Article

Anti-Idiotypic Regulatory Responses Induced by Vaccination with DNA Encoding Murine TCR Vα5 and Vβ2

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There is evidence suggesting that anti-idiotypic regulation against T cells plays a role in maintaining homeostasis in the immune system, although its mechanism is not fully understood. By using DNA constructs encoding the TCR Vα5.2 and Vβ2.1 chains derived from an ovalbumin (OVA)-specific T cell clone (OVA-T), we herein demonstrated that vaccination with TCR-DNA effectively induced anti-idiotypic cellular as well as humoral responses. Serum samples from the TCR-DNA-vaccinated BALB/c mice were able to stain T cells in an idiotype-specific manner. $CD4^+$ T cells from the TCR-DNA-vaccinated mice proliferated in response to stimulation with irradiated syngeneic OVA-T cells and secreted interferon-γ but very little IL-4. Splenocytes from the TCR-DNA-vaccinated mice showed strong idiotype-specific CTL activity against the OVA-T cells. Furthermore, adoptive transfer of the $CD4^+$ or $CD8^+$ T cells from the TCR-DNA-vaccinated mice resulted in hyporesponsiveness of syngeneic recipients. These results demonstrated that vaccination with DNA encoding TCR can effectively activate anti-idiotypic regulatory responses *in vivo* and thus providing a useful way for immunological intervention. *Cellular & Molecular Immunology*. 2007; 4(4):287-293.

Key Words: T cell vaccination, immunoregulation, anti-T-cell antibody

Introduction

T cell receptors (TCRs) are essential for antigen recognition and activation of T lymphocytes. Certain $CD4^+$ and $CD8^+$ anti-idiotypic regulatory T cells recognize clonotypic or Vα/Vβ-specific determinants of the TCR presented by autologous T cells (1-3) and are capable of down-regulating T cell reactivity in an idiotype-specific manner (4). Such self-reactive, anti-TCR regulatory T cells may be useful in therapeutic attempts to selectively downregulate pathogenic, autoaggressive T-cell responses *in vivo*.

The use of DNA immunization is an attractive approach to investigate possible ways of regulating the immunological homeostasis, since it induces strong cellular and humoral immunity and long-lived memory lymphocytes capable of modulating autoimmune conditions (5-8). Such DNA immunization can evoke both $CD8^+$ and $CD4^+$ T cell responses restricted by class I and class II MHC molecules respectively, as well as humoral responses (9, 10). The plasmid vectors also contain immunostimulatory nucleotide sequences with unmethylated CpG motifs that can activate antigen presenting cells (APCs), e.g. dendritic cells (DCs), and result in their secretion of IL-12 (11,12).

Vaccination with DNA encoding TCR (TCR-DNA) has been investigated as a specific immunosuppressive therapy in several settings of autoimmune disorders. For example, injection of DNA encoding Vβ8.2 of an autoreactive TCR led to effective protection from experimental autoimmune encephalomyelitis (EAE) in mice (13). In order to further investigate the role of anti-idiotypic cellular and humoral responses in maintaining immunohomeostasis, we have here prepared expression vectors (pcDNA3) harboring the TCR Vα5 or Vβ2 genes expressed by an ovalbumin-specific T cell clone, OVA-T, from BALB/c mice. BALB/c mice were then vaccinated with the DNA constructs and their anti-idiotypic cellular and humoral responses characterized. The results arising from this study will enable us to better understand the mechanisms of immunoregulation mediated by anti-idiotypic regulatory T cells and also antibodies (Abs).

Materials and Methods

Mice

Female BALB/c mice ($H\text{-}2^d$), 6 to 8 weeks of age, were purchased from the Animal Breeding Center of Peking

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University Health Science Center and maintained at the animal facilities in the Department of Immunology, Peking University Health Science Center.

Plasmid constructions

Vα5.1 TCR chain and Vβ2.1 TCR chain of the OVA-T cell clone were cloned into pcDNA3.1 plasmid vector which was then used for transfection of JM109 strain. Colonies were selected and used for miniprep. DNA from colonies with the right length insert was amplified and then sequenced to verify the insertion of the right gene with an appropriated open reading frame.

