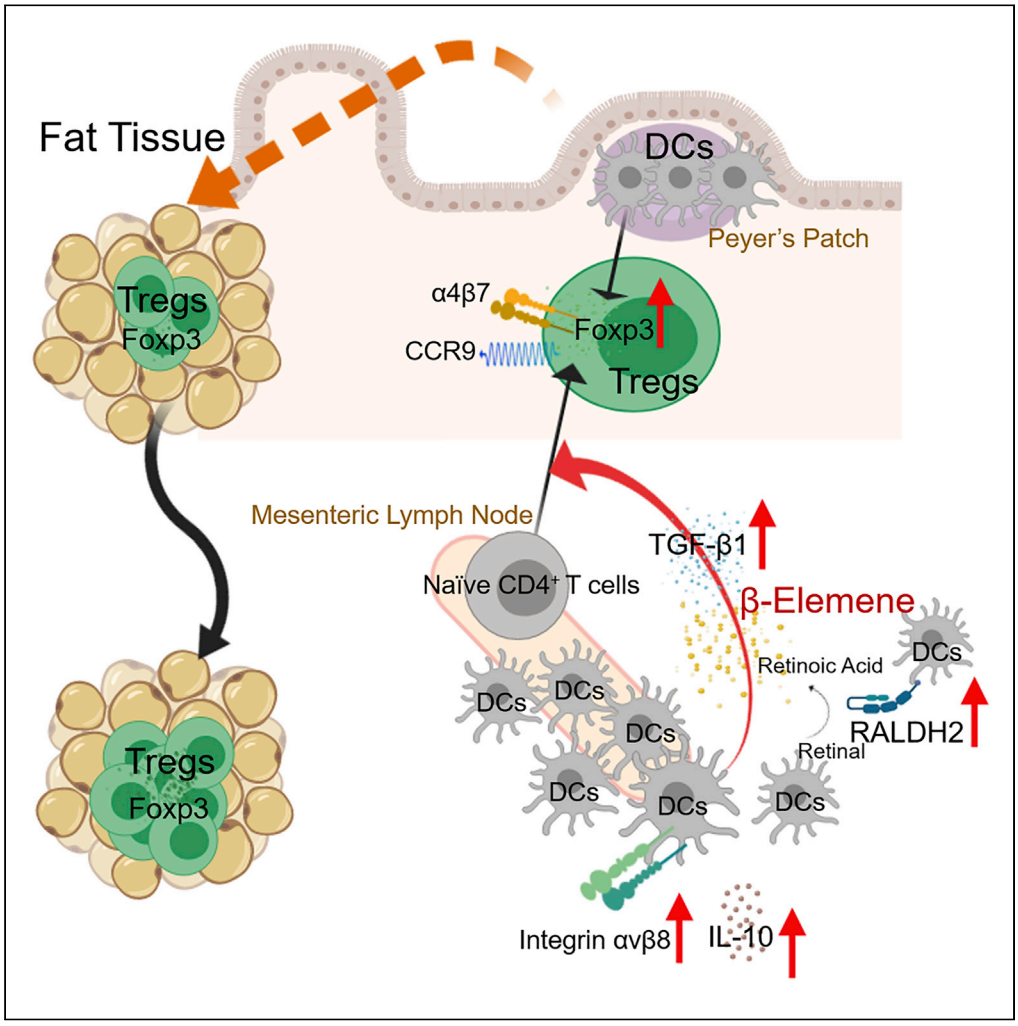
iScience

CellPress
OPEN ACCESS

Article

Intestinal regulatory T cell induction by β-elemene alleviates the formation of fat tissue-related inflammation



Yingyu Zhou, Tomohiro Takano, Yimei Wang, ..., Masaru Tanokura, Takuya Miyakawa, Satoshi Hachimura

atmiya@mail.ecc.u-tokyo.ac.jp (T.M.)
ahachi@mail.ecc.u-tokyo.ac.jp (S.H.)

HIGHLIGHTS

β-elemene downregulated inflammatory cytokines of adipose tissue of obese mice

β-elemene increased Tregs of adipose tissue of obese mice

β-elemene enhanced the expression of molecules for Treg induction in intestinal DCs

Zhou et al., iScience 24, 101883
January 22, 2021 © 2020
https://doi.org/10.1016/j.isci.2020.101883

iScience

CellPress
OPEN ACCESS

Article

Intestinal regulatory T cell induction by β-elemene alleviates the formation of fat tissue-related inflammation

Yingyu Zhou,[1,2] Tomohiro Takano,[1,2] Yimei Wang,[1,2] Xuyang Li,[2] Rong Wang,[1,2] Yoshio Wakatsuki,[3] Haruyo Nakajima-Adachi,[1,2] Masaru Tanokura,[1,2] Takuya Miyakawa,[2,*] and Satoshi Hachimura[1,2,4,*]

SUMMARY

The role of the intestinal immune system in the inhibition of fat tissue-related inflammation by dietary material is yet to be elucidated. Oral administration of β-elemene, contained in various foodstuffs, downregulated expressions of inflammatory cytokines and increased Foxp3$^+$CD4$^+$ T cells in adipose tissue of obese mice. However, β-elemene did not affect the inflammatory response of adipose tissue *in vitro*, suggesting that the inhibition observed *in vivo* was not due to direct interactions of adipose tissue with β-elemene. Instead, β-elemene increased Foxp3$^+$CD4$^+$ T cell population enhancing gene expressions of transforming growth factor β 1, retinaldehyde dehydrogenase 2, integrin αvβ8, and interleukin-10 in intestinal dendritic cells (DCs) *in vivo* and *in vitro*. Taken together, this study suggested the therapeutic effects of β-elemene on treating experimental obesity-induced chronic inflammation by adjusting the balance of immune cell populations in fat tissue through the generation of regulatory T cells in the intestinal immune system by modulating DC function.

INTRODUCTION

In 2006, Hotamisgil originally summarized the concept of "metabolically triggered inflammation (meta-inflammation)", which is a long-term, low-grade, chronic persistent inflammation caused by overnutrition or metabolic load (Hotamisligil, 2006; Saltiel and Olefsky, 2017). As a kind of chronic low-grade inflammation, obesity can trigger lesions in the peripheral circulation, including specific cytokines generation, metabolic imbalance of acute response factors, and numerical or functional polarization of various immune cells (Goldfine and Shoelson, 2017; Crewe et al., 2017). Obvious differences have been observed between the obese state and lean state in immune cell subsets. Clinical or experimental data have already shown that the properly proportional regulatory T cells (Tregs) are the basis for avoiding chronic inflammatory diseases (Lumeng et al., 2007; Cosmi et al., 2014).

The intestine is an organ that ingests nutrients or other food components. Mesenteric lymph nodes (MLNs) and Peyer's patches (PPs), play important roles in maintaining homeostasis in the intestinal immune system (Mclaughlin et al., 2017; Abreu, 2010). Kwon et al. reported that the conversion of T cells into Foxp3$^+$ Treg cells by probiotics is directly mediated by MLN CD11c$^+$ dendritic cells (DCs) that highly express transforming growth factor β (TGF-β) and interleukin-10 (IL-10), the immune process has been tested to treat inflammatory bowel disease (Kwon et al., 2010; O'Garra et al., 2004). TGF-β is reported to take part in the process of naive T cells' differentiation into the Tregs expressing Foxp3 (Kwon et al., 2010; Shiokawa et al., 2017). In particular, TGF-β1, an important member of the TGF-β family, can be activated by the degradation and alter-conformation of latency-associated protein. This process is mainly mediated by some proteins, such as αvβ8 integrin, which is expressed on DCs (Li and Flavell, 2008; Mu et al., 2002). In addition, MLN DCs highly induce Tregs by expressing retinaldehyde dehydrogenase 2 (RALDH2), an enzyme that can catalyze the synthesis of retinoid acid (RA), a vitamin A metabolite (Benson et al., 2007; Coombes et al., 2007). These responses enables Tregs to regulate the intestinal immune system, which emerged as a site altering diet-induced obesity (Winer et al., 2017). Thus, clearly clarifying the role of intestinal immune reactions mentioned above on the formation or treatment of obesity is necessary and has not been reported previously.

[1]Research Center for Food Safety, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

[2]Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

[3]Department of Clinical Bio-regulatory Science, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan

[4]Lead Contact

*Correspondence: atmiya@mail.ecc.u-tokyo.ac.jp (T.M.), ahachi@mail.ecc.u-tokyo.ac.jp (S.H.)

https://doi.org/10.1016/j.isci.2020.101883

β-elemene is a sesquiterpene, which can be found in herbs, spices, and root vegetables, and its efficacy has been reported in treating a series of tumors (Zhai et al., 2019). Mantovani et al. had already clarified a strong relationship between inflammation and tumors, highlighting that the tumor microenvironment is primarily caused by inflammatory cells and mediators (Mantovani et al., 2008). Therefore, we raised a question that whether β-elemene would inhibit chronic inflammation if it has a strong effect on treating tumors. In this article, a kind of obese mouse model was utilized to help us solve this question. We focused on Tregs in epididymal adipose tissue (EAT) and mesenteric adipose tissue (MAT), which seemed like the important parts composed for the white adipose tissue. Then, to investigate the intrinsic mechanism of β-elemene in treating obesity-induced inflammation, we studied its function in the induction of Tregs by intestinal DCs.

RESULTS

β-elemene did not influence mouse body weight but modified inflammation-related cytokines and increased Foxp3$^+$CD4$^+$ T cells in EAT and MAT

C57BL/6 male mice were fed a normal diet or diet with 60 kcal% fat (HFD) separately for 12 weeks to establish the obese mouse model. Milli-Q water or β-elemene dissolved in Milli-Q water was administered to the mice by gavage during the last 3 weeks. As shown in Figure 1A, the body weights of the mice in the HFD group increased and became significantly different from those of the control group beginning at the first week. The weights of EAT and MAT were obviously increased in the HFD group, suggesting that the white adipose tissue depots of the mice were increased by feeding HFD (Figures S1A and S1B). However, it seemed that body weight and white adipose tissue weight were not influenced by gavage of β-elemene (Figures 1A, S1A, and S1B). We then measured the expressions of classical inflammation-related cytokines in the EAT and MAT of mice by quantitative PCR (qPCR). Our data showed that β-elemene downregulated HFD-induced expressions of tumor necrosis factor alpha (TNF-α), IL-1β, and IL-6 in EAT of obese mice (Figures 1B–1D) and a similar tendency was observed for MAT (Figures S1C–S1E). These cytokines are inflammatory mediators that are involved in the pathogenesis and progression of obesity (Calle and Fernandez, 2012). Therefore, we hypothesized that β-elemene alleviated obesity-induced inflammation. Stromal vascular cells (SVCs) consist of fibroblasts, blood vessel cells, white blood cells, macrophages, pre-adipocytes, etc. SVCs are usually used to study the characteristics of immune cell subsets in adipose tissue both *in vivo* and *in vitro* (Hauner et al., 1989; Deslex et al., 1987). As shown in Figures 1E–1J, oral administration of β-elemene inhibited the gene expressions of inflammatory cytokines TNF-α, IL-1β, IL-6, and chemokine (C-C motif) ligand 2 (CCL2), and promoted that of the anti-inflammatory cytokines TGF-β1 and IL-10 in the SVCs of EAT. Similar tendency was observed for MAT (Figures S2F–S2K). The results provided evidence that β-elemene had immunoregulatory effects on alleviating experimental obesity-induced chronic inflammation. In SVCs, CD4$^+$ T cells are involved in the reparative process of chronic inflammatory diseases in adipose tissue (Gregor and Hotamisligil, 2011). Tregs are the major subset of CD4$^+$ T cells that maintain immune homeostasis and prevent the occurrence of aberrant immune responses (Cosmi et al., 2014). We analyzed Foxp3$^+$CD4$^+$ T cells expression in white adipose tissue and spleen of mice using the flow cytometry in the processes shown in the Figures S2A and S2B. Compared with the control group, the HFD group showed a reduced number of CD4$^+$ T cells in lymphocytes of EAT and MAT, as shown in Figures 2A, 2C, 2E, and 2G. This result is consistent with the previous observation (Nishimura et al., 2009). At the same time, the ratio of CD4$^+$ T cells expressing transcription factor Foxp3, which is one of the most reliable markers for Tregs, was also tested (Feuerer et al., 2009). We found that β-elemene clearly upregulated the ratio of Foxp3$^+$CD4$^+$ T cell ratio in the CD4$^+$ T cell population (Figures 2B, 2D, 2F, and 2H), which suggested the anti-inflammatory effect of β-elemene on the adipose tissue of obese mice. In addition, β-elemene could increase the ratios of Foxp3$^+$CD4$^+$ T cells in the spleen (Figures 3A–3D) and alleviate the inflammatory cytokines of obese mice serum (Figures 3E and 3F). All the results mentioned above suggest that β-elemene may affect systemic immunity *in vivo* with increased Tregs in the spleen.

