**Cyclopia Extracts Enhance Th1-, Th2-, and Th17-type T Cell Responses and Induce Foxp3+ Cells in Murine Cell Culture**

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**ABSTRACT**
*Cyclopia genistoides*, one of the traditional South African medicinal plants, and other species of the same genus offer noteworthy phenolic profiles, in particular high levels of the anti-allergic xanthone mangiferin. Hot water and 40% ethanol-water (v/v) extracts, prepared from *C. genistoides*, *Cyclopia subternata*, and *Cyclopia maculata*, were tested for immunoregulating activity *in vitro* using murine splenocytes and mesenteric lymph node cells. The 40% ethanol-water extracts of *C. genistoides* and *C. subternata* significantly enhanced production of several types of cytokines, including IL-4, IL-17, and IFN-γ, by antigen-stimulated splenocytes. A concentration-dependent response was observed, noticeably for IFN-γ production. The activity of the extracts did not correlate with the content of any of the major phenolic compounds, indicative that other extract constituents also play a role in immunomodulation. Additionally, the increased ratio of CD4⁺CD25+Foxp3⁺ Treg cells to total CD4⁺ cells induced induction of Foxp3⁺ cells when mesenteric lymph node cells were cultured in the presence of these two extracts. This study is the first reporting immunostimulatory activity for *Cyclopia*, which are widely consumed as the herbal tea known as honeybush, underpinning further investigations into the potential use of its extracts as adjuvants for mucosal immunotherapy.

**Introduction**
During the past few decades, the number of patients suffering from immune disorders such as allergy, autoimmune diseases, and relapsed infections has shown a steady increase worldwide [1–3]. An urgent need exists for efficient prevention and/or treatment methods as evidenced by the many attempts to develop a novel therapy for immune diseases. Ye et al. [4] pointed out that improved strategies and targets for immunomodulation of allergic diseases should consider aspects such as fewer side effects, antigen-specific modulation for long-term effects, and non-injection routes. Mucosal immunotherapy is therefore an ideal choice based on these considerations. The health-promoting functions of some phytochemical constituents are receiving increasing attention with many studies revealing that their daily intake contributes to reducing the number of patients with diseases such as allergy, arteriosclerosis, and cancer [5–7]. Some of the immunomodulating effects might be achieved through enhancement of
a particular response, while others would depend on the promotion of comprehensive immune responses. On the other hand, inhibition of some particular responses may also be relevant. Although plant constituents generally elicit a weaker response than medicines in vivo [8–10], daily intake of such immune-modulating plant constituents, some commonly found in food, may form an acceptable safe therapy to prevent or mitigate diseases caused by immune disorders [11].

Polyphenols have been identified as potential immune-modulating agents capable of affecting multiple biological pathways and immune cell functions in the immune response [12, 13]. Our focus in this study was on the functions of selected species, out of the 23 known species, from the genus Cyclopia Vent. (Family: Leguminosae; Tribe: Podalyrieae) [14], medicinal plants endemic to South Africa and currently economically exploited as herbal tea known as honeybush [15]. Cyclopia extracts offer an interesting combination of phenolic compounds, with members of the xantheme, benzophenone, dihydrochalcone, flavanone, and flavone subclasses predominant and with interspecies differences [16–18]. Recent interest focused on the antidiabetic and antiobesity properties of Cyclopia extracts, in particular hot water extracts of Cyclopia maculata (Andrews) Kies and Cyclopia subternata Vogel [19–21]. Even though mangiferin, the major xanthone present in Cyclopia and in particular Cyclopi genistoides (L.) Vent., has been reported to have some immunomodulatory activity [8, 9], Cyclopia extracts have not yet been investigated for potential immunomodulatory activity.