DNA vaccination and blood sample collection

For DNA vaccination, BALB/c mice were injected with 50 μl of 0.25% bupivacaine two days before the DNA injection as this was known to enhance the efficiency of DNA plasmid vaccination. Plasmid DNA (50 μg dissolved in 50 μl PBS) was then injected into each tibialis anterior muscle of the mice. This injection was repeated three times with fortnight intervals.

For OVA immunization, mice were injected sub-cutaneously at the base tail with 100 μg OVA emulsified in 5% saponin (Sigma). The immunization was boosted 3 weeks later with the OVA in IFA. Mice were bled for serum samples at different time points after the immunization.

Proliferation assays

Freshly prepared draining lymph node cells and splenocytes (4×10^5) from mice, following OVA immunization or TCV, T cells from long-term culture lines, were incubated in 96-well flat-bottomed plates (Nuce, Rosklide, Denmark) in the presence, or absence, of irradiated stimulatory T cells in a total volume of 200 μl of RPMI-1640 (Sigma). Triplicate wells were dispensed for each group. The cells were cultured at 37°C with 5% CO_2 for 3 days. In the last 8 h of incubation, 0.5 μCi ^{3}H-thymidine (^{3}H-TdR, BRM Inc., Beijing) was added into each well. The cells were then harvested, using a 96-well plate harvester (Tomtec, USA) onto fiberglass filters and radioactivity on the filters was counted in a MicroBeta Trilux LSC counter (EG & G Wallac, Turky, Finland).

Synthetic peptides

The each V region of the TCR α (Vα5.2-Jα2) and β (Vβ2.1-Jβ1) chains of OVA-T cells contains a CTL epitope restricted by H-2^d. Peptides representing these fragments, p11 (VADYSNNRLTL) and p12 (QYFGPGTRLLVL), respectively, were synthesized by Hanyu Biotech Co. Ltd., (Shenzhen, China). The resultant peptides were HPLC purified to purities higher than 80% and supplied as lyophilized powder. The peptides were dissolved in distilled water (10 mg/ml), filter sterilized, aliquoted and stored at -80°C, and they were diluted with culture medium before use.

Cytotoxic T-lymphocyte assays (JAM test)

Splenocytes from OVA- or TCV-immunized mice were co-cultured in 24-well plates with irradiated OVA-T cells at a ratio of 10:1 for 5 days. After washing with RPMI medium, cells were counted and serially diluted into 96-well round-bottom microtiter plates against various [^{3}H]-thymidine-pulsed (2.5-5 μCi/ml per 2.5-5 × 10^5 cells) target cells. The target cells (10^4) were incubated with effector cells at varying E/T cell ratios at 37°C for 6 h. For peptide pulse, P815 (H-2^d) cells were incubated with peptide p11 (10 μg/50 μl), or p12 peptide, or medium alone, for 1 h at 37°C followed by 2 washes. Specific lysis was determined by the amounts of ^{3}H-retainment in the chromosomal DNA of the target cells. The percentage of specific lysis was calculated as: [(spontaneous release - experimental release) / spontaneous release] × 100% while spontaneous release was defined as the mean cpm counted from three replicates of 1×10^4 labeled cells incubated in medium alone (14, 15).

Enzyme-linked immunosorbent assay (ELISA)

ELISA plates were coated with ovalbumin (10 μg/ml) in carbonate buffer (pH 9.6) overnight at 4°C , and then washed four times with PBS containing 0.05% Tween 20 (PBS-T) to remove unbound antigen. Plates were subsequently incubated with blocking solution (2% milk powder in PBS) overnight at 4°C. After further washes with PBS-T, the plates were incubated with serial diluted sera for 2 h at room temperature, followed by 3 washes to remove unbound antibody. The plates were then incubated with HRP-labeled goat anti-mouse IgG antibody for 1 h at 37°C. The *orthophenylenediamine* substrate (Sigma, USA, 100 μl/well) was added after 5 washes with PBS-T and the wells incubated for 2 min at room temperature. Stop solution (50 μl, 2 M H_2SO_4) was added to each well and the optical density at 492 nm was read immediately.