β-elemene did not directly suppress LPS-induced SVC inflammation *in vitro*

To evaluate the direct effects of β-elemene on the SVCs in EAT and MAT, we mimicked the inflammatory environment *in vitro* using lipopolysaccharide (LPS). LPS is known as a bacterial component that is usually used to induce inflammation. In this part of the experiment, 10 μg/mL LPS and different concentrations of β-elemene (1, 2, 5 and 10 μg/mL) were added to the SVCs of EAT and MAT, respectively. As shown in Figure 4, only IL-1β, TGF-β1, and IL-10 in EAT SVCs and CCL2 in MAT SVCs had significant differences compared with those of the LPS group *in vitro* in response to 10 μg/mL β-elemene treatment. In addition, we supplemented the mRNA expressions of TGF-β1 and IL-10 in the culture system of EAT SVCs and different concentrations of β-elemene without LPS stimulation as shown in the Figure S3. We found that

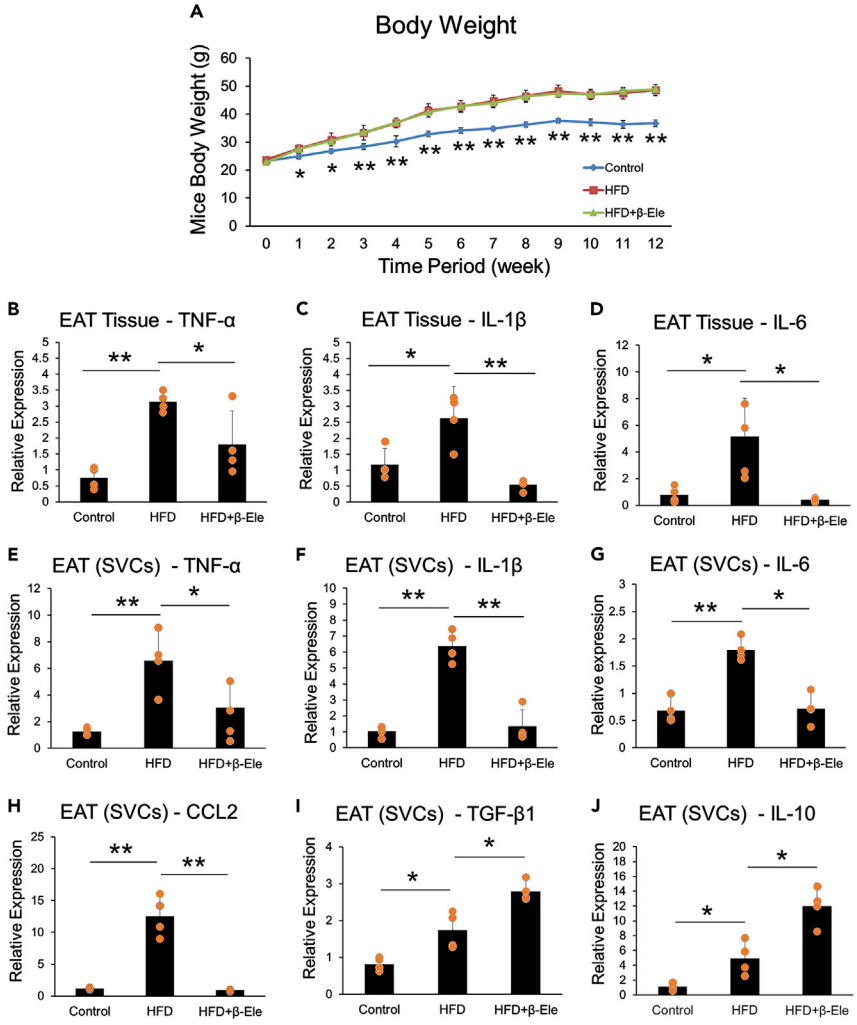


Figure 1. β-elemene Did Not Influence Mouse Body Weight but Regulated Inflammation-related Cytokine Expressions in EAT of Obese Mice

The body weight of each mouse was measured every week until they were sacrificed. After obtaining tissue RNA or SVCs RNA from EAT of the mice, qPCR was used to measure the relative expressions of inflammatory cytokines (GAPDH expression was the standard).

(A) β-elemene did not influence mice body weight.

(B–D) Expressions of the cytokines TNF-α (B), IL-1β (C), and IL-6 (D) in EAT.

(E–J) Expressions of the cytokines TNF-α (E), IL-1β (F), IL-6 (G), CCL2 (H), TGF-β1 (I), and IL-10 (J) in EAT SVCs.

The results are shown as the mean ± SEM. *$p < 0.05$; **$p < 0.01$ versus the HFD group (assessed using one-way ANOVA with Tukey's multiple comparisons). HFD, high-fat diet; HFD+β-Ele, HFD-induced obese mice under treatment with β-elemene. $n = 4$; the results represent one of two independent experiments.

the mRNA expressions of TGF-β1 and IL-10 were hardly changed by the addition of β-elemene, which suggested that β-elemene did not have the direct effects on the adipose tissue of obese mice. The results were also consistent with Figure 4. Therefore, we wondered why β-elemene had obvious effects on treating inflammation *in vivo*, while the effects on inflammation *in vitro* were subtle. To answer this question, we hypothesized that other immunoregulatory functions of β-elemene may exist in the treatment of experimental obesity-induced chronic inflammation.

β-elemene increased Foxp3$^+$CD4$^+$ T cells proportion in the intestinal immune system through enhanced expressions of TGF-β1, RALDH2, integrin αvβ8, and IL-10 in DCs

Intestinal DCs are involved in T cell-mediated immune regulation by inducing the differentiation of CD4$^+$ Tregs, which is mediated by TGF-β, RALDH2, integrin αvβ8, and IL-10 (Bakdash et al., 2015; Seeger et al.,

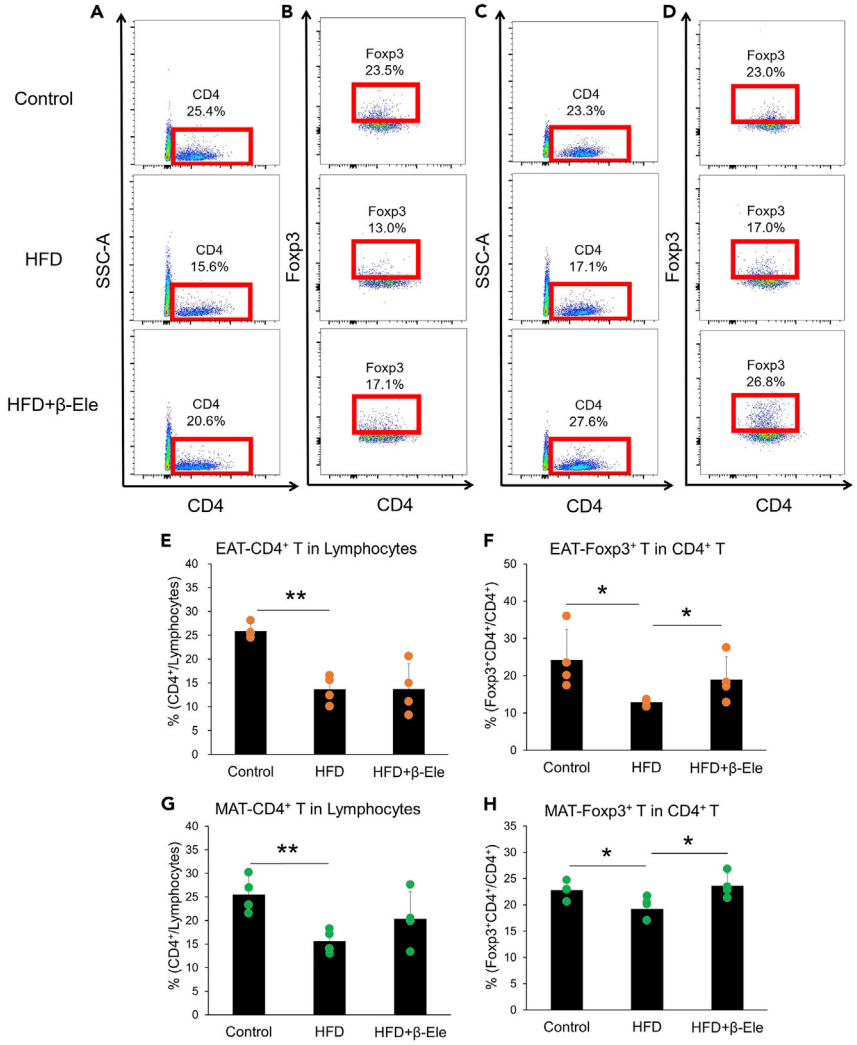


Figure 2. The Effect of β-elemene on Regulating the T Cells in EAT and MAT of Obese Mice

After obtaining EAT and MAT SVCs of each mouse, APC-conjugated anti-CD4 and PE-conjugated anti-Foxp3 antibodies were used to identify $CD4^+$ T cells in lymphocytes and Foxp3 expression in $CD4^+$ T cells using flow cytometry.

(A) $CD4^+$ T cells in lymphocytes of EAT SVCs.

(B) $Foxp3^+CD4^+$ T cells in $CD4^+$ T cells of EAT SVCs.

(C) $CD4^+$ T cells in lymphocytes of MAT SVCs.

(D) $Foxp3^+CD4^+$ T cells in $CD4^+$ T cells of MAT SVCs.

(E) Ratio of $CD4^+$ T cells in lymphocytes of EAT SVCs.

(F) Ratio of $Foxp3^+CD4^+$ T cells in $CD4^+$ T cells of EAT SVCs.

(G) Ratio of $CD4^+$ T cells in lymphocytes of MAT SVCs.

(H) Ratio of $Foxp3^+CD4^+$ T cells in $CD4^+$ T cells of MAT SVCs.

The results are shown as the mean ± SEM. *$p < 0.05$; **$p < 0.01$ versus the HFD group (assessed using one-way ANOVA with Tukey's multiple comparisons). HFD, high-fat diet; HFD+β-Ele, HFD-induced obese mice under treatment with β-elemene. $n = 4$; the results represent one of two independent experiments.

2015). Based on the effects of β-elemene on Tregs in the fat tissue of mice, we explored the functions of β-elemene in upregulating $Foxp3^+CD4^+$ T cells in the intestinal immune system, which seemed to modulate obesity-induced inflammation. After obtaining MLNs and PPs, the proportion of $CD4^+$ T cells in lymphocytes and the proportion of $Foxp3^+CD4^+$ T cells in $CD4^+$ T cells were measured by flow cytometry, respectively. As shown in Figures 5A–5D and Figures S4A–S4D, MLNs and PPs tended to increase ratio of $Foxp3^+CD4^+$ T cells in the β-elemene group than in the HFD group (the analysis processes of $Foxp3^{+-}CD4^+$ T cells expression in MLN or PP of mice as shown in the Figure S2C). This phenomenon suggested

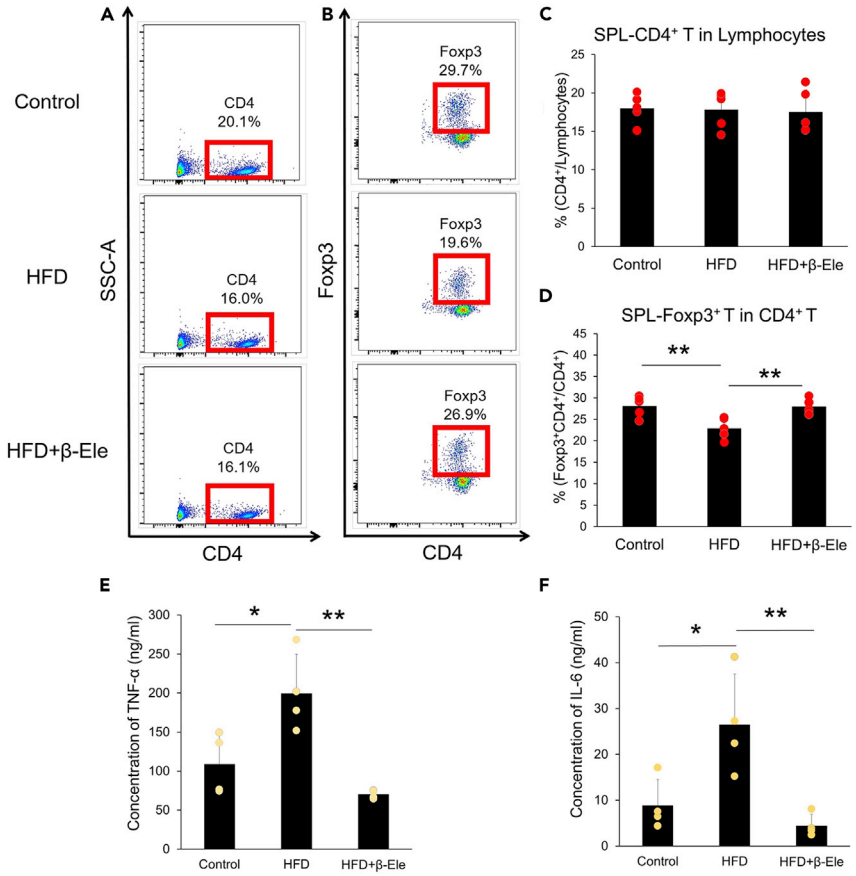


Figure 3. The Effects of β-elemene on Systemic Informatory Responses of Obese Mice

(A–D) After obtaining cells from mouse spleen, APC/Cy7-conjugated CD4 and PE-conjugated anti-Foxp3 antibodies were used to identify $CD4^+$ T cells in lymphocytes and Foxp3 expression in $CD4^+$ T cells by flow cytometry.

(A) $CD4^+$ T cells in lymphocytes of SPLs.

(B) $Foxp3^+CD4^+$ T cells in $CD4^+$ T cells of SPLs.

(C) Ratio of $CD4^+$ T cells in lymphocytes of SPLs.

(D) Ratio of $Foxp3^+CD4^+$ T cells in $CD4^+$ T cells of SPLs.

(E and F) The effects of β-elemene on regulating the protein expressions of TNF-α (E) and IL-6 (F) of serums from different mice group.

The results are shown as the mean ± SEM. *$p < 0.05$; **$p < 0.01$ versus the HFD group (assessed using one-way ANOVA with Tukey's multiple comparisons). HFD, high-fat diet; HFD+β-Ele, HFD-induced obese mice under treatment with β-elemene. n = 4–5; the results represent one of two independent experiments.

that β-elemene induced $Foxp3^+$ Tregs in the intestinal immune system of HFD mice. Furthermore, we tested the mRNA expressions of TGF-β1, RALDH2, integrin αvβ8, and IL-10 in the DCs of MLNs and PPs and found that β-elemene increased the expressions of these molecules, as shown in Figures 5E–5I and S4E–S4I. These results indicate that β-elemene induces $Foxp3^+$ Tregs in the intestinal immune system through inducing expressions of TGF-β1, RALDH2, integrin αvβ8, and IL-10 in DCs.