It is accepted that the Th1 and Th2 cells are responsible for coordinating the immune system and that the Th1/Th2 balance is critical to control not only immunoprotection against infections and cancer, but also the onset of allergy and autoimmune diseases [22]. Therefore, regulation of the Th1/Th2 balance by plant constituents could play an important and effective role in the prevention of such diseases. In addition, recent studies have revealed that Th1 and Th2 cells might be involved in inflammation, an underlying cause of many types of diseases, for example, arteriosclerosis, diabetes, ulcerative colitis, and Alzheimer’s disease [23–26]. Other types of cells such as Th17 and macrophages [27, 28] may also play critical roles in the inflammatory response, while some regulatory cells may inhibit inflammation. The Foxp3 regulatory T cell is one of the important cell types responsible for controlling the onset of the diseases mentioned [29]. The induction of the number or activity of these cells is an attractive target for flavonoids [30].

The murine splenocyte culture is one of the models used to investigate the immune-regulating effects of potential agents because it can be easily prepared [31]. On the other hand, it is often the case that cells prepared from different organs respond to some agents in distinct ways, i.e., lactic acid bacteria induced regulatory T cells by mainly affecting the function of dendritic cells in the mesenteric lymph node but not those in the spleen [32]. Aside from the splenocytes, mesenteric lymph node cells were used to determine the immune-regulating activity of Cyclopia extracts to provide novel findings in support of further investigations into their potential use as adjuvants for mucosal immunotherapy. For each Cyclopia species, both a hot water extract, representing a food ingredient extract, and a 40% ethanol-water extract, for enhanced phenolic content, were investigated.

Results

Qualitative and quantitative differences in the phenolic composition of the extracts, depending on the Cyclopia species and solvent, were demonstrated (►Table 1). Mangiferin, isomangiferin, iriophenone-3-C-glucoside, vicenin-2, and hesperidin were present in all extracts. Of these compounds, the hesperidin and mangiferin content of the extracts increased the most by using 40% ethanol instead of hot water. Mangiferin was the major compound in all extracts, except in the hot water extract of C. subternata. Its content varied between 2.3 and 9.3% for the hot water extract of C. subternata and 40% ethanol extract of C. genistoides, respectively. Similarly, isomangiferin, differing from mangiferin only in the position of the glucose moiety on the 9H-xanthene-9-one structure, was present in the highest (2.5%) and lowest (0.6%) levels in these two extracts, respectively. Iriophenone-3-C-glucoside–4-O-glucoside was the major compound in the hot water extract of C. subternata. ▶Fig. 1 depicts the phenolic profiles of the respective 40% ethanol extracts with the major compounds, common to C. genistoides, C. subternata, and C. maculata, indicated on the chromatograms.

The extracts were screened for their Th1/Th2 modulating effects to select those Cyclopia extracts having the best immunomodifying potential. Of the six extracts tested, both the 40% ethanol and hot water extracts of C. genistoides and C. subternata enhanced IFN-γ and IL-4 production from spleen cells of DO11.10 mice at 25 µg/mL (►Fig. 2). On the basis of these results, the latter Cyclopia species, and in particular their 40% ethanol extracts, were selected for further study at a range of concentrations (►Fig. 3). IFN-γ production was enhanced at extract concentrations of 10 µg/mL (0 µg/mL, 5.37 ± 0.55 ng/mL and 10 µg/mL, 6.39 ± 0.55 ng/mL for C. genistoides; 0 µg/mL, 7.01 ± 0.19 ng/mL and 10 µg/mL, 7.95 ± 0.39 ng/mL for C. subternata), while the effects were diminished at higher concentrations. Moreover, at the highest concentration of 250 µg/mL, the effects were rather inhibited compared to the untreated group (►Fig. 3). However, only C. genistoides significantly increased IL-4 production (0 µg/mL, 62.17 ± 2.25 ng/mL and 2 µg/mL, 74.55 ± 4.41 ng/mL). The concentration of each cytokine in the supernatants from the wells in which cells were maintained for 3 days in the absence of OVA is shown as “No antigen” (►Fig. 3).
Testing the effect of the 40% ethanol extracts of *C. genistoides* and *C. subternata* on the proliferation of splenocytes indicated that both extracts significantly enhanced proliferation at low concentrations, but inhibited proliferation at high concentrations (▶Fig. 4a). This result is consistent with that of the cytokine responses. Surprisingly, the viable cell number did not change even at the highest concentration (▶Fig. 4b), suggesting that the suppression of cell proliferation was not due to the apparent cytotoxicity of the extracts.

