FOOD & FUNCTION

Compound CVT-E002 attenuates allergen-induced airway inflammation and airway hyperresponsiveness, in vivo

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Immune modulation has been a sought after means of therapy for atopic diseases. CVT-E002 is an extract derived from North American Ginseng shown to promote T-helper-1-like responses. We determined what effect CVT-E002 could have in a mouse model of atopic asthma. We report that oral CVT-E002 inhibited the development of allergic airway inflammation and airway hyperresponsiveness. This correlated with an increased presence of interferon-γ in the lung, and also increased regulatory T cells and IL-10. The ability of CVT-E002 to induce regulatory T-cell development was also seen in human in vitro co-cultures.

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People with allergic diseases like asthma have airway hyperresponsiveness (AHR) and inflammation characterized by T-helper-2-like lymphoctyes and accompanying eosinophils [1]. Immune modulation would be an attractive means to treat allergic asthma. For instance, T-helper-1 (Th1)-like responses inhibit those of T-helper-2 responses [2, 3]. Glucocorticosteriods, the main therapy for asthma, appear to promote some of their anti-asthma effects by this mechanism [4]. CVT-E002 is an extract derived from North American ginseng (*Panax quinquefolius* L., Fam. Araliaceae). CVT-E002 is marketed as an immune modulator. It can induce Th1 immune response in mice [5, 6] and in clinical studies decreases the incidence and duration of common cold symptoms [7, 8].

Given this, we hypothesized that CVT-E002 could act as an immune modulator in an animal model of asthma. In the present study, we show that when CVT-E002 is administered to allergen-sensitized BALB/c mice by oral gavage before allergen challenge, the pathological features of atopic asthma are significantly attenuated.

Male BALB/c mice were sensitized via intraperitoneal injection on days 1 and 7 with 10 μg ovalbumin (OVA) and 2 mg $Al(OH)_3$ (Fig. 1A). Seven days following the final sensitization, mice were gavaged with 200 mg/kg CVT-E002 or sham vehicle alone for 7 consecutive days; 24 h following the final gavage, the mice were challenged twice by intranasal OVA or saline (control) on alternating days. Twenty-four hours after OVA challenge all mice were assessed for AHR and airway inflammation. Sensitized mice given sham treatments before OVA challenge had AHR and airway eosinophilia, with a strong lymphocyte infiltration, compared with similarly treated non-sensitized mice (Fig. 1B and C). In contrast, sensitized mice gavaged with CVT-E002 before challenge did not develop AHR and had lower airway eosinophil and lymphocyte numbers than those seen in sham-treated sensitized mice. The non-sensitized CVT-E002-treated mice did not show differences in airway responsiveness or bronchoalveolar lavage (BAL) cell counts compared with non-sensitized mice not fed CVT-E002.

The 200 mg/kg dose of CVT-E002 was chosen based on both previous human and mouse data after back calculating

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Abbreviations: AHR, airway hyperresponsiveness; **BAL,** bronchoalveolar lavage; **DC,** dendritic cell(s); **moDC,** monocyte-derived DC; **OVA,** ovalbumin; **Th1,** T-helper-1; **T_{reg},** regulatory T cell(s)