In the *in vitro* experiments, $CD11c^+$ DCs were obtained from MLNs or PPs of mice and were cultured with 1–10 μg/mL β-elemene, and mRNA levels were then measured in the collected DCs. We found that unlike the SVCs in mouse adipose tissue, β-elemene clearly increased the expressions of TGF-β1, RALDH2, integrin αvβ8, and IL-10 in MLN-$CD11c^+$ DCs (Figures 5J–5N). In addition, RALDH activity of MLN DC subsets was analyzed by flow cytometry using ALDEFLUOR reagent. We also found that β-elemene significantly increased the $ALDH^+$ DCs of MLNs *in vitro* (Figures 5O and 5P). The analysis processes of ALDEFLOUR *in vitro* was as shown in the Figure S2D. These findings indicate that MLN DCs were more sensitive to β-elemene stimulation than SVCs of adipose tissue with highly expressions of TGF-β1, RALDH2, Integrin αvβ8, and IL-10 in DCs *in vitro*. Similar results were also shown in the PP-$CD11c^+$ DCs (Figures S4J–S4N).

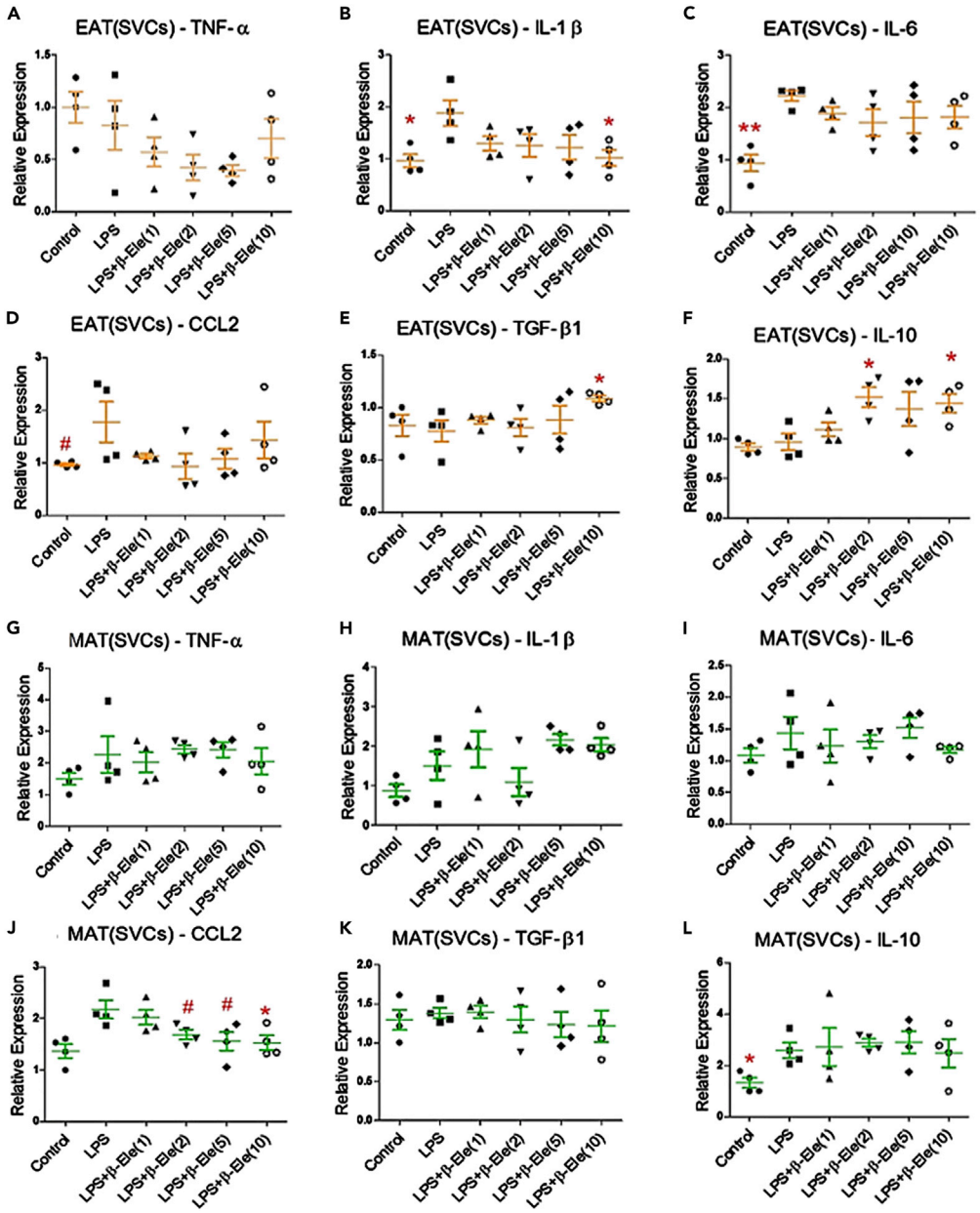


Figure 4. Effects of Different Concentrations of β-elemene on Regulating Cytokine mRNA Expressions in SVCs of EAT and MAT *In Vitro* under LPS Stimulation

(A–F) Gene expressions of the cytokines TNF-α (A), IL-1β (B), IL-6 (C), CCL2 (D), TGF-β1 (E), and IL-10 (F) in EAT SVCs. (G–L) Gene expressions of the cytokines TNF-α (G), IL-1β (H), IL-6 (I), CCL2 (J), TGF-β1 (K), and IL-10 (L) in MAT SVCs. LPS: SVCs (5×10^5 cells/well) of EAT and MAT were cultured with LPS (10 μg/mL); LPS+β-Ele: SVCs were cultured with LPS (10 μg/mL) and β-elemene (1, 2, 5 and 10 μg/mL); after culture for 24 hr, qPCR was used to measure the gene expressions of inflammation-related cytokines as shown in the figures. The data are obtained from individual wells of each sample. The results are shown as the mean ± SEM. $^{\#}p < 0.1$; $^{*}p < 0.05$; $^{**}p < 0.01$ versus the LPS group (assessed using one-way ANOVA with Tukey's multiple comparisons). $n = 4$; the results represent one of two independent experiments.

β-elemene enhanced the induction of Foxp3$^+$CD4$^+$ T cells in the MLN DC and splenic T cell culture system under different treatment conditions *in vitro*

The αvβ8-integrin mediates activation of TGF-β in MLN DCs (Worthington et al., 2011). Therefore, to examine the mechanism whether β-elemene enhances Foxp3$^+$CD4$^+$ T cell induction by the interaction between DCs and

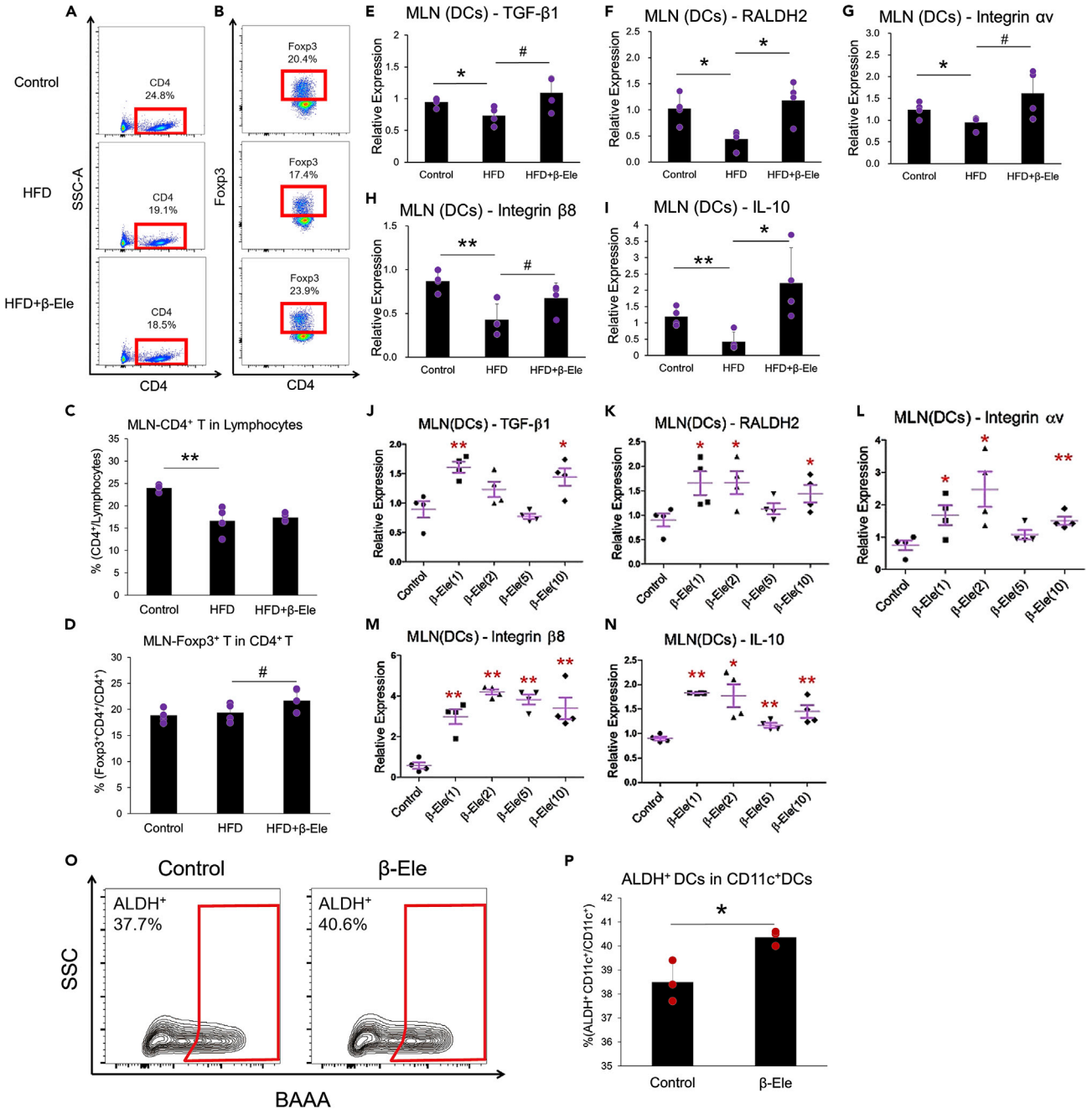


Figure 5. β-elemene Increased the Proportion of Foxp3$^+$CD4$^+$ T Cells in the Intestinal Immune System through Enhanced Expressions of TGF-β1, RALDH2, Integrin αvβ8, and IL-10 in MLN-DCs

(A–D) After obtaining mouse MLN cells, APC-conjugated anti-CD4 and PE-conjugated anti-Foxp3 antibodies were used to identify the CD4$^+$ T cell and Foxp3$^+$CD4$^+$ T cell populations by flow cytometry. HFD, high-fat diet; HFD+β-Ele, HFD-induced obese mice under treatment with β-elemene.

(A) CD4$^+$ T cells in MLN lymphocytes.

(B) Foxp3$^+$CD4$^+$ T cells in CD4$^+$ T cells of MLNs.

(C) Ratio of CD4$^+$ T cells in MLN lymphocytes.

(D) Ratio of Foxp3$^+$CD4$^+$ T cells in CD4$^+$ T cells of MLNs. (E–I) β-elemene increased the mRNA expressions of TGF-β1 (E), RALDH2 (F), integrin αv (G), integrin β8 (H), and IL-10 (I) in MLN DCs of obese mice. (J–N) MLN DCs were cultured with β-elemene (1, 2, 5 and 10 μg/mL) for 24 hr, β-elemene increased the mRNA expressions of TGF-β1 (J), RALDH2 (K), Integrin αv (L), Integrin β8 (M), and IL-10 (N) in MLN DCs *in vitro*. (O and P) RALDH activity of MLN DC subsets were analyzed by flow cytometry using ALDEFLUOR reagent. (O) ALDH$^+$ DCs in CD11c$^+$ DCs of MLNs. (P) Ratio of ALDH$^+$ DCs in CD11c$^+$ DCs of MLNs. The results

Figure 5. *Continued*

are shown as the mean ± SEM. $^{\#}p < 0.1$; $^{*}p < 0.05$; $^{**}p < 0.01$ versus the HFD group (A–I) or the control group (J–P) (assessed using one-way ANOVA with Tukey's multiple comparisons for A-N; assessed using two-tailed paired Student's *t*-test for O and P). *n* = 3–4; the results represent one of two independent experiments.