We then evaluated the effects of the samples on other cytokines, IL-10 and IL-17A, and found that the 40% ethanol extracts of both *C. genistoides* and *C. subternata* upregulated IL-10 (▶Fig. 5c) and IL-17A (▶Fig. 5d) similarly to IL-4 and IFN-γ. We also confirmed the effect of Con A as a positive control for the T cell activation agent, and showed that Con A enhanced all cytokines tested in a dose-dependent manner (▶Fig. 5a–d).

The 40% ethanol extracts of *C. genistoides* and *C. subternata* significantly enhanced IFN-γ production in mesenteric lymph node cells similarly to the splenocytes (▶Fig. 6). However, the effect of the extracts on Th2 cells from mesenteric lymph node cells could not be evaluated, since we could not detect IL-4 production by any of the groups. On the other hand, culturing of mesenteric lymph node cells in the presence of the 40% ethanol extracts of *C. genistoides* and *C. subternata* significantly induced Foxp3+ Treg cells similar to TGF-β, used as a positive control (▶Fig. 7). No Treg-inducing activity was observed for Con A (data not shown) in spite of its ability to enhance production of IL-4, IL-10, IL-17, and IFN-γ (▶Fig. 5a–d).

**Discussion**

The burden on health services, resulting from the rising prevalence of allergies including food allergy in both developed and developing countries [33], demands intervention. Paradoxically, plant-based food and beverages are also known as potential sources of anti-allergic agents [12]. Utilization of polyphenol-rich plant products offers the opportunity to prevent or mitigate allergic reactions [11]. The aim of the present study was to demonstrate the multifarious immune-regulating activity, including the anti-allergy activity described above, of *Cyclopia* extracts in vitro as a first step in their continuing evaluation as sources of health-promoting constituents or potential use as nutraceutical ingredients. Given the current honeybush industry, *C. genistoides* and *C. subternata* were selected for investigation as these species form the bulk of the cultivated crop. *C. maculata* is considered one of the *Cyclopia* species with potential for cultivation [15]. Selection of extract type for investigation was governed by extracts with commercial application and/or potential. Hot water extraction is used by industry to produce the food grade ingredient extracts added to various food products, while the 40% ethanol extraction procedure was previously optimized for extraction of xanthones and benzophenones [34].

*In vitro* screening of the extracts using splenocytes of TCR transgenic mice indicated that extracts produced from *C. genistoides* and *C. subternata* showed immune-stimulating activity in terms of IL-4 and IFN-γ production. Furthermore, the extent of cytokine production depended on the *Cyclopia* species rather than the solvent used for extraction. The qualitative and quantitative phenolic differences between extracts and species may explain the distinct activity of the extracts. Neither mangiferin content

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**Table 1** Phenolic content (g/100 g extract) of 40% ethanol-water and hot water extracts of *C. genistoides*, *C. subternata*, and *C. maculata*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>40% ethanol</th>
<th>40% ethanol</th>
<th>40% ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C. genistoides</td>
<td>C. subternata</td>
<td>C. maculata</td>
</tr>
<tr>
<td></td>
<td>water</td>
<td>water</td>
<td>water</td>
</tr>
<tr>
<td>Iriflophenone-3-C-glucoside-4-O-glucoside</td>
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<td>0.9</td>
<td>2.3</td>
</tr>
<tr>
<td>Iriflophenone-3-C-glucoside</td>
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<td>1.3</td>
<td>0.7</td>
</tr>
<tr>
<td>Mangiferin</td>
<td>9.3</td>
<td>8.1</td>
<td>3.0</td>
</tr>
<tr>
<td>Isomangiferin</td>
<td>2.5</td>
<td>2.4</td>
<td>0.7</td>
</tr>
<tr>
<td>Maclurin-3-C-glucoside</td>
<td>0.6</td>
<td>0.5</td>
<td>–</td>
</tr>
<tr>
<td>Vicenin-2</td>
<td>0.4</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Scolymoside</td>
<td>–</td>
<td>–</td>
<td>1.1</td>
</tr>
<tr>
<td>3-Hydroxy-phloretin-3’,5’-di-C-hexosideb</td>
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<td>–</td>
<td>0.5</td>
</tr>
<tr>
<td>Phloretin-3’,5’-di-C-glucoside</td>
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<td>–</td>
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</tr>
<tr>
<td>Hesperidin</td>
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<td>1.1</td>
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</tr>
<tr>
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<td>0.3</td>
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</tr>
<tr>
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<td>0.2</td>
<td>–</td>
</tr>
<tr>
<td>Naringenin-O-deoxyhexose-O-hexoside Bc</td>
<td>1.3</td>
<td>1.1</td>
<td>–</td>
</tr>
<tr>
<td>Eriodictyol-O-glucosidee</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*aNot detected or present in trace quantities. bQuantified as phloretin-3,5-di-glucoside equivalents. cQuantified as eriocitrin equivalents. dQuantified as naringin equivalents. eQuantified as narirutin equivalents.*