Flow cytometric analysis

For indirect immunofluorescence staining, OVA-T cells (10^6 cells/100 μl PBS) were treated with mouse antisera for 30 min at 4°C, followed by three washes with PBS containing 1% bovine serum albumin (BSA). The cells were then incubated with FITC-conjugated goat anti-mouse IgG (γ-specific, 1:1000 dilution) for 45 min on ice. After washes, the cells were analysed on a fluorescence-activated cell sorter (FACScan; Becton-Dickinson, NJ). For direct staining, the spleen cells were incubated with FITC- or PE-labelled mAb for 30 min at 4°C, washed three times with PBS containing 1% BSA and then analyzed for CD8, CD3, CD4, Vβ2, Vβ4 expression on FACScan. The Vβ2 and Vβ4 mAb were purchased from PharMingen (USA).

Separation of $CD4^+$ and $CD8^+$ T cells and their adoptive transfer

Spleens from BALB/c mice were gently ground on stainless steel sieves in 5 ml of PBS. After centrifugation at 1,500 g for 5 min, erythrocytes in the pellet were lysed using erythrocyte lysis buffer (Sigma). The remaining cells were incubated with FITC-conjugated goat anti-mouse CD4 and PE-conjugated goat anti-mouse CD8 mAb for 30 min on ice. The cells were then subjected to flow cytometric separation

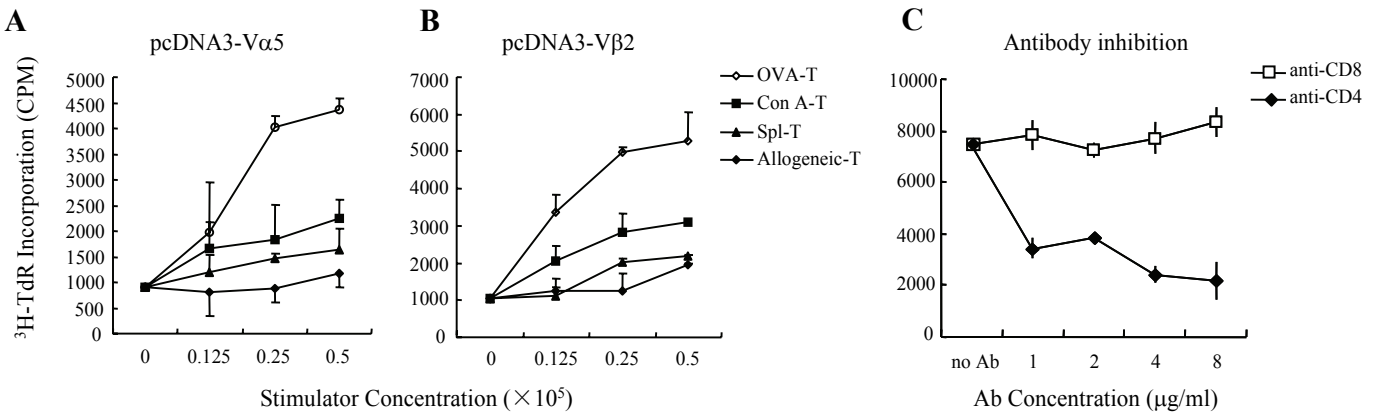

Figure 1. CD4$^+$ T cell responses induced by TCR-DNA vaccination. Groups of BALB/c mice were vaccinated with constructs pcDNA-Vα5.2 (A) or pcDNA-Vβ2.1 (B) and sacrificed for spleens 10 days after the last immunization. The splenocytes were co-cultured with irradiated OVA-T cells, Con A-T cells, syngeneic spleen cells (Spl-T) or allogeneic T cells from BXSB mice. (C) Splenocytes from the pcDNA-Vα5.2 group were also stimulated with irradiated OVA-T cells in the presence of monoclonal Abs against mouse CD4 or CD8. The cells were cultured for 3 days and ^{3}H-TdR was added 8 h before harvesting. The results are expressed as ^{3}H-TdR incorporation into the DNA of responder cells (CPM), the mean ± SD are from triplicate wells.

using a flow cytometry (FACScan, BD). The purities of CD4$^+$ and CD8$^+$ cells thus obtained were more than 96%. For passive transfer experiments, recipient mice were given CD4$^+$ or CD8$^+$ cells (5×10^6 cells/mouse) from the pcDNA-Vα5.2-immunized mice *via* the tail vein, which was followed by *s.c.* immunization with OVA (100 μg) emulsified in 5% saponin at the base of tail the following day.

Statistical analysis

All experiments were repeated at least three times and representative results are shown. Student's *t* test was performed for comparison of the data; significance was defined as *p* value is no more than 0.05% ($p < 0.05$).