T cells, we cultured splenic $CD4^+$ T cells from RAG2-deficient DO11.10 mice and MLN $CD11c^+$ DCs from BALB/c mice in the absence or presence of OVA peptide (10 nM), RA (1 μM), hTGF-β1 (2 ng/mL) and β-elemene (10 μg/mL). The results were shown in Figure 6. We confirmed that TGF-β was the major inducer of the increase in $Foxp3^+$ Tregs compared with that of RA, but TGF-β1 and RA have a synergistic effect on Treg production. β-elemene enhanced the induction of $Foxp3^+CD4^+$ T cells both in the presence and absence of TGF-β. However, β-elemene did not exert significant effects in the presence of high levels of exogenous RA (Figure 6B). These results suggested that β-elemene enhanced the expression of RALDH2, promoting the production of RA, which cooperatively induced $Foxp3^+$ Tregs with TGF-β. The upregulated RA level was suggested by the increased proportion of RA dependent $\alpha4\beta7^+CCR9^+$ T cells in β-elemene group compared with that of the non-β-elemene group both *in vitro* and *in vivo* as shown in the Figures S5 and S6 (Hammerschmidt et al., 2008). The analysis processes of $Foxp3^+CD4^+$ T cells and $\alpha4\beta7^+CCR9^+$ T cells expression *in vitro* was as shown in the Figure S2E and the analysis processes of $\alpha4\beta7^+CCR9^+$ T cells expression in the SPL, MLN or PP of mice was as shown in the Figures S2B and S2F. In addition, β-elemene tended to increase the induction of $Foxp3^+CD4^+$ T cells in the absence of TGF-β, which may be due to the enhancement of TGF-β production or αvβ8-mediated activation. These results revealed the function of β-elemene in modulating DC-T cell responses in intestinal immunity, which enhanced the induction of $Foxp3^+CD4^+$ T cells and alleviated experimental obesity-induced chronic inflammation. In addition, we also examined the direct effects of β-elemene on differentiation of $CD4^+$ T cells (Figure S7). There was no significant effect on differentiation of Th1 or Th2 cells in the absence of antigen-presenting cells both in the proteins or mRNA expressions. Concerning Th17 cells, an increase of IL-17 mRNA expression was observed, but there was no significant difference in the protein level. Concerning Treg differentiation, there were increases in the expressions of Foxp3 and TGF-β1 mRNA. In addition to effects via DCs, β-elemene may also have direct effects on enhancing Treg induction by upregulation of TGF-β.

DISCUSSION

In mice, the paired gonadal and mesenteric deposits are the important parts and are composed of white adipose tissue, which is usually used for energy storage and has various insulin receptors (Birbrair et al., 2013). We found that β-elemene did not have effect on decreasing mice bodyweight, which was unexpected. However, inflammatory cytokines in mouse adipose tissue including TNFα, IL-1β and IL-6, which directly participate in obesity-related insulin resistance were obviously inhibited by β-elemene treatment particularly in EAT (Hotamisligil et al., 1993). Inflammatory cytokine levels in serum were also decreased by β-elemene administration. The results suggested that β-elemene had some effects on treating obesity-induced inflammation. To further explore the mechanism of the anti-inflammatory function of β-elemene, we focused on the SVCs of EAT and MAT of obese mice, which are usually used to study the characteristics of adipose tissue, especially immune cell subsets that are related chronic inflammatory disease. The proinflammatory cytokines TNFα, IL-1β, IL-6, and CCL2 of SVCs in adipose tissue of mice, EAT in particular, were downregulated by oral administration of β-elemene while the anti-inflammatory cytokines IL-10 and TGF-β1 were upregulated. TGF-β can work together with IL-10 to escape the extra inflammatory reaction which will induce self-tissue damage (Derynck and Zhang, 2003). TGF-β deficient mice develop multi-focal inflammatory diseases, which support the effects of TGF-β on maintaining the normal immune function for the body (Brunkow et al., 2001). Besides TGF-β signaling, the $CD4^+$ $Foxp3^+$ Tregs alleviated inflammation. It is also reported that the Foxp3 deficient mice are similar to the TGF-βRII (the important receptor of TGF-β signaling) deficient mice that results in T cell dependent inflammatory phenotype (Brunkow et al., 2001; Fontenot et al., 2017). In the lymphocytes of each mouse group, we studied the $CD4^+$ T cells in adipose tissue. Winer et al. indicated that the $CD4^+$ T lymphocyte subsets have protective effects against the occurrence of obesity and insulin resistance (Winer et al., 2009). Our data showed a similar decrease in $CD4^+$ T cells proportion in HFD-fed mice. We found that even though the decreased $CD4^+$ T cells in lymphocytes caused by HFD in EAT and MAT was not recovered by β-elemene treatment, the Foxp3 expression was significantly upregulated. These results provide evidence that oral administration of β-elemene induced $Foxp3^+$ Tregs in adipose tissue of obese mice.

To evaluate the direct effect of β-elemene on the SVCs in EAT and MAT, we mimicked the inflammatory environment *in vitro* using LPS, which is a bacterial endotoxin and highly circulate in the intestine of obese

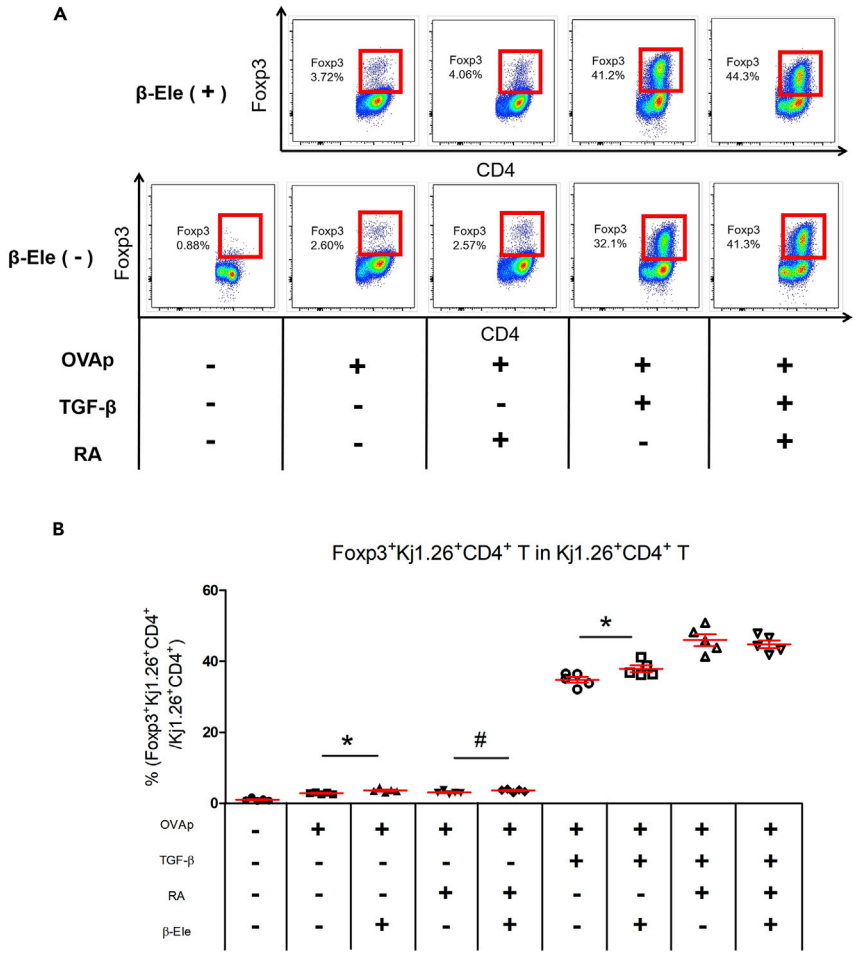


Figure 6. β-elemene Increased the Antigen-Specific Foxp3$^+$CD4$^+$ T Cell Proportion in the Antigen-specific T Cell Population under Different Treatment Conditions *In Vitro*

The culture system consisted of splenic CD4$^+$ T cells (2×10^5 cells/well) from RAG2-deficient DO11.10 mice and MLN CD11c$^+$ DCs (2×10^4 cells/well) from BALB/c mice was conducted in the absence or presence of OVA peptide (10 nM), RA (1 μM), hTGF-β1 (2 ng/mL) and β-elemene (10 μg/mL). After 72 hr culture, APC/Cy7-conjugated anti-CD4, PE/Cy7-conjugated anti-DO11.10 TCR and PE-conjugated Foxp3 antibodies were used to identify the antigen-specific CD4$^+$ T cell and Foxp3$^+$CD4$^+$ T cell populations using flow cytometry.

(A) The effect of β-elemene on increasing the proportion of Foxp3$^+$CD4$^+$ T cells in CD4$^+$ T cells in the culture system.

(B) Ratio of Foxp3$^+$CD4$^+$ T cells in Kj1.26$^+$CD4$^+$ T cells in the culture system. The data are obtained from individual wells of each sample.

The results are shown as the mean ± SEM. $^{\#}p < 0.1$; $^{*}p < 0.05$ between wells with β-elemene added and those without β-elemene (assessed using two-tailed paired Student's *t*-test). $n = 5$; the results represent one of two independent experiments.

status (Leu et al., 2006; Rui et al., 2007). LPS stimulation induces the production of various inflammatory cytokines such as TNFα, IL-1β, IL-6, IFN-γ, etc (De Groote et al., 1992), all of them involved in the development of insulin resistance (Wunderlich et al., 2013) and indirectly interfere with insulin signaling pathways by promoting ER stress and oxidative stress (Ilangumaran and Ferbeyre, 2016; Özcan et al., 2004). However, we found that the anti-inflammatory effects were minimal even with high concentrations of β-elemene (10 μg/mL) treatment under the LPS induced inflammation. Based on these results, we hypothesized that adipose tissue inflammation was not inhibited by the direct effect of β-elemene and that other immunoregulatory functions were executed in treating obesity-induced inflammation.

β-elemene was orally administered, so we considered the possibility that it may modulate the intestinal immune system. We found that β-elemene induced Foxp3$^+$CD4$^+$ Tregs in MLNs and PPs. DCs in the two

lymphoid organs are capable of producing RA, which is dependent on the expression of RALDH2 mediating vitamin A metabolism (Bakdash et al., 2015). Furthermore, it has been demonstrated that integrin αvβ8 (encoded by the integrin αv and integrin β8 genes) plays a fundamental role in TGF-β activation and acts with RA to participate in TGF-β1-mediated development of Foxp3$^+$ Tregs. In addition, Turovskaya et al. showed that IL-10 acts on Tregs with high expression of Foxp3 and suppressive functions in mice with colitis (Turovskaya et al., 2009). The three molecules mentioned above are involved in Foxp3$^+$ Treg production, which appears to be the key point in alleviating inflammatory immune responses (Fenton et al., 2017). In our present study, β-elemene increased Foxp3$^+$CD4$^+$ T cells in the intestinal immune system through enhanced expressions of TGF-β1, RALDH2, integrin αvβ8, and IL-10 derived from DCs *in vivo* and *in vitro*, the latter was under low concentrations of β-elemene treatment. The phenomena demonstrated that intestinal DCs were more sensitive to β-elemene than SVCs of EAT and MAT and suggested that the induction of Foxp3$^+$ T cells observed *in vivo* was the direct effects of β-elemene on intestinal DCs, although there was a possibility of direct effect on T cell differentiation. In the previous studies, several molecular targets of β-elemene in cancer cells have been identified (Zhai et al., 2019). However, the molecular mechanisms of its effects on immune cells are mostly unknown, although its effect on balancing CD4$^+$ Foxp3$^+$ regulatory T cells, Th17 helper cells and Th1 helper cells through down-regulating expressions of IL-6, IL-23, RORγt signaling had been reported (Zhang et al., 2010, 2011). Concerning the effects on tumors, Zhang et al. clarified that β-elemene could inhibit the proliferation and induce apoptosis of human hepatocarcinoma cell HepG-2, affecting the tumor cell-cycle arrest at S phase via downregulating α-tubulin and microtubular polymerization (Zhang et al., 2013). In addition, the cell death receptor pathway, the mitochondrial pathway and the ER stress pathway which guide the tumor cell apoptosis are also controlled by β-elemene intervention (Su et al., 2013; Dai et al., 2013). These molecules may be targets in DCs as well. In relation to cancer treatment, synergistic effects to induce Th1 and cytotoxic T-lymphocyte immune responses are observed when β-elemene combines with IL-23-transfected DC vaccine (Tan et al., 2007). This supports the possibility that β-elemene may act on DCs.

To clarify the intrinsic effect of β-elemene on the induction of Foxp3$^+$ T cells by DCs, we cultured CD11c$^+$ DCs and CD4$^+$ T cells together in the absence or presence of RA and hTGF-β1. We found that β-elemene promoted Treg induction in the presence of TGF-β1. The results also suggested the upregulation of RALDH2 expression by β-elemene in the culture system, which would produce RA, and the produced RA could work together with TGF-β1 to promote the expression of Tregs. In addition to promoting TGF-β-mediated Treg induction, RA directs the expressions of α4β7-integrin and CCR9 in the intestine (Benson et al., 2007; Hammerschmidt et al., 2008). We also found that β-elemene increased the proportion of α4β7$^+$CCR9$^+$ T cells in DC-T culture system. This was further evidence that β-elemene enhanced the production of RA.

Currently, there is growing evidence that establishes a relationship between the intestinal immune system and metabolic diseases. In addition to adipose tissue, regulating the intestinal system represents a novel strategy for alleviating obesity-induced inflammation (Winer et al., 2016). Our results suggested that Foxp3$^+$ Tregs generated in the intestinal immune system migrate to adipose tissue and inhibit inflammation. This may be the reason there was only a tendency in increase of Foxp3$^+$CD4$^+$ T cells in MLN. Collectively, we suggested the therapeutic effects of β-elemene on treating experimental obesity-induced chronic inflammation by enhancing the generation of Tregs in the intestinal immune system through modulation of DC function, further alleviating inflammatory symptoms in the fat tissue of mice. Although feeding β-elemene did not affect body weight, it may possibly improve insulin resistance and ameliorate blood glucose levels through its anti-inflammatory effects on adipose tissue.

Limitations of the study

In the animal experiments, there are some interobserver variabilities in the manual segmenting. These anatomic structures which are included in the study depend to some extents on subjective interpretation.

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Satoshi Hachimura (ahachi@mail.ecc.u-tokyo.ac.jp).

Materials availability

This study did not include any new unique reagents and all reagents generated in this study are available from the Lead Contact without restriction.

Data and code availability

The data sets supporting the current study are available from the Lead Contact upon reasonable request.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101883.

ACKNOWLEDGMENTS

This research was supported by a grant from the Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research (B) (Grant number 18H02152).