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nor that of the other compounds common to all extracts correlated with the observed effects (enhanced production of IFN-γ and IL-4 at 25 µg/mL). Additionally, at higher concentrations (250 µg/mL), the extracts inhibited cytokine production for both Th1 and Th2 types, and this activity also did not correlate with any of the identified compounds (▶ Table 1). Opposite effects induced by different compounds and their levels in the extracts may have confounded results. Mangiferin, present in C. genistoides extracts at the highest concentration, was reported to enhance Th1-type responses such as IFN-γ production and to inhibit Th2-type responses such as IL-4 production [9]. Conversely, hesperidin, proportionally of greater importance in C. maculata extracts than the others, enhanced both IL-4 and IFN-γ production in a mouse splenocyte model [35]. However, the activity observed in this present study did not conform to those results. The flavones scolymoside, present only in the C. subternata extracts, but in substantial quantities, and vicenin-2, with the highest concentration in C. genistoides extracts, elicited anti-inflammatory activity in various systems [36, 37]. Nevertheless, their concentration in the extracts also did not correlate with their immune-regulating activity, suggesting that some other compounds or synergistic effects of several molecules would contribute to the activity observed in our experiments. Actually, a major compound could be less effective as an antioxidant than a minor compound, although it has not been determined whether the antioxidant activity is linked to the immune-regulating activities or not. A study by Kawai et al. [12] to delineate the structure-activity relationship of flavonoids investigated the inhibition of IL-4 production from basophils by flavonoids. Structural features essential for maximal inhibition of IL-4 production were reported to be hydroxylation of positions 7 and 4′ on the A and B rings, respectively. Additional features to enhance inhibition are hydroxylation of either position 3 or 5, while glycosylation of C-3 decreases activity. These features are also relevant for the structure-activity relationship of flavonoids relating to anti-inflammatory activity [38]. On the other hand, the structural features of polyphenols responsible for the enhancing of T cell responses have not yet been clarified. A future focus would be to identify the compounds contributing to the confounded immune-regulating activity of Cyclopia, and to demonstrate struc-

![Fig. 1](https://example.com/fig1.png)  
**Fig. 1** HPLC-DAD chromatograms of the 40% ethanol-water extracts of (a) *C. genistoides*, (b) *C. subternata*, and (c) *C. maculata*, captured at 288 (solid lines) and 320 nm (dashed lines). Injection volumes were varied to accommodate quantitative differences between these extracts, particularly with regard to the mangiferin content of the extracts.