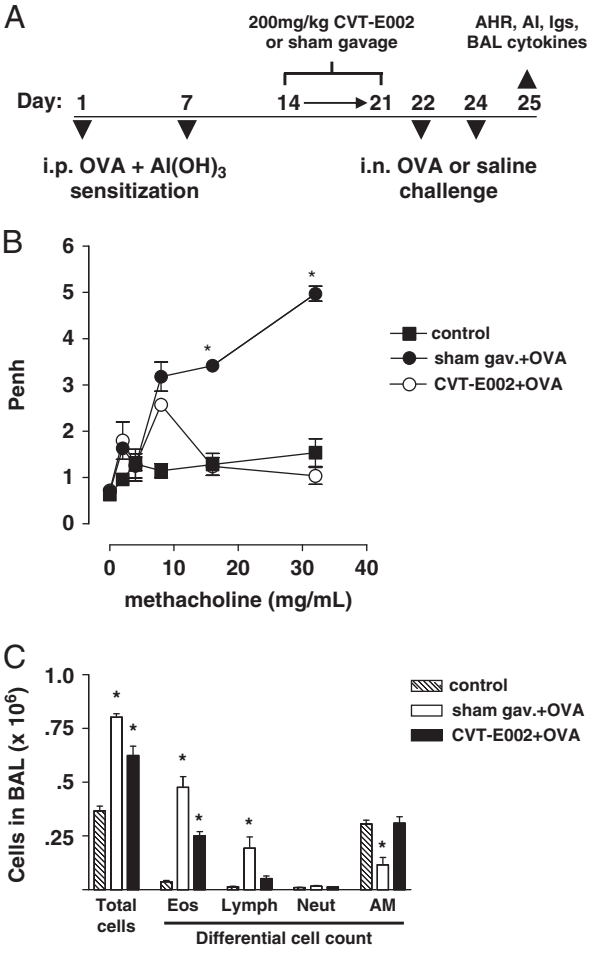

Figure 1. (A) Experimental time course. i.n., Intranasal; i.p., intraperitoneal; OVA, ovalbumin; AHR, airway hyperresponsiveness; AI, airway inflammation. View Within Article (B) Enhanced pause (Penh) was measured by whole-body plethysmography to determine AHR in response to methacholine challenge ($n = 10$). $^*p < 0.05$ compared with control or sham gavage with OVA challenge. (C) Airway inflammation was determined by numbers of inflammatory cells (Eos, eosinophils, Lymph, lymphocytes, Neut, neutrophils, AM, alveolar macrophages) in the (BAL) fluid ($n = 10$). $^*p < 0.05$ compared with control or sham gavage with OVA challenge. All results are shown as mean ± SEM.

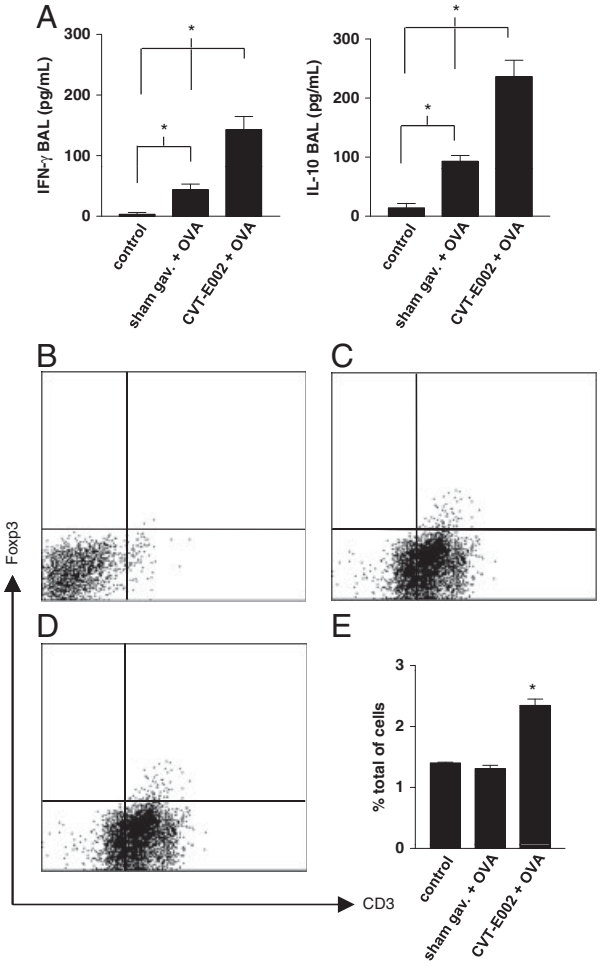

Figure 2. Twenty-four hours after the final challenge BAL samples were taken and the levels of IFN-γ and IL-10 were determined by ELISA (A) ($n = 5$). In addition following OVA challenge, the lungs were excised from the mice, digested, and single cell suspensions were stained for CD3 and Foxp3 expression. Shown are isotype control antibodies (B), the expression of CD3 and Foxp3 in sham-treated mice (C) versus CVT-E002-treated mice (D). The percentage of Foxp3 expression is shown for sham- versus CVT-E002-treated groups (E) ($n = 3$). $^*p < 0.05$ compared with control or sham gavage with OVA challenge. All results are shown as mean ± SEM.

an equivalent amount of ginseng root to CVT-E002 extract [9]. Please note that mouse and human doses cannot be directly translated. When the FDA does a rough translation for safety, they estimate that humans are 15 times more sensitive to medications than mice. The general guidelines for dose conversions are outlined in http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatory Information/Guidances/ucm078932.pdf, 2005. Thus, dose conversions between humans and mice must be made [10].