AUTHOR CONTRIBUTIONS

S.H., M.T. and T.M. conceived this study. Y.Y.Z. designed the research studies as well as analyzed the data and wrote the manuscript. Y.Y.Z., T.T., Y.M.W., R.W., X.Y.L. and H.N.A. conducted the experiments. Y.W. provided the RAG2-deficient DO11.10 mice. S.H., H.N.A., M.T. and T.M. reviewed the manuscript. All the authors read and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: June 19, 2020
Revised: October 20, 2020
Accepted: November 25, 2020
Published: January 22, 2021

REFERENCES

Abreu, M.T. (2010). Toll-like receptor signalling in the intestinal epithelium: How bacterial recognition shapes intestinal function. Nat. Rev. Immunol. *10*, 131–144.

Bakdash, G., Vogelpoel, L.T.C., Van Capel, T.M.M., Kapsenberg, M.L., and De Jong, E.C. (2015). Retinoic acid primes human dendritic cells to induce gut-homing, IL-10-producing regulatory T cells. Mucosal Immunol. *8*, 265–278.

Benson, M.J., Pino-Lagos, K., Rosemblatt, M., and Noelle, R.J. (2007). All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. J. Exp. Med. *204*, 1765–1774.

Birbrair, A., Zhang, T., Wang, Z.-M., Messi, M.L., Enikolopov, G.N., Mintz, A., and Delbono, O. (2013). Role of pericytes in skeletal muscle regeneration and fat accumulation. Stem Cells Dev. *22*, 2298–2314.

Brunkow, M.E., Jeffery, E.W., Hjerrild, K.A., Paeper, B., Clark, L.B., Yasayko, S.A., Wilkinson, J.E., Galas, D., Ziegler, S.F., and Ramsdell, F. (2001). Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat. Genet. *27*, 68–73.

Calle, M.C., and Fernandez, M.L. (2012). Inflammation and type 2 diabetes. Diabetes Metab. *38*, 183–191.

Coombes, J.L., Siddiqui, K.R.R., Arancibia-Cárcamo, C.V., Hall, J., Sun, C.M., Belkaid, Y., and Powrie, F. (2007). A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-β -and retinoic acid-dependent mechanism. J. Exp. Med. *204*, 1757–1764.

Cosmi, L., Maggi, L., Santarlasci, V., Liotta, F., and Annunziato, F. (2014). T helper cells plasticity in inflammation. Cytom. Part A. *85*, 36–42.

Crewe, C., An, Y.A., and Scherer, P.E. (2017). The ominous triad of adipose tissue dysfunction: inflammation, fibrosis, and impaired angiogenesis. J. Clin. Invest. *127*, 74–82.

Dai, Z.J., Tang, W., Lu, W.F., Gao, J., Kang, H.F., Ma, X. Bin, Min, W.L., Wang, X.J., and Wu, W.Y. (2013). Antiproliferative and apoptotic effects of β-elemene on human hepatoma HepG2 cells. Cancer Cell Int. *13*, 1–10.

De Groote, D., Zangerle, P.F., Gevaert, Y., Fassotte, M.F., Beguin, Y., Noizat-Pirenne, F., Pirenne, J., Gathy, R., Lopez, M., Dehart, I., et al. (1992). Direct stimulation of cytokines (IL-1β, TNF-α, IL-6, IL-2, IFN-γ and GM-CSF) in whole blood. I. Comparison with isolated PBMC stimulation. Cytokine *4*, 239–248.

Derynck, R., and Zhang, Y.E. (2003). Smad-dependent and Smad-independent pathways in TGF-β family signalling. Nature *425*, 577–584.

Deslex, S., Negrel, R., Vannier, C., Etienne, J., and Ailhaud, G. (1987). Differentiation of human adipocyte precursors in a chemically defined serum-free medium. Int. J. Obes. *11*, 19–27.

Fenton, T.M., Kelly, A., Shuttleworth, E.E., Smedley, C., Atakilit, A., Powrie, F., Campbell, S., Nishimura, S.L., Sheppard, D., Levison, S., et al. (2017). Inflammatory cues enhance TGFβ activation by distinct subsets of human intestinal dendritic cells via integrin αvβ8. Mucosal Immunol. *10*, 624–634.

Feuerer, M., Herrero, L., Cipolletta, D., Naaz, A., Wong, J., Nayer, A., Lee, J., Goldfine, A.B., Benoist, C., Shoelson, S., et al. (2009). Lean, but not obese, fat is enriched for a unique population

of regulatory T cells that affect metabolic parameters. Nat. Med. *15*, 930.

Fontenot, J.D., Gavin, M.A., and Rudensky, A.Y. (2017). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. J. Immunol. *4*, 330–336.

Goldfine, A.B., and Shoelson, S.E. (2017). Therapeutic approaches targeting inflammation for diabetes and associated cardiovascular risk. J. Clin. Invest. *127*, 83–93.

Gregor, M.F., and Hotamisligil, G.S. (2011). Inflammatory mechanisms in obesity. Ann. Rev. Immunol. *29*, 415–445.

Hammerschmidt, S.I., Ahrendt, M., Bode, U., Wahl, B., Kremmer, E., Förster, R., and Pabst, O. (2008). Stromal mesenteric lymph node cells are essential for the generation of gut- Homing T cells in vivo. J. Exp. Med. *205*, 2483–2490.

Hauner, H., Entenmann, G., Wabitsch, M., Gaillard, D., Ailhaud, G., Negrel, R., and Pfeiffer, E.F. (1989). Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. J. Clin. Invest. *84*, 1663–1670.

Hotamisligil, G.S. (2006). Inflammation and metabolic disorders. Nature *444*, 860–867.

Hotamisligil, G.S., Shargill, N.S., and Spiegelman, B.M. (1993). Adipose expression of tumor necrosis factor-α: direct role in obesity-linked insulin resistance. Science *259*, 87–91.

Ilangumaran, S., and Ferbeyre, G. (2016). Editorial: Cytokines in Inflammation, Aging, Cancer and Obesity. Cytokine *82*, 1–3.

Kwon, H.K., Lee, C.G., So, J.S., Chae, C.S., Hwang, J.S., Sahoo, A., Nam, J.H., Rhee, J.H., Hwang, K.C., and Im, S.H. (2010). Generation of regulatory dendritic cells and CD4+Foxp3 + T cells by probiotics administration suppresses immune disorders. Proc. Natl. Acad. Sci. U S A *107*, 2159–2164.

Leu, T.H., Charoenfuprasert, S., Yen, C.K., Fan, C.W., and Maa, M.C. (2006). Lipopolysaccharide-induced c-Src expression plays a role in nitric oxide and TNFα secretion in macrophages. Mol. Immunol. *43*, 308–316.

Li, M.O., and Flavell, R.A. (2008). Contextual regulation of inflammation: a Duet by transforming growth factor-β and interleukin-10. Immunity *28*, 468–476.

Lumeng, C.N., Bodzin, J.L., Saltiel, A.R., Lumeng, C.N., Bodzin, J.L., and Saltiel, A.R. (2007). Obesity induces a phenotypic switch in adipose tissue macrophage polarization Find the latest version : obesity induces a phenotypic switch in adipose tissue macrophage polarization. J. Clin. Invest. *117*, 175–184.

Mantovani, A., Allavena, P., Sica, A., and Balkwill, F. (2008). Cancer-related inflammation. Nature *454*, 436–444.

Mclaughlin, T., Ackerman, S.E., Shen, L., and Engleman, E. (2017). Role of innate and adaptive immunity in obesity-associated metabolic disease. J. Clin. Invest. *127*, 5–13.

Mu, D., Cambier, S., Fjellbirkeland, L., Baron, J.L., Munger, J.S., Kawakatsu, H., Sheppard, D., Courtney Broaddus, V., and Nishimura, S.L. (2002). The integrin αvβ8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-β1. J. Cell Biol. *157*, 493–507.

Nishimura, S., Manabe, I., Nagasaki, M., Eto, K., Yamashita, H., Ohsugi, M., Otsu, M., Hara, K., Ueki, K., Sugiura, S., et al. (2009). CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. Nat. Med. *15*, 914.

O'Garra, A., Vieira, P.L., Vieira, P., and Goldfeld, A.E. (2004). IL-10-producing and naturally occurring CD4+Tregs: limiting collateral damage. J. Clin. Invest. *114*, 1372–1378.

Özcan, U., Cao, Q., Yilmaz, E., Lee, A.H., Iwakoshi, N.N., Özdelen, E., Tuncman, G., Görgün, C., Glimcher, L.H., and Hotamisligil, G.S. (2004). Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science *306*, 457–461.

Rui, Y., Liu, X., Li, N., Jiang, Y., Chen, G., Cao, X., and Wang, J. (2007). PECAM-1 ligation negatively regulates TLR4 signaling in macrophages. J. Immunol. *179*, 7344–7351.

Saltiel, A.R., and Olefsky, J.M. (2017). Inflammatory mechanisms linking obesity and metabolic disease. J. Clin. Invest. *127*, 1–4.

Seeger, P., Musso, T., and Sozzani, S. (2015). The TGF-β superfamily in dendritic cell biology. Cytokine Growth Factor. Rev. *26*, 647–657.

Shiokawa, A., Kotaki, R., Takano, T., Nakajima-Adachi, H., and Hachimura, S. (2017). Mesenteric lymph node CD11b− CD103+ PD-L1High dendritic cells highly induce regulatory T cells. Immunology *152*, 52–64.

Su, J., Zhou, L., Kong, X., Yang, X., Xiang, X., Zhang, Y., Li, X., and Sun, L. (2013). Endoplasmic reticulum is at the crossroads of autophagy, inflammation, and apoptosis signaling pathways and participates in the pathogenesis of diabetes mellitus. J. Diabetes Res. *2013*, 193461.

Tan, G., Wang, Z., Che, L., and Yin, S. (2007). Immunotherapeutic effects on murine pancreatic carcinoma by β-elemene combined with dendritic cells modified with genes encoding interleukin-23. Front. Med. China *1*, 41–45.

Turovskaya, O., Kim, G., Cheroutre, H., Kronenberg, M., and Madan, R. (2009). Interleukin 10 acts on regulatory t cells to maintain expression of the transcription factor foxp3 and suppressive function in mice with colitis. Nat. Immunol. *10*, 1178–1184.

Winer, D.A., Luck, H., Tsai, S., and Winer, S. (2016). The intestinal immune system in obesity and insulin resistance. Cell Metab. *23*, 413–426.

Winer, D.A., Dranse, H.J., Lam, T.K.T., Winer, D.A., Winer, S., Dranse, H.J., and Lam, T.K.T. (2017). Immunologic impact of the intestine in metabolic disease Find the latest version : Immunologic impact of the intestine in metabolic disease. J. Clin. Invest. *127*, 33–42.

Winer, S., Chan, Y., Paltser, G., Truong, D., Tsui, H., Bahrami, J., Dorfman, R., Wang, Y., Zielenski, J., Mastronardi, F., et al. (2009). Normalization of obesity-associated insulin resistance through immunotherapy. Nat. Med. *15*, 921.

Worthington, J.J., Czajkowska, B.I., Melton, A.C., and Travis, M.A. (2011). Intestinal dendritic cells specialize to activate transforming growth factor-β and induce Foxp3+ regulatory T cells via integrin αvβ8. Gastroenterology *141*, 1802–1812.

Wunderlich, C.M., Hövelmeyer, N., and Wunderlich, F.T. (2013). Mechanisms of chronic JAK-STAT3-SOCS3 signaling in obesity. JAK-STAT *2*, e23878.

Zhai, B., Zhang, N., Han, X., Li, Q., Zhang, M., Chen, X., Li, G., Zhang, R., Chen, P., Wang, W., et al. (2019). Molecular targets of β-elemene, a herbal extract used in traditional Chinese medicine, and its potential role in cancer therapy: a review. Biomed. Pharmacother. *114*, 108812.

Zhang, J., Mao, Y., Hou, L., and Cui, X. (2013). The effect of beta-elemene on alpha-tubulin polymerization in human hepatoma HepG2 cells. Chin. J. Cancer Res. *25*, 770.

Zhang, R., Tian, A., Shi, X., Yu, H., and Chen, L. (2010). Downregulation of IL-17 and IFN-γ in the optic nerve by β-elemene in experimental autoimmune encephalomyelitis. Int. Immunopharmacol. *10*, 738–743.

