interactions with multiple molecules on the surface of or secreted by Tregs and their corresponding receptors of target cells. During interaction, OVA-ITF might play a key role in determining the immunoregulatory function of $CD4^+CD25^+$ T cells.

Cytokine levels in lymphocyte supernatant also could reflect difference between OVA-ITF and $CD4^+CD25^+$ T cells. The IL-10 level in lymphocyte supernatant was significant lower in mice received OVA-ITF than those received $CD4^+CD25^+$ T cells. But no difference was observed in the level of TGF-β_1. A recent report indicates that the main action of IL-10 produced by Treg cells was to inhibit DC maturation, leading to reduction of MHC class II and co-stimulatory ligand expression (Houot *et al*., 2006). The immature Dcs would secrete IL-10 to induce the proliferation of $CD4^+CD25^+$ T cells that down-regulate the response of Ag-primed $CD4^+$ T cells to normal mature DCs (Wakkach *et al*., 2003; Mahnke *et al*., 2007). In the study, adoptive transfer of $CD4^+CD25^+$ T cells could increase IL-10 and TGF-β_1 levels dramatically whereas only TGF-β_1 level was increased with OVA-ITF treated. These results suggested that the OVA-ITF would mainly affect level of TGF-β_1 cytokine which agreed with previous studies that TGF-β was required for Treg cells to mediate their suppressor function in the periphery (Nakamura *et al*., 2004; Marie *et al*., 2005; Li *et al*., 2006).

CONCLUSION

The smaller soluble antigen-specific factor (OVA-TIF), extracted from $CD4^+CD25^+$ T Cells of OVA tolerant mice was a novel and low MW factor and totally different from other suppressive components reported previously. OVA-TIF was produced by $CD4^+CD25^+$ T cell subset and could induce OVA-specific peripheral tolerance independently *in vivo* with TGF-β_1 as its main suppressive cytokine in recipient mice. These results have important implications for finding potential therapeutic route of prevention and control of graft rejection, autoimmune and related diseases. However, considering the clinical application of these disease-related tolerated factors, purification and physical evidence of the OVA-TIF would be further recognized using some other biochemical studies in the future.

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