To determine the potential mechanism of CVT-E002, we looked at alteration in sensitization or production of immune-modulating Igs and cytokines. Twenty-four hours after final challenges, circulating OVA-specific Igs (IgE, IgG_1, and IgG_{2a}) and BAL fluid IL-4, 5, 10, 13, and interferon (IFN)-γ levels were measured by ELISA as described previously [11]. There were no differences in the levels of IgE, IgG1, or IgG2a in the sensitized mice irrespective of CVT-E002 treatment (Supporting Information Fig. 1). However, sensitized mice given CVT-E002 showed significantly higher levels of both IFN-γ and IL-10 in their BAL fluid compared with sham-treated mice (Fig. 2A). Levels of IL-4, 5, and 13 showed no differences.

Given the IL-10 data, we hypothesized that CVT-E002 could in part be exerting its inhibitory effect through the

induction of regulatory T cells (T_{reg}). We measured the number of Foxp3-expressing T cells in the lungs. Twenty-four hours following the OVA challenge, lungs were excised excluding any lymph node tissue. Parenchyma was cut into pieces and digested. The numbers of $CD3^+Foxp3^+$ cells were determined by flow cytometry versus isotype control (Fig. 2B). Sensitized mice receiving sham-gavage treatments before OVA challenge had lower numbers of Foxp3-expressing T cells (Fig. 2C and E) compared with CVT-002-treated sensitized mice (Fig. 2D and E).

To determine whether our murine data were applicable to humans, we performed co-culture experiments with human T cells and dendritic cells (DC), with or without CVT-E002. Blood was taken from consenting donors. After red cell sedimentation, the leukocyte-rich supernatants were subjected to density centrifugation. The mononuclear cells were placed in six-well plates, and after incubation, unattached cells were removed and run through a nylon wool column. The eluted lymphocyte-enriched population was stored at −80°C. Adherent cells remaining in the six-well plates were washed and GM-CSF and IL-4 were added to generate monocyte-derived DC (moDC). The moDC were irradiated prior to further use. T cells were thawed and added to 96-well plates with or without moDC with or without CVT-002 (500 μg/mL). Plates containing various combinations of cells were incubated for 7 days and stained for the expression of Foxp3 with isotype control (Fig. 3A).

T cells incubated alone served as a baseline measure of Foxp3 expression (Fig. 3B). The number $Foxp3^+$ T cells were similar in wells of T cells with or without CVT-E002 treatment (Fig. 3C and D). Further, adding moDC to the T cells without CVT-E002 did not increase the number of $Foxp3^+$ T cells compared with T cells alone. However, when CVT-E002 was added to T cells incubated with moDC the number of Foxp3-expressing T cells increased significantly (Fig. 3E and F). These Foxp3-expressing T cells were all $CD4^+$ $CD25^{hi}$.

T cells were isolated as above and frozen in two aliquots. T-cell aliquot ♯1 was used for the generation of T_{reg} using autologous moDC with or without CVT-E002 as described above. After 7 days, these cells were washed and resuspended. Viability of cells was similar between treatment groups. T-cell aliquot ♯2 was stimulated by CD3/CD28 beads. After 5–7 days, both T-cell aliquots ♯1 and ♯2 were combined 1:1 in 96-well plates. After 72 h T cells were assessed for annexinV staining by flow cytometry or BrdU uptake. We found that CD3/CD28 stimulated T cells added to T cells with prior CVT-E002 treatment showed more apoptosis and less proliferation (Fig. 3G and H). From this we concluded that the CVT-E002-generated T_{reg} were indeed functional.

In this report, we demonstrate a potential new immune modulating agent for the treatment of allergic diseases. We hypothesized that CVT-E002 would exert an inhibitory effect in our mouse model of allergic asthma. We were surprised that in addition to the induction of IFN-γ,