Results

CD4$^+$ T cell responses induced by TCR-DNA vaccination

Groups of BALB/c mice were given 4 intramuscular injections, with fortnight intervals, with the vector pcDNA3 alone or pcDNA3 constructs harboring the gene encoding for mouse TCR Vα5.1 (pcDNA-Vα5) or Vβ2.1 (pcDNA-Vβ2) chains. Expression of TCR Vα5.1 and Vβ2.1 in the local tissues of the pcDNA-Vα5- and pcDNA-Vβ2-vaccinated animals was verified by PCR detection of the relevant mRNA and also immuno-histochemical staining using appropriate monoclonal Abs (data not shown). The mice were sacrificed for spleens 7 to 14 days after the last dose of immunization and the splenocytes were stimulated, in proliferation assays, with irradiated stimulator cells including unstimulated or Con A-activated syngeneic splenocytes (ConA-T) and also OVA-T cells. OVA-T cells induced vigorous proliferation of the splenocytes from both the pcDNA-Vα5 and pcDNA-Vβ2 groups (Figure 1). These responder splenocytes also proliferated in response to stimulation with ConA-T cells, but the magnitude was much less impressive. As shown in Figure 1C, the proliferation of the responding splenocytes was inhibited almost completely by mAb against murine CD4, but not CD8, indicating that the proliferation herein was

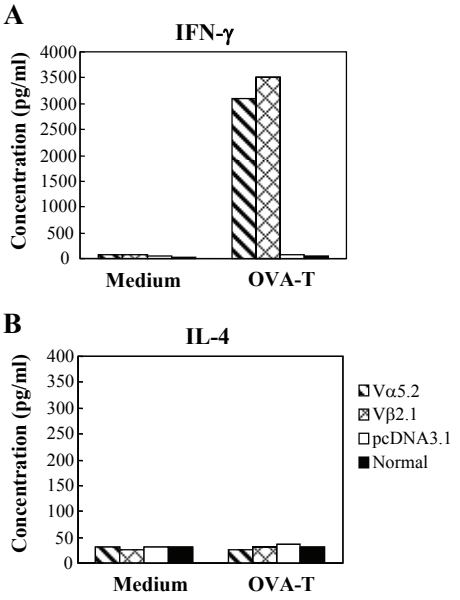

Figure 2. Cytokine secretion of the anti-idiotypic T cells. Groups of BALB/c mice were vaccinated with pcDNA3, pcDNA-Vα5.2 or pcDNA-Vβ2.1, and then sacrificed for spleens 10 days after the last immunization. The splenocytes were also stimulated with irradiated OVA-T cells for 48 h. Splenocytes from naïve BALB/c mice were also included as control (normal). The culture supernatant was then harvested and assayed for IFN-γ (A) and IL-4 (B) by ELISA. The results are expressed as concentration of cytokines (pg/ml).

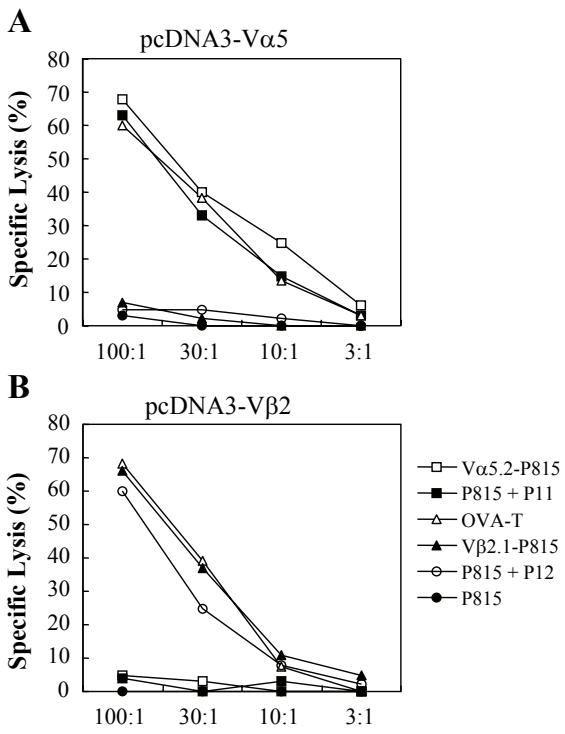

Figure 3. CTL responses induced by TCR-DNA immunization. Groups of BALB/c mice were vaccinated with constructs pcDNA-Vα5.2 (A) or pcDNA-Vβ2.1 (B) and sacrificed for spleens 10 days after the last immunization. The splenocytes were co-cultured with irradiated OVA-T cells for 5 days then titrated against different target cells in a 6 h JAM test. The results are expressed as specific lysis (%) of the target cells at the indicated effector/target (E/T) ratio.