![Fig. 2](https://example.com/fig2.png)  
**Fig. 2** Effects of Cyclopia extracts on cytokine production. Spleenocytes from DO11.10 mice were stimulated with OVA (100 µg/mL) and each extract (25 or 250 µg/mL) for 3 days. Concentrations of (a) IFN-γ and (b) IL-4 in the supernatants were measured by ELISA. N = 3 for each group. The data are representative of two distinct sets of experiments. *P < 0.05 vs. the negative control group (OVA alone) evaluated by Dunnett’s test.
Natural features responsible for enhancing activity. Even though the mechanisms of the cytokine-modulating activity of *Cyclopia* extracts have not been investigated, it could be considered that they directly affect T cells given that some flavonoids directly affect IL-4 and IL-13 production from human basophils [39]. Although anti-allergic constituents other than polyphenols in *Cyclopia* have not been identified, this cannot be excluded. T cell activation by mitogenic lectins such as Con A, which is derived from the jack bean [40], and the immune-regulating activity of polysaccharides from plants [41] exemplify the role of plant constituents other than polyphenols.

The 40% ethanol extracts of *C. genistoides* and *C. subternata* were subsequently selected as candidates for further investigation. Their concentration effect on IL-4 and IFN-γ production in splenocytes and IFN-γ production in mesenteric lymph node cells from the same TCR transgenic mice were confirmed. The sites where the immune cells are influenced by phytochemicals, including *Cyclopia* extracts, have not yet been fully clarified, but results from a previous study using mice fed with lactic acid bacteria having an immune-modulating activity indicated the mesenteric lymph node as a potential site [32]. Therefore, our data from mesenteric lymph node cells lend strong support to *Cyclopia* extracts.
as potential immune enhancers in vivo. We also showed that these extracts enhanced the production of IL-10 and IL-17A in splenocytes. IL-10 is known to be produced by Th2 and some regulatory T cells such as Th3 or Treg, while IL-17A is produced by Th17, indicating that Cyclopia extracts affect various types of T cells but not some special T cells. This finding might indicate that Cyclopia extracts enhance the production of each cytokine by direct stimulation of T cell activity but not via enhancing the differentiation of T cells into Th1, Th2, and Th17 either directly or through the modification of the functions of antigen-presenting cells.

The induction of Foxp3+ Treg cells by the 40% ethanol extracts of C. genistoides and C. subternata when culturing mesenteric lymph node cells in their presence was evident from the increased ratio of CD4+CD25+Foxp3+ Treg cells to total CD4+ cells, especially at low concentrations. The question of which type of cell is dominantly induced under a certain condition would be determined by the balance of differentiation into each subset. Generally, a substance or a mixture of some substances, which has both activating and suppressing effects on immune responses, is likely to induce productive responses such as Th1, Th2, and Th17 at a high concentration, but inhibitory responses such as Th3 and Treg at a low concentration. The reason has not been clarified, but it can be considered that the conditions for inducing Th1 or Th2 differentiation would be too strong to induce Treg, because Treg differentiation would be suppressed under such conditions. We previously showed that the induction of Treg cells by a probiotic could occur through increasing the number of dendritic cells expressing RALDH at the mesenteric lymph node [32]. This type of cell produces retinoic acid, which induces Treg cells [42]. Treg cells are known to inhibit excessive immune responses and suppress some immune disorders such as autoimmune diseases, allergy, and lifestyle-related diseases [29]. We have not yet investigated the mechanism responsible for enhancing Treg induction by Cyclopia extracts. Some constituents of the Cyclopia extracts might enhance RALDH activity in dendritic cells of the mesenteric lymph node, while some might be able to induce the differentiation into Treg cells via other pathways. For example, it is known that the signal from the AhR induces Treg cells [43], and polyphenols have been demonstrated to stimulate AhR [44]. Future research would include clarification of the molecular mechanism and the structure-activity relationship of the phenolic compounds whereby Cyclopia extracts induce Treg cells.

We also observed that Cyclopia extracts did not affect any T cell responses in the absence of antigen stimulation (data not shown), suggesting that Cyclopia extracts could modulate T cell responses in an antigen-specific manner. The results of the present study confirmed that Cyclopia extracts merit further investigation of their immunomodulatory effects. It is expected that the daily intake of honeybush, either as a nutraceutical ingredient or as herbal tea, could aid in the prevention of infectious diseases by enhancing Th1- and Th17-type responses, and lifestyle-related diseases and allergies by simultaneously inhibiting excessive immune responses through IL-10 and Treg induction.
Materials and Methods