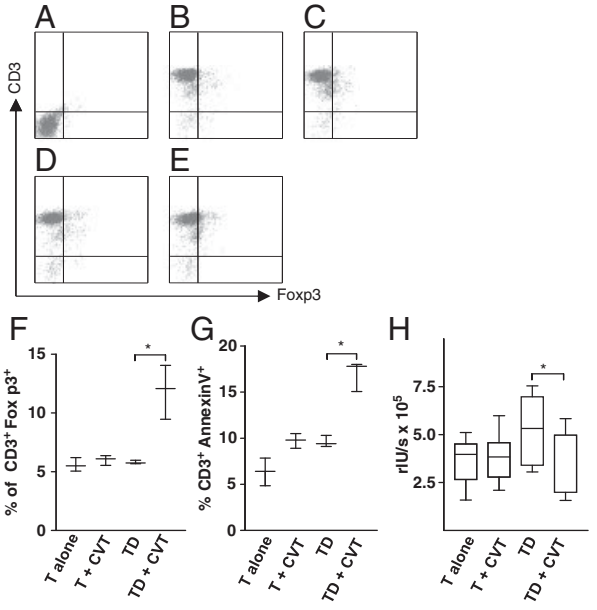

Figure 3. CVT-E002 induces the functional $CD3^+$, $Foxp3^+$ T_{reg} in human T cell, DC co-cultures, in vitro. Human T cells were exposed or not to CVT-E002 (500 μg/mL, for 7 days) in the presence or absence of autologous irradiated moDC. Shown are the untreated T cells alone, stained either with isotype control antibodies (A) or the CD3- and Foxp3-specific antibodies (B). CVT-E002-treated T cells without moDC are shown as (C). T cells with moDC are shown without CVT-E002 (D) or with CVT-E002. The percentage of $CD3^+Foxp3^+$ cells is shown for each group ($n = 3$) (F). To assess T_{reg} function, some of the T-cell/moDC co-cultures above were washed and resuspended. These cells were added to T cells along with CD3, CD28, CD2 stimulatory microbeads for 72 h. T-cell apoptosis and proliferation were measured by annexinV staining (G; $n = 4$) and BrdU uptake measured in relative light units (rlU) (H; $n = 4$), respectively. $^*p < 0.05$ compared with controls. All results are shown as mean ± SEM.

CVT-E002 treatment also induced increased IL-10 levels in the BAL with recruitment of T_{reg} into the lung. Thus, in addition to its reported induction of a Th1-like immune phenotype [5, 6], we find that there is also a potential regulatory immune modulating effect.

Precisely where in the body CVT-E002 exerts its effects remains to be determined. We believe there are two equally plausible hypotheses. The first is that the CVT-E002, being introduced orally, could be dispersed throughout the body systemically, such that it could impact the resident immature DCs in the lungs as well as DC recruited to the lungs during allergen challenge. The second hypothesis is that the CVT-E002 is affecting DC in the gastrointestinal tract, which induces the development of T_{reg} in the mesenteric lymph nodes. These T_{reg} then circulate systemically and are present in the lung during allergen challenge to inhibit inflammatory responses.

Although we believe that T_{reg} responses are induced in our model, we have not confirmed whether these regulatory

cells are naturally ocurring T_{reg}, OVA-specific T_{reg}, or CVT-E002-specific T_{reg}. Mice were given CVT-E002 1 wk following OVA sensitization when OVA should not be present. Thus, it is unlikely that CVT-E002-treated DC could have acquired OVA and processed it for presentation to OVA-specific T cells. Further, in the human co-culture studies, T_{reg} development occurred without need for the presence of cognate antigen. We are now assessing whether the CVT-E002 treatments induce the expansion of natural T_{reg} or induced T_{reg}.

Similar to other groups, we see that ginseng extracts effect DC function [12]. IL-10-differentiated DC can induce the outgrowth of T_{reg} from T effector cells of atopic asthmatic individuals [13]. The question remains how CVT-E002 treatment affects moDC. CVT-E002 contains >80% polyfuranosyl-pyranosyl-saccharides (carbohydrates) and 10% protein. We reported that the active component may be the carbohydrate portion [14]. We do not think the effect is through TLR-4 as all preparations of CVT-E002 were confirmed LPS-free, and we have data that anti-TLR4 treatment does not inhibit the effect on moDC (unpublished observations). There are reports of anti-inflammatory actions using Panax ginseng [15], although they did not cite the production of T_{reg} as the mechanism. We hypothesize that CVT-E002 binds to a yet to be determined receptor on DCs, which alters their phenotype to promote T_{reg} differentiation. An example of this is pro-biotic bacteria cell wall carbohydrates inhibiting experimental asthma through the induction of T_{reg} [16].

Overall, we found that CVT-E002 not only inhibited the development of allergen-induced AHR and airway inflammation in a mouse model of asthma, but also induced functional human T_{reg}. We suggest that further development of CVT-E002 could lead to an attractive alternative to the anti-inflammatory medicines currently available.

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