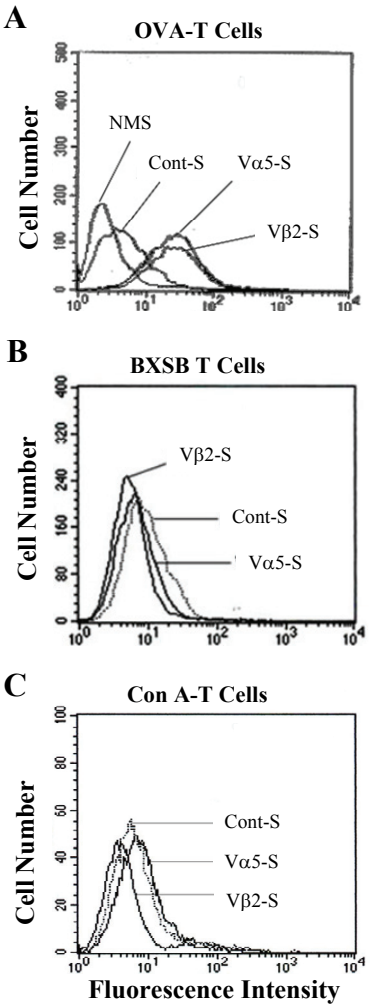

Figure 4. TCR-specific Abs induced by TCR-DNA vaccination. Serum samples from naïve BALB/c mice (NMS) or BALB/c mice after vaccination with pcDNA3 (Cont-S), pcDNA-Vα5.2 (Vα5-S) or pcDNA-Vβ2.1 (Vβ2-S) were used, as primary Abs, to treat OVA-T cells (A), BXSB T cells (B) and Con A-T cells (C) for 30 min at 4°C. The cells were then stained with FITC-labeled goat anti-mouse IgG and analyzed on a flow cytometer.

mainly mediated by $CD4^+$ T cells.

Cytokine profiles of the T cells induced by TCR-DNA vaccination

In order to assess the cytokine secretion profiles of the OVA-T-specific T cells induced by TCR-DNA vaccination, splenocytes (responders) from the vaccinated mice were cultured together with irradiated OVA-T cells (stimulators) and the supernatant assayed 48 h later for IL-4 and IFN-γ by ELISA. As shown in Figure 2, the responder T cells produced substantial amount of IFN-γ but very little IL-4, suggesting that T cell response induced by TCR-DNA vaccination was dominated by Th1 cells.

CTL responses induced by TCR-DNA vaccination

To determine whether anti-idiotypic CTL responses were also elicited in BALB/c mice following TCR-DNA immunization, splenocytes from the vaccinated animals were tested in killing assays against the following target cells: OVA-T cells; P815 cells stably transfected with plasmids pcDNA3 (as control), or pcDNA-Vα5 (P815-Vα5.2), or pcDNA-Vβ2 (P815-Vβ2.1); and P815 pulsed with synthetic peptide p11 (amino acid sequence: VADYSNNRLTL) or peptide p12 (amino acid sequence: QYFGPGTRLLVL) representing $H\text{-}2^d$-restricted T cell epitopes in the TCR α and β chain V regions of OVA-T cells, respectively. As illustrated in Figure 3, splenocytes from mice immunized with pcDNA-Vα5 showed strong cytotoxicity against OVA-T cells, P815-Vα5.2 cells and p11-pulsed P815 cells. Splenocytes from mice inoculated with pcDNA-Vβ2 lysed specifically OVA-T cells, P815-Vβ2.1 cells and p12-pulsed P815 cells. It is thus clear that TCR-DNA vaccination of BALB/c mice effectively induced TCR idiotypic-specific CTL responses *in vivo*.