Phenolic standards and solvents
Authentic reference standards (purity > 95%) were sourced from Extrasynthese (eriocitrin, narirutin), Sigma-Aldrich (mangiferin, hesperidin, iriflophenone-3-C-glucoside, scolymoside), Chemos GmbH (isomangiferin), and Phytolab (vicenin-2). Maclurin-3-C-glucoside (95% purity by LC-MS) and iriflophenone-3-C-glucoside-4-O-glucoside (99% purity by LC-MS) were previously isolated from *C. genistoides* [45], while phloretin-3′,5′-di-C-glucoside (94% purity by LC-MS) was isolated from *C. subternata*. When no authentic reference standard was available, the compound was quantified in terms of equivalents of the most similar available standard. HPLC grade solvents were supplied by Merck Millipore. Ethanol for extraction was obtained from Servochem.

Plant material and preparation of extracts
Plant material samples of *C. genistoides*, *C. subternata*, and *C. maculata* were sourced from commercial plantations located on farms (−34.24052, 020.47272; −33.95480, 022.11712; −34.04722, 19.51765, respectively) in the Boland and Overberg regions of the Western Cape region to Province of South Africa. Original identification of these species for seed collection in the wild (by J. H. de Lange, SANBI Kirstenbosch) was carried out according to the taxonomic revision of the *Cyclopia* genus by Schutte [14]. The harvested shoots (consisting of both leaves and stems) were dried intact in a temperature-controlled cross-flow drier at 40 °C to a moisture content < 10%, followed by coarse milling using a rotary mill, equipped with a 1-mm sieve (Retsch Gmbh). The hot water and 40% ethanol (40% ethanol-water, v/v) extracts were prepared from the same batches of milled plant material as described by de Beer et al. [16] and Bosman et al. [34], respectively. Briefly, this entailed a 30-min extraction of the plant material using a 1:10 solid: solvent ratio (m/v) with the extraction temperature maintained at 93 °C for the hot water extraction and at 70 °C for the 40% ethanol extraction. The ethanol was subsequently evaporated and the remaining aqueous layer as well as the hot water extract was freeze-dried. The freeze-dried extracts were ball-milled (Fritsch GmbH), and the powders were stored in sealed containers at 5 °C until used. Retention samples of the 40% ethanol-water extracts of *C. genistoides*, *C. subternata*, and *C. maculata* were coded CGN_L0083_0190B1_1307, CSB_L0084_0192B1_1303 and CMC_L0086_0194B1_1305, respectively, while their water extracts were coded CGN_L0083_0191B1_1307, CSB_L0084_0193B1_1303 and CMC_L0086_0195B1_1305, respectively.

Phenolic content of extracts
The phenolic compounds in the extracts were quantified by high-performance liquid chromatography with diode-array detection (HPLC-DAD) using an Agilent 1200 HPLC system (Agilent Technologies Inc.). Duplicate analysis of the extracts were performed according to the validated species-specific methods for *C. genistoides* [17], *C. subternata* [16], and *C. maculata* [18].

![Fig. 7](image-url) Effect of *Cyclopia* extracts on Foxp3+ cell induction. Mesenteric lymph node cells from DO11.10 mice were stimulated with ovalbumin (OVA) (33 µg/mL) and the 40% EtOH extracts from *C. genistoides* and *C. subternata* (0.01–10 µg/mL) for 3 days. The cells were then harvested from the wells and stained with CD4-FITC, CD25-biotin, and Foxp3-PE. CD25 was detected with PE-Cy5-streptavidin. The cells were analyzed by flow cytometry, and the CD4+CD25+Foxp3+ cells were calculated as a ratio of the total CD4+ cells. **a** The representative charts for each group; No antigen, OVA alone, OVA + *C. genistoides* (0.1 µg/mL), and OVA + *C. subternata* (0.01 µg/mL). Each panel shows the cells gated for CD4+ cells. **b** The average of the ratio of CD4+CD25+Foxp3+ cells to total CD4+ cells is reflected. N = 6 for each group. The data are representative of two distinct sets of experiments. *P < 0.05 vs. the negative control (OVA alone) evaluated by Dunnett’s test.
Animals