Zhang, R., Tian, A., Zhang, H., Zhou, Z., Yu, H., and Chen, L. (2011). Amelioration of experimental autoimmune encephalomyelitis by β-elemene treatment is associated with Th17 and Treg cell balance. J. Mol. Neurosci. *44*, 31–40.

iScience, Volume *24*

Supplemental Information

Intestinal regulatory T cell induction by β-elemene alleviates the formation of fat tissue-related inflammation

Yingyu Zhou, Tomohiro Takano, Yimei Wang, Xuyang Li, Rong Wang, Yoshio Wakatsuki, Haruyo Nakajima-Adachi, Masaru Tanokura, Takuya Miyakawa, and Satoshi Hachimura

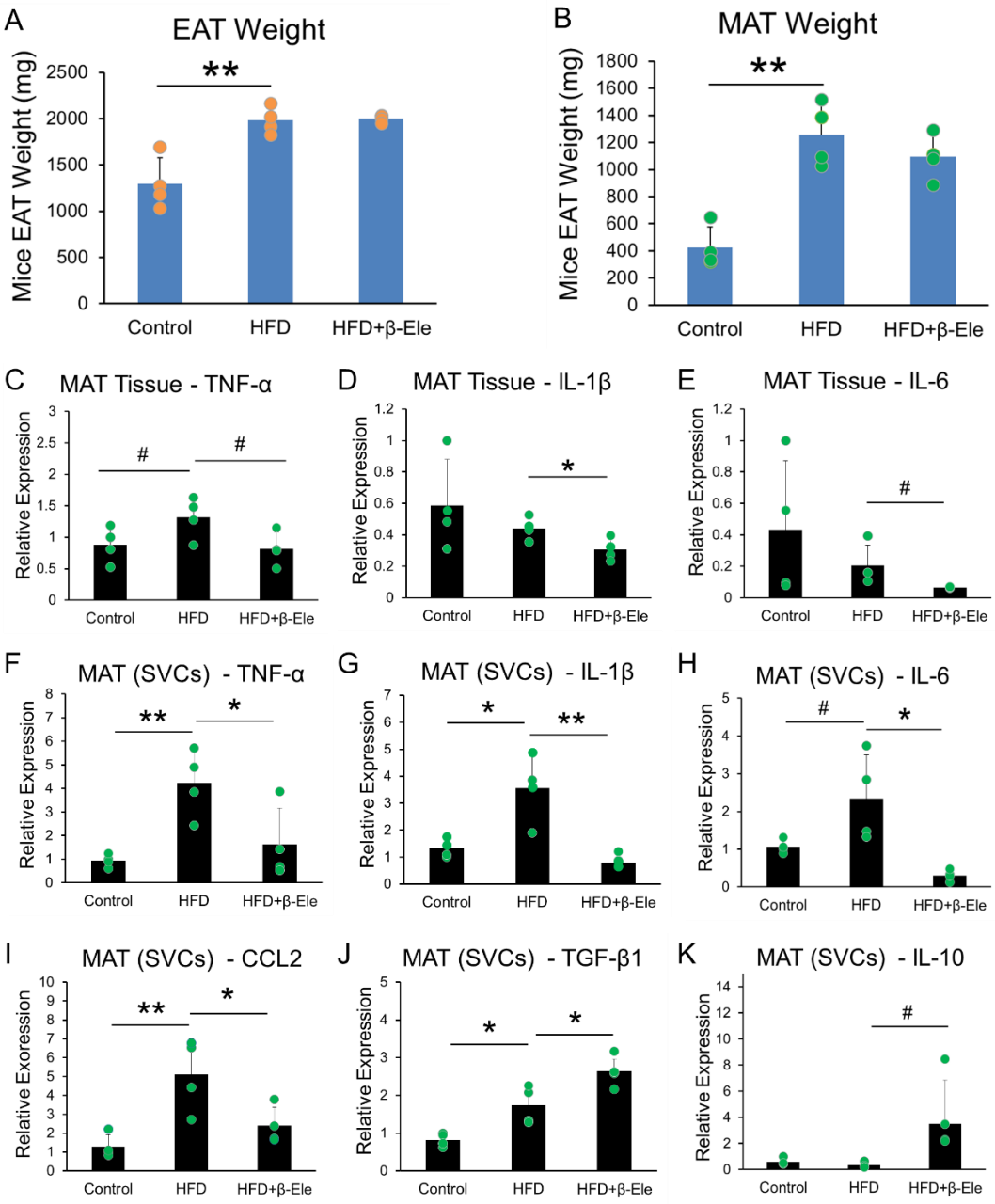


Figure S1. β-Elemene Did Not Influence Mouse Fat Tissue Weight but Regulated Inflammation-related Cytokine Expressions in MAT of Obese Mice, Related to Figure 1. (**A**) EAT weight of mice. (**B**) MAT weight of mice. After obtaining the MAT or SVCs from MAT of each mouse, qPCR was used to measure the gene expressions of inflammation-related cytokines which are shown in the figures (GAPDH expression was the standard). (**C-E**) Expressions of the cytokines TNF-α (**C**), IL-1β (**D**), and IL-6 (**E**) in MAT. (**F-K**) Expressions of the cytokines TNF-α (**F**), IL-1β (**G**), IL-6 (**H**), CCL2 (**I**), TGF-β1 (**J**), and IL-10 (**K**) in MAT SVCs. The results are shown as the mean±SEM. $^{\#}p<0.1$; $^{*}p<0.05$; $^{**}p<0.01$ versus the HFD group (assessed using one-way ANOVA with Tukey's multiple comparisons). HFD, high-fat diet; HFD+β-Ele, HFD-induced obese mice under treatment with β-elemene. $n=4$; the results represent one of two independent experiments.

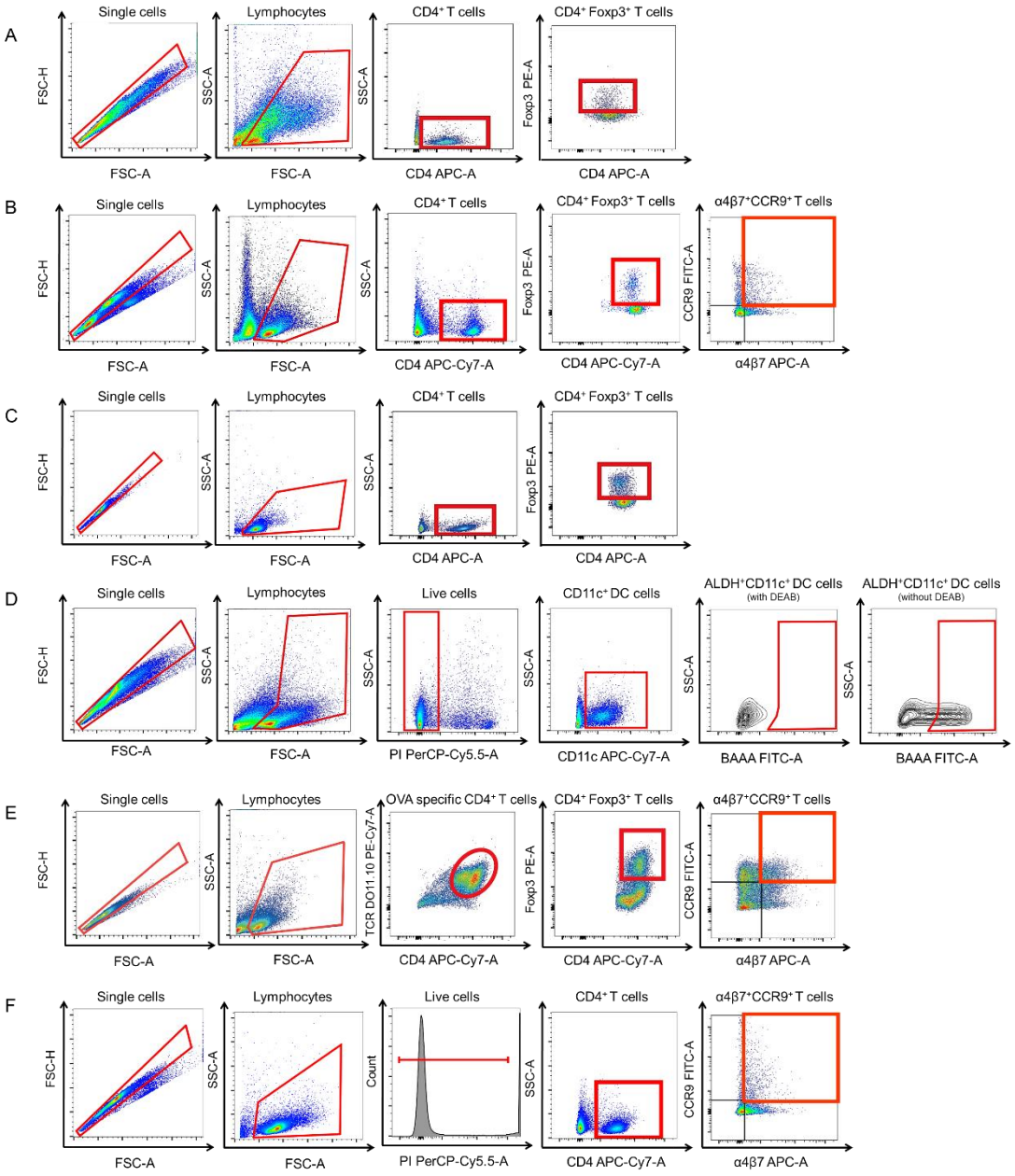


Figure S2. Analysis Processes of Flow Cytometry Related Experiments, Related to Figure 2, Figure 3, Figure 5 and Figure 6. **(A)** The analysis processes of Foxp3⁺CD4⁺ T cells expression in white adipose tissue of mice. **(B)** The analysis processes of Foxp3⁺CD4⁺ T cells and α4β7⁺CCR9⁺ T cells expression in the spleen of mice. **(C)** The analysis processes of Foxp3⁺CD4⁺ T cells expression in MLN or PP of mice. **(D)** The analysis processes of ALDEFLOUR *in vitro*. **(E)** The analysis processes of Foxp3⁺CD4⁺ T cells and α4β7⁺CCR9⁺ T cells expression *in vitro*. **(F)** The analysis processes of α4β7⁺CCR9⁺ T cells expression in the MLN or PP of mice.

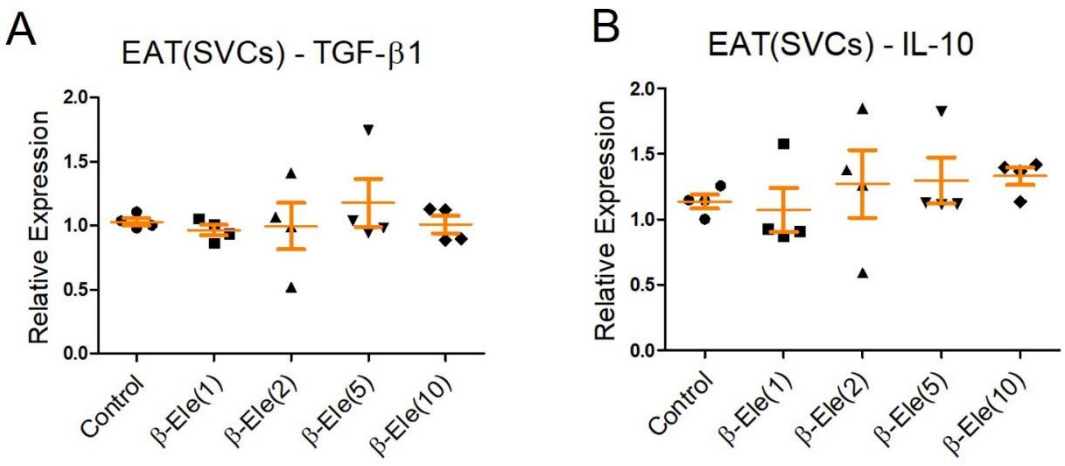


Figure S3. Effects of Different Concentrations of β-Elemene on Regulating Cytokine mRNA Expressions in SVCs in EAT and MAT *in Vitro* in Absence of LPS, Related to Figure 4. Gene expressions of the cytokines TGF-β1 (**A**), and IL-10 (**B**) in EAT SVCs. β-Ele: SVCs (5×10^5 cells/well) were cultured with β-elemene (1, 2, 5 and 10 μg/mL); after culture for 24 h, qPCR was used to measure the gene expressions of inflammation-related cytokines as shown in the figures. The data are obtained from individual wells of each sample. The results are shown as the mean±SEM (versus the control group; assessed using one-way ANOVA with Tukey's multiple comparisons). *n*=4; the results represent one of two independent experiments.