TCR-specific Abs induced by TCR-DNA vaccination

Serum samples were collected from mice 2 weeks post-vaccination and employed in indirect immunofluorescence staining of OVA-T cells followed by flow cytometric analysis.

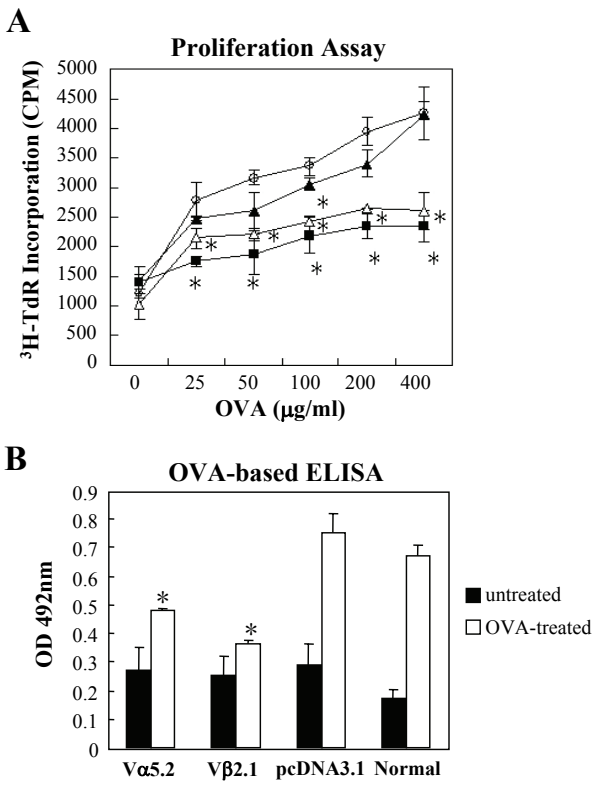

Figure 5. Hyporesponsiveness of BALB/c mice following TCR-DNA vaccination. Groups of BALB/c mice were vaccinated with pcDNA3 (solid triangle), pcDNA-Vα5.2 (solid square) or pcDNA-Vβ2.1 (open triangle) and then *s.c.* immunized with OVA emulsified in CFA. Naïve BALB/c mice were also immunized with OVA/CFA as control (open square). Draining lymph nodes were collected from the mice 10 days after the OVA immunization and lymph node cells stimulated with OVA for 3 days in standard proliferation assays (A). The results are expressed as ^{3}H-TdR incorporation into the DNA of responder cells (CPM). Serum samples were also collected from the DNA-vaccinated animals immunized with (open histogram), or without (solid histogram), OVA and assayed for OVA-specific IgG Abs by ELISA (B). Results are represented as the mean ± SD from five mice in each group. *, $p < 0.05$, compared with normal control group.

As shown in Figure 4, OVA-T cells were positively stained by the sera from the pcDNA-Vα5- or pcDNA-Vβ2-vaccinated groups, but not that from the pcDNA3 control group. None of the serum samples was able to stain allogeneic CD4 T cell clone (BXSB-T) expressing a different set of TCRα and β chains, implying that TCR-DNA vaccination elicited TCR- specific humoral responses *in vivo*.

Hyporesponsiveness induced by TCR-DNA vaccination of BALB/c mice

BALB/c mice were subjected to TCR-DNA vaccination and then assessed for ability to respond to *s.c.* OVA immunization. Their draining lymph node cells were harvested 7 days after OVA immunization and then stimulated with OVA in standard proliferation assays. As shown in Figure 5A, lymph node cells from the pcDNA-Vα5- and pcDNA-Vβ2-vaccinated mice responded poorly to OVA stimulation *in vitro* in comparison to cells of the pcDNA3 control group. In addition, OVA-specific serum IgG levels of these groups were also significantly lower than that of the control mice (Figure 5B).