OVA-specific TCR transgenic DO11.10 mice were obtained from Jackson Laboratory. The T cells of DO11.10 mice recognize OVA 323–339 restricted to I-Ab. The TCR transgenic mice were bred and maintained on irradiated food and autoclaved distilled water. Their offspring were used for experiments at 6–20 weeks of age. All the mice were maintained and used in accordance with the guidelines for the care and use of experimental animals of Tokyo University of Agriculture and Technology. All experiments were approved by the ethical committee of Tokyo University of Agriculture and Technology (27–2, April 17th, 2015; 28–2, April 19th, 2016).

Cell culture

Cells isolated from DO.11.10 mice were cultured in 96-well plates at 5 × 10^5 cells/well for splenocytes or 2 × 10^6 cells/well for mesenteric lymph node cells in RPMI 1640 (Nissui Pharmaceutical) containing 10% fetal calf serum (Sigma-Aldrich), 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 5 × 10^{-5} mol/L of 2-mercaptoethanol in the presence of OVA (100 µg/mL for the cytokine and proliferation assays, and 33 µg/mL for the Foxp3 assay). Each Cyclopia extract was dissolved in 40% ethanol and added to the culture at various concentrations. The final ethanol concentration was adjusted to 0.4% for all wells. Tween 20 (Wako) was used as a negative control (0.2%), and Con A (0.1 or 1 µg/mL; Sigma) and TGF-β (2 ng/mL; PEPROTECH) were used as positive controls for the cytokine and the Foxp3 assays, respectively. The supernatant was collected from each well 3 days after stimulation and used for cytokine determination, while the remaining cells were used for the cell proliferation assay. Other plates were similarly prepared, and cells were harvested from the wells 3 days after the stimulation for cell counting following staining with trypan blue or for the Foxp3 assay.

Cytokine measurement by ELISA

For measurement of IL-4, IL-10, and IFN-γ concentrations in the culture supernatant, Maxisorp immunoplates were coated with purified 11B11 rat anti-mouse IL-4 (BD Pharmingen), purified JESS-2A5 rat anti-mouse IL-10 (BD Pharmingen), or purified R4-6A2 rat anti-mouse IFN-γ (BD Pharmingen) monoclonal antibodies. Samples and standards were added after washing and blocking. Bound IL-4, IL-10, and IFN-γ were respectively detected using BVD6-24G2 biotinylated rat anti-mouse IL-4 (BD Pharmingen), SXC-1 biotinylated rat anti-mouse IL-10 (BD Pharmingen), or XMG1.2 biotinylated rat anti-mouse IFN-γ (BD Pharmingen) before incubation with alkaline phosphatase-streptavidine. The enzyme substrate (p-nitrophenol phosphate) was added and the absorbance was measured at 405 nm. IL-17A was measured using the Mouse IL-17A ELISA MAX Standard Set (BioLegend) according to manufacturer’s protocol.

Cell proliferation

Cell proliferation was evaluated using the BrdU ELISA kit (Roche Diagnostics) according to the manufacturer’s instructions.

Induction of Foxp3+ cells

Cells were stained with FITC-labeled anti-mouse CD4 (eBioscience) and biotinylated anti-mouse CD25 antibodies (eBioscience). CD25 was detected using streptavidin-conjugated PE-Cy5 (Biolegend). Foxp3 was stained with PE using the Foxp3 staining kit (eBioscience) according to the manufacturer’s protocol. The cells were analyzed by flow cytometry (EPICS Elite ESP Flow Cytometer, Beckman Coulter). CD4+CD25+Foxp3+ cells were identified as regulatory T cells (Treg), and the ratio of these cells to total CD4+ cells was calculated to indicate induction of Foxp3+ Treg cells.

Statistical analysis

Statistical analysis was performed by one-way ANOVA or Kruskal-Wallis’s test, and Dunnett’s test between the control (treated with OVA alone) and each treatment group. P values < 0.05 were considered significant.

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Conflict of Interest

The authors declare no conflict of interest.

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