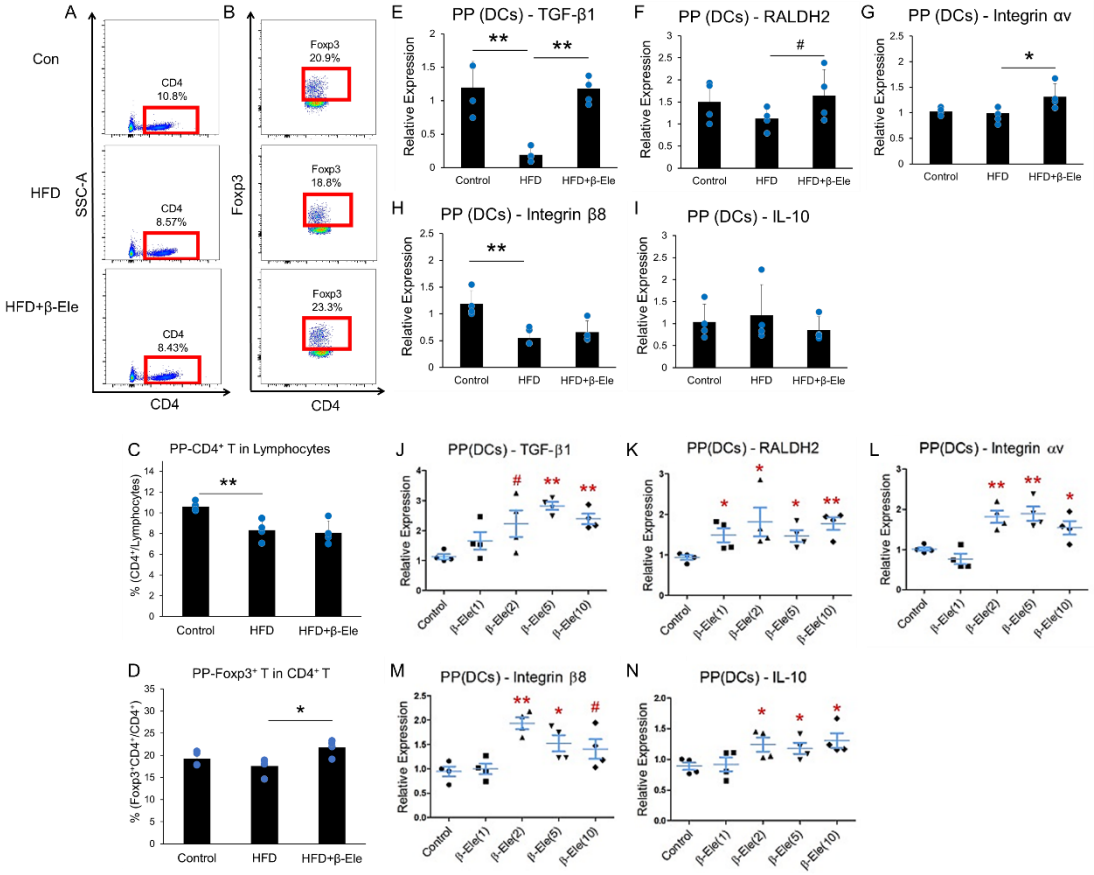


Figure S4. β-Elemene Increased the Proportion of Foxp3⁺CD4⁺ T Cells in the Intestinal Immune System through Enhanced Expressions of TGF-β1, RALDH2, Integrin αvβ8, and IL-10 in PP-DCs, Related to Figure 5. After obtaining mouse PP cells, APC-conjugated anti-CD4 and PE-conjugated anti-Foxp3 antibodies were used to identify the CD4⁺ T cell and Foxp3⁺CD4⁺ T cell populations by flow cytometry. HFD, high-fat diet; HFD+β-Ele, HFD-induced obese mice under treatment with β-elemene. (**A**) CD4⁺ T cells in PP lymphocytes. (**B**) Foxp3⁺CD4⁺ T cells in CD4⁺ T cells of PPs. (**C**) Ratio of CD4⁺ T cells in PP lymphocytes. (**D**) Ratio of Foxp3⁺CD4⁺ T cells in CD4⁺ T cells of PPs. The mRNA expressions of TGF-β1(**E**), RALDH2 (**F**), integrin αv (**G**), integrin β8 (**H**), and IL-10 (**I**) in PP DCs of mice. PP DCs were cultured with β-elemene (1, 2, 5 and 10 μg/mL) for 24 h, the mRNA expressions of TGF-β1 (**J**), RALDH2 (**K**), Integrin αv (**L**), Integrin β8 (**M**) and IL-10 (**N**) in PP DCs *in vitro*. $^{\#}p<0.1$; $^{*}p<0.05$; $^{**}p<0.01$ versus the HFD group (**A-I**) or the control group (**J-N**) (assessed using one-way ANOVA with Tukey's multiple comparisons). $n=4$; the results represent one of two independent experiments.

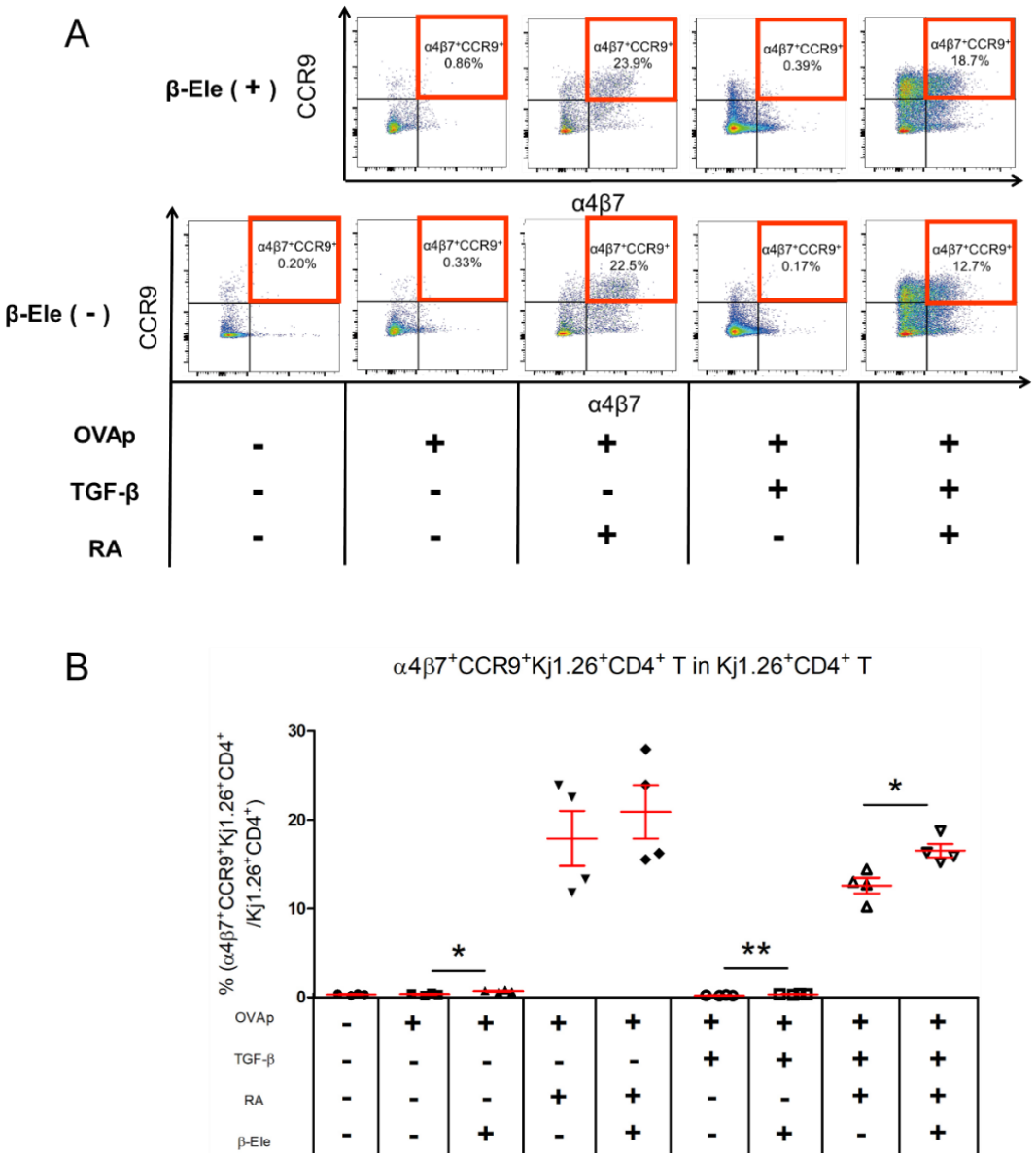


Figure S5. β-Elemene Increased the Proportion of α4β7⁺CCR9⁺ T Cells in the Antigen-specific T Cell Population under Different Treatment Conditions *in Vitro*, Related to Figure 6. The culture system consisted of splenic CD4⁺ T cells (2×10^5 cells/well) from RAG2-deficient DO11.10 mice and MLN CD11c⁺ DCs (2×10^4 cells/well) from BALB/c mice, was conducted in the absence or presence of OVA peptide (10 nM), RA (1 μM), hTGF-β1 (2 ng/ml), and β-elemene (10 μg/ml). After 72 h culture, APC/Cy7-conjugated anti-CD4, PE/Cy7-conjugated anti-DO11.10 TCR (Kj1.26), FITC-conjugated anti-CCR9 and APC-conjugated anti-α4β7 antibodies were used to identify the antigen-specific CD4⁺ T cell and α4β7⁺CCR9⁺ T cell populations by flow cytometry. (**A**) The effect of β-elemene on increasing the proportion of α4β7⁺CCR9⁺ T cells in CD4⁺ T cells in the culture system. (**B**) Ratio of α4β7⁺CCR9⁺ T cells in Kj1.26⁺CD4⁺ T cells of the culture system. The data are obtained from individual wells of each sample. The results are shown as the mean±SEM. $^{*}p<0.05$; $^{**}p<0.01$ between wells with β-elemene added and those without β-elemene (assessed using two-tailed paired Student's *t*-test). $n=4$; the results represent one of two independent experiments.

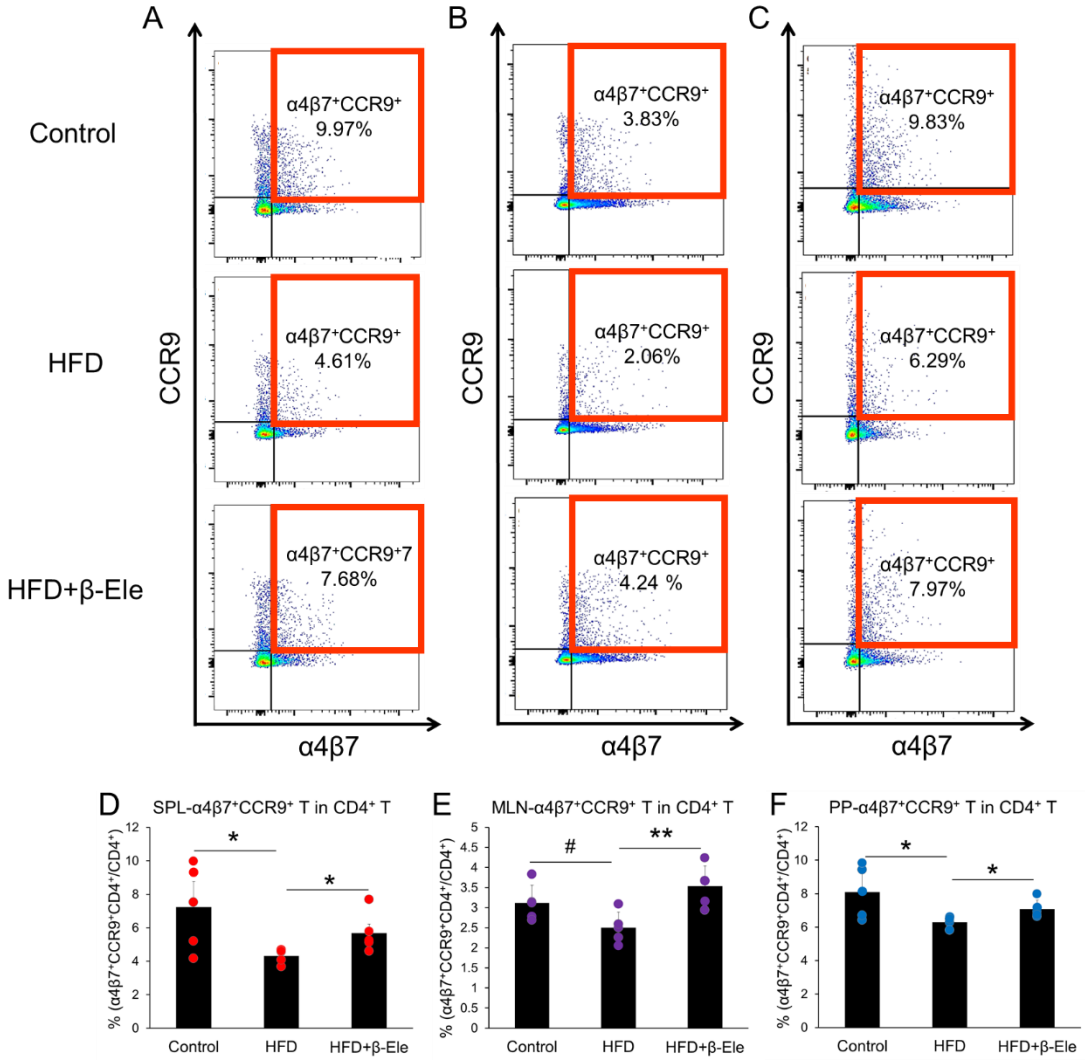


Figure S6. β-Elemene Increased the Proportion of α4β7⁺CCR9⁺ T Cells in SPL, MLN and PP of Obese Mice, Related to Figure 6. After obtaining cells from mouse SPLs, MLNs and PPs, APC/Cy7-conjugated CD4, FITC-conjugated CCR9 and APC-conjugated α4β7 antibodies were used to identify the CD4⁺ T cell and α4β7⁺CCR9⁺ T cell populations by flow cytometry. α4β7⁺CCR9⁺ T cells in CD4⁺ T cells of SPLs (**A**), MLNs (**B**) and PPs (**C**). Ratio of α4β7⁺CCR9⁺ T cells in CD4⁺ T cells of SPLs (**D**), MLNs (**E**) and PPs (**F**). The results are shown as the mean±SEM. $^{\#}p<0.1$; $^{*}p<0.05$; $^{**}p<0.01$ versus the HFD group (assessed using one-way ANOVA with Tukey's multiple comparisons). HFD, high-fat diet; HFD+β-Ele, HFD-induced obese mice under treatment with β-elemene. $n=5$; the results represent one of two independent experiments.