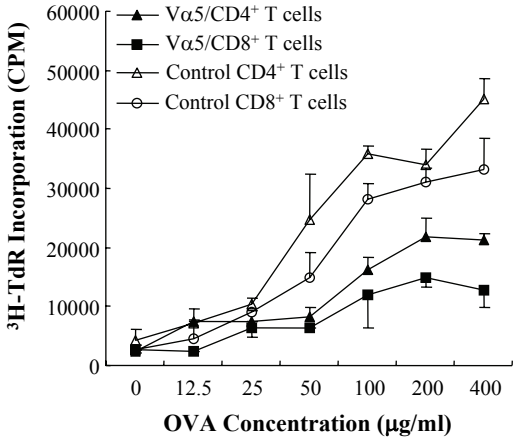

Figure 6 Immunosuppression mediated by CD4$^+$ and CD8$^+$ cells from BALB/c mice after TCR-DNA vaccination. CD4$^+$ and CD8$^+$ T cells were fractionated from the splenocytes of BALB/c mice that had been vaccinated with pcDNA3 (control) or construct pcDNA-Vα5 (Vα5). Naïve syngeneic recipients were injected *i.v.* with 2×10^6 purified T cells and then immunized *s.c.* with OVA at the base of the tail. Draining lymph node cells were collected 10 days later and stimulated with OVA for 3 days in standard proliferation assays. The results are expressed as ^{3}H-TdR incorporation (CPM).

T cells from the TCR-DNA-vaccinated mice exert immunosuppression in naïve recipients

To assess whether anti-idiotypic T cells were responsible for mediating the hyporesponsiveness in TCR-DNA vaccinated mice, CD4$^+$ and CD8$^+$ T cells were fractionated from the vaccinated animals and then passively transferred to naïve syngeneic recipients. The recipient mice were subsequently immunized *s.c.* with OVA and their draining lymph node cells tested for ability to respond to OVA re-stimulation in proliferation assays. The recipient animals responded less well to subsequent OVA challenge compared with the control mice (Figure 6), suggesting that the passively transferred T cells exerted suppressive effect *in vivo*.

Discussion

Our results indicate that TCR-DNA vaccination induces anti-idiotypic responses mediated by Abs, CD4$^+$ and CD8$^+$ T cells, which lead to an Ag-specific hyporesponsiveness (Ag-specific tolerance) *in vivo*. TCR-DNA vaccination elicited MHC class I-restricted CD8$^+$ CTL capable of lysing OVA-T cells, Vα5- or Vβ2-transfectant P815 cells, and also TCR peptide-pulsed P815 cells (Figure 3). Previous studies

have also demonstrated that autoimmune TCR epitopes are presented by MHC-I molecules and the $CD8^+$ T cells appear to be major suppressors of the autoimmune T cells (16, 17). It is possible that fragments of the TCR can be presented by MHC-I molecules and serve as targets for recognition by anti-idiotypic MHC-I-restricted $CD8^+$ CTL. There are also examples where Abs can specifically recognize the idiotypic epitopes in TCR and play a role in regulating T cell activity. However, the mechanism for recognition of OVA-T cells by $CD4^+$ Th1 type T cells is not yet clear. Murine T lymphocytes express MHC class I but not class II molecules. Direct and specific murine T-T interactions in a syngeneic/autologous system are expected to be class I but not class II restricted. Previous investigators have proposed that DC and macrophages can process and present the T cell determinants in a class I or class II MHC context (5, 18, 19). In any case, anti-idiotypic Th responses may be of importance for the generation of regulatory CTL and humoral response. Recently, two different groups have demonstrated that $CD4^+$ T cell help *via* a class II MHC-dependent pathway is required for the efficient generation of an effective CTL and humoral response following DNA immunization (20, 21). Evidence for the existence of anti-idiotypic suppressive $CD4^+$ T cells was also obtained in T cell vaccination studies using myelin basic protein-specific T cells or its immunogenic peptides, and in TCR peptide-mediated T-T interaction between myelin basic protein-specific $CD4^+$ T cell clone and their putative regulator T cells (22, 23).

Although strong anti-idiotypic CTL responses were elicited by TCR-DNA vaccination in mice, it is unlikely that such CTLs caused substantial elimination of TCR Vα5- or Vβ2-bearing T cells from the periphery, because the percentage of TCR-Vβ2-positive T cells in the splenocyte population from the pcDNA-Vβ2 group was not significantly different from that of the control group, as evidenced by immunofluorescence staining using PE-labeled anti-TCR-Vβ2 mAb (data not shown).

In conclusion, TCR-DNA vaccination can trigger anti-idiotypic regulatory responses against T cells, thereby leading to down-regulating adaptive immune responses in a selective manner. This study underlines a possibility of using TCR-DNA vaccination as an effective means of modulating immune responses *in vivo*.

Acknowledgements

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