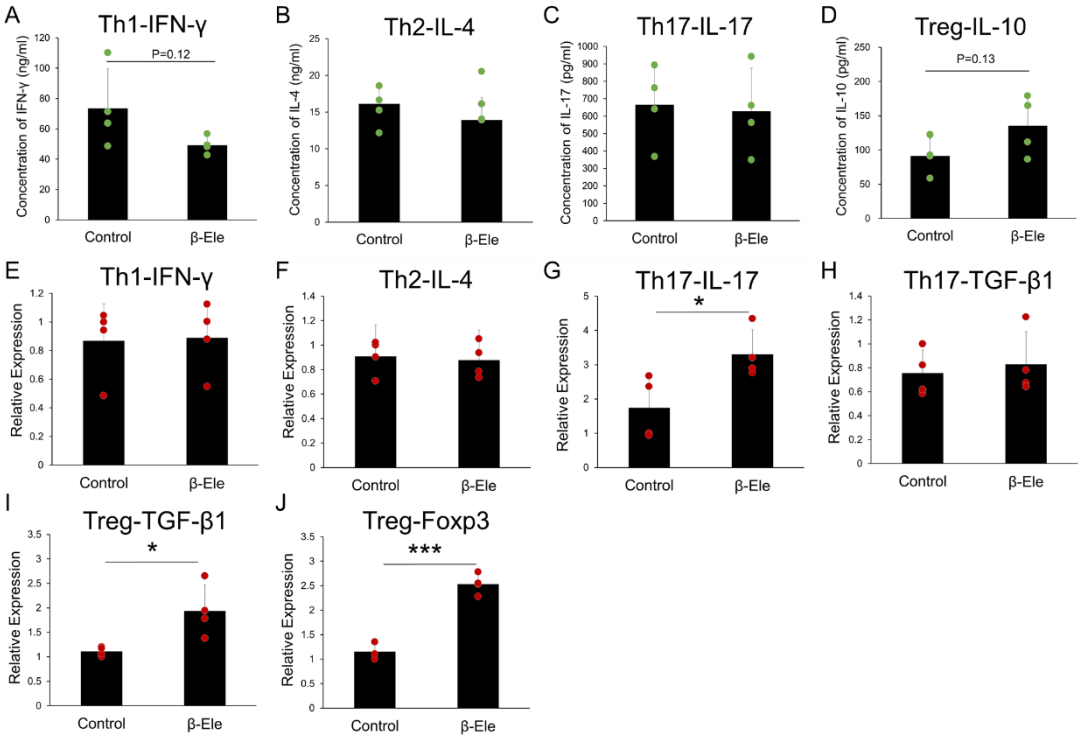


Figure S7. The Direct Effects of β-Elemene on Regulating Differentiation of Naive CD4⁺ T Cells to Th1, Th2, Th17, and Treg, Related to Figure 6. Naïve CD4⁺ T cells (2×10^5 cells/well) were activated with immobilized anti-CD3 and soluble anti-CD28 in the presence of different cytokines and antibodies as described below for 2 day to allow for naïve CD4⁺ T cells to differentiate into different effector lineages. For Th1 differentiation, the cultures were supplemented with interleukin-12 (IL-12) and anti-interleukin-4 (IL-4). For Th2 differentiation, the cultures were supplemented with IL-4 and anti-IFN-γ. For Th17 differentiation, IL-6, TGF-β, anti-IFN-γ and anti-IL-4 were included. For Treg differentiation, the cultures were supplemented with TGF-β. Protein levels of IFN-γ (**A**), IL-4 (**B**), interleukin-17 (IL-17) (**C**), and IL-10 (**D**) were used as hallmarks for Th1, Th2, Th17, and iTreg, respectively using ELISA. the mRNA expressions of IFN-γ (**E**) for Th1, IL-4 (**F**) for Th2, IL-17 (**G**) and TGF-β (**H**) for Th17, TGF-β (**I**) and Foxp3 (**J**) for iTreg were determined as hallmarks using qPCR. n=3-4. Results are shown as the mean±SEM. $^{*}p<0.05$; $^{**}p<0.01$ between wells with β-elemene added and those without β-elemene (assessed using two-tailed paired Student's t-test).

Figure S8. Schedule of Obesity Mouse Model, Related to Figure 1, Figure 2, Figure3 and Figure 5.

Table S1. Primer Sequences for Quantitative PCR, Related to Figure 1, Figure 4 and Figure 5.

Targets	qPCR primers
GAPDH	5-AGGTCGGTGTGAACGGATTTG-3' (forward)
	5'-GGGGTCGTTGATGGCAACA-3' (reverse)
IL-4	5' -GGTCTCAACCCCCAGCTAGT-3' (forward)
	5'-GCCGATGATCTCTCTCAAGTGAT-3'(reverse)
IL-17A	5'-TCAGCGTGTCCAAACACTGAG -3' (forward)
	5'-CGCCAAGGGAGTTAAAGACTT-3' (reverse)
Foxp3	5'-CACCTATGCCACCCTTATCCG-3' (forward)
	5'-CATGCGAGTAAACCAATGGTAGA-3' (reverse)
IL-6	5'-CTGCAAGAGACTTCCATCCAG-3' (forward)
	5'-AGTGGTATAGACAGGTCTGTTGG-3' (reverse)
TNF-α	5'-CAGGCGGTGCCTATGTCTC-3' (forward)
	5'-CGATCACCCCGAAGTTCAGTAG-3' (reverse)
IFN-γ	5'-GCCACGGCACAGTCATTGA-3' (forward)
	5'-TGCTGATGGCCTGATTGTCTT-3' (reverse)
CCL2	5'-TTAAAAACCTGGATCGGAACCAA-3' (forward)
	5'-GCATTAGCTTCAGATTTACGGGT-3' (reverse)
IL-1β	5'-GAAATGCCACCTTTTGACAGTG-3' (forward)
	5'-TGGATGCTCTCATCAGGACAG-3' (reverse)
IL-10	5'-CTTACTGACTGGCATGAGGATCA-3' (forward)
	5'-GCAGCTCTAGGAGCATGTGG-3' (reverse)
TGF- β1	5'-CCACCTGCAAGACCATCGAC-3' (forward)
	5'-CTGGCGAGCCTTAGTTTGGAC-3' (reverse)
RALDH2	5'-GTGGGAGAGTGTTCCCTGTCT-3' (forward)
	5'-TGCCTTGTCTATATCCACCTTGT-3' (reverse)
Integrin αv	5'-CGGGTCCCGAGGGAAGTTA-3' (forward)
	5'-TGGATGAGCATTCACATTTGAGA-3' (reverse)
Integrin β8	5'-TGCATGTTGTAACGTCAAGTGA-3' (forward)
	5'-GATGCTGACACATCAACCAGATA-3' (reverse)

Transparent Methods

Mice. C57BL/6 male mice (8 weeks old, weighing 20±3 g) were purchased from Charles River Laboratories Japan (Yokohama, Japan) and were maintained in appropriate temperature (23±2°C) and humidity (50±5%) with a 12 h light/dark cycle. For *in vivo* experiments, the mice were administered a normal diet (AIN-93G; Oriental Yeast Corporation, Tokyo, Japan) or diet with 60 kcal% fat (HFD-60; Oriental Yeast Corporation) separately for 12 weeks. Oral administration was started in the final 3 weeks, and distilled Milli-Q water or distilled Milli-Q-dissolved β-elemene (7.5 mg/kg/d; 0.2 ml) was administered by gavage. A detailed schedule was shown in **Figure S8**. For *in vitro* experiments, all mice were sacrificed at 8-12 weeks of age. BALB/c female mice were purchased from Charles River Laboratories Japan. RAG2-deficient DO11.10 female mice that express the OVA-specific T cell receptor were maintained and obtained from Sankyo Labo Service Corporation (Tokyo, Japan). All the experimental protocols were approved by the Experimental Animal Ethics Committee of the Graduate School of Agricultural and Life Sciences of the University of Tokyo.

Reagents. (−)-β-Elemene analytical standard was brought from Sigma-Aldrich (St. Louis, MO, United States). Lipopolysaccharide (LPS), from *E.coli* O26 (by phenol extraction) was bought from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). OVA peptide (OVAp) corresponding the OVA 323-339 sequence ISQAVHAAHAEINEAGR was provided by the Medical Proteomics Laboratory at the Institute of Medical Science of the University of Tokyo (Tokyo, Japan). All-*trans* retinoic acid (RA) (FUJIFILM Wako Pure Chemical Corporation) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) at a concentration of 10 mM, and human TGF-β1 (R&D system) was reconstituted at 10 μg/ml in sterile 4 mM HCl containing 1% bovine serum albumin (FUJIFILM Wako Pure Chemical Corporation) before use. For flow cytometry analysis, following antibodies: anti-CD16/32 (93), APC-conjugated anti-CD4 (GK-1.5), APC/Cy7-conjugated anti-CD4 (RM4-5), PE/Cy7-conjugated anti-TCR DO11.10 (KJ1-26), FITC-conjugated anti-CCR9 (CW-1.2), APC-conjugated anti-α4β7 (RATK32) and APC/Cy7-conjugated anti-CD11c (N418) were purchased from BioLegend (San Diego, CA, United States); PE-conjugated anti-Foxp3 (FJK-16s) was purchased from eBioscience (SanDiego, CA, United States).

Cell Preparation and Cell Culture. The MLNs and PPs of mice were treated with 1 mg/ml collagenase I (FUJIFILM Wako Pure Chemical Corporation) with 10 μg/ml DNase I (Roche Diagnostics GmbH, Mannheim, Germany) for 60- 90 mins to obtain single cell suspensions of MLN and PP cells after filtering with a 70-μm cells trainer (Corning, New York, NY, United States) and washing with 10% fetal calf serum (FCS) in RPMI medium. The EAT and MAT of mice were dissociated with 1 mg/ml collagenase type II (Sigma-Aldrich) for 40 mins. After filtering with a 114 μm nylon mesh (TOKYO SCREEN, Tokyo, Japan) and centrifuging, the SVCs were extracted from the cell suspensions after treatment with red blood cell lysis buffer, which is made from ammonium chloride, potassium carbonate, and EDTA (Lumeng et al., 2007). Magnetic-activated cell sorting (MACS) system (Miltenyi Biotec, Bergisch Gladbach, Germany) was used to isolate $CD11c^+$ DCs using CD11c MicroBeads Ultrapure (Miltenyi Biotec) from the obtained cells, including MLNs and PPs. In addition, 1×10^5 DCs or 5×10^5 SVCs of C57BL/6 male mice were cultured with LPS (10 μg/ml) or β-elemene (1, 2, 5, and 10 μg/ml) in 96-well flat bottom plates (Corning), and the cells were collected after 24 h to analyze by quantitative PCR (qPCR). Splenocytes from mice were filtered with 86-μm nylon mesh (TOKYO SCREEN), followed by the MACS system using CD4 Micro Beads (Miltenyi Biotec) to obtain splenic $CD4^+$ T cells. Splenic $CD4^+$ T cells (2×10^5 cells/well) from RAG2-deficient DO11.10 mice and MLN $CD11c^+$ DCs (2×10^4 cells/well) from BALB/c mice were cultured in

the absence or presence of OVA peptide (10 nM), RA (1 μM), hTGF-β1 (2 ng/ml) and β-elemene (10 μg/ml) in 96-well flat-bottom plates for 72 h in a 5% CO_2 humidified atmosphere at 37°C. For cell preparation and culture, RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) containing 100 U/ml penicillin G potassium (Meiji Seika Pharma, Tokyo, Japan), 100 μg/ml streptomycin sulfate (Meiji Seika Pharma), 50 μM 2-mercaptoethanol (Tokyo Chemical Industry, Tokyo, Japan), 0.03% L-glutamine (FUJIFILM Wako Pure Chemical Corporation), and 0.2% sodium hydrogen carbonate (FUJIFILM Wako Pure Chemical Corporation) was prepared with 10% heat-inactivated FCS (Thermo Fisher Scientific, Darmstadt, Germany).

Enzyme-Linked ImmunoSorbent Assay (ELISA). Purified antibodies of IFN-γ, IL-4, IL-17, IL-6, IL-10 and TNF-α (BD Bioscience, Franklin Lakes, NJ, United States) were diluted by 1000 times in 0.1 M sodium hydrogen phosphate (FUJIFILM Wako Pure Chemical Corporation) at 96-well flat bottom plates (Corning). After storing at 4 °C overnight, the plates were blocked by addition of 1% BSA/PBS (100 μl/well) at room temperature for 1 h. Samples and standards were diluted by 1% BSA/PBS-Tween 20 and incubated for 2 h at room temperature. Biotinylated antibodies (BD Bioscience) were diluted by 1% BSA/PBS-Tween 20 (1000 times dilution; 50 μl/well) and the plates were incubated at room temperature for 1 h. Then, Streptavidin-alkaline phosphatase (BD Bioscience) was diluted by 1% BSA/PBS-Tween 20 by 5000 times and added into plates for 50 μl/well for 30 mins at room temperature. 2-Methyl-6-nitroaniline (FUJIFILM Wako Pure Chemical Corporation) was dissolved in diethanolamine (FUJIFILM Wako Pure Chemical Corporation) to the concentration of 1.0 mg/ml. The solution was added to plates for 50 μl/well, and the plates were incubated in dark at 37 °C for 30-40 min. Absorbance of all the samples were measured by a microplate reader (Bio-Rad, California, United States).

T Cell Purification and Differentiation. MACS system (Miltenyi Biotec) was used to isolate $CD4^+$ T cells using CD4 MicroBeads Ultrapure (Miltenyi Biotec) from the splenic cells of C57BL/6 male mice. Naïve $CD4^+$ T cells (2×10^5 cells/well in RPMI1640 medium with 10 % FBS) were activated with immobilized anti-CD3 (BD Bioscience; 5 μg/ml) and soluble anti-CD28 (BD Bioscience; 1 μg/ml) in the presence of different cytokines and antibodies as described below for 2 day to allow for naïve $CD4^+$ T cells to differentiate into different effector lineages. For Th1 differentiation, the cultures were supplemented with IL-12 (10 ng/ml) and anti-IL-4 (10 μg/ml). For Th2 differentiation, the cultures were supplemented with IL-4 (10 ng/ml) and anti-IFN-γ (10 μg/ml). For Th17 differentiation, IL-6 (20 ng/ml), TGF-β (5 ng/ml), anti-IFN-γ (10 μg/ml) and anti-IL-4 (10 μg/ml) were included. For Treg differentiation, the cultures were supplemented with TGF-β (5 ng/ml). Protein levels of IFN-γ, IL-4, IL-17 and IL-10 were used as hallmarks for Th1, Th2, Th17, and iTreg, respectively using ELISA. Similar, the intracellular mRNA expressions of IFN-γ for Th1, IL-4 for Th2, IL-17 and TGF-β for Th17, TGF-β and Foxp3 for iTreg were determined as hallmarks using qPCR.

Flow Cytometry Analysis. All the cells mentioned in the "Cell Preparation and Culture" section, including MLNs, PPs, EAT SVCs, MAT SVCs, splenic cells, MLN $CD11c^+$ DCs, and splenic $CD4^+$ T cells, were used for flow cytometry. Cell staining for flow cytometry was performed at 4°C for 20 mins after Fc-block (anti-CD16/32) for 15 mins with the following monoclonal antibodies: APC-conjugated anti-CD4, PE-conjugated anti-Foxp3, APC/Cy7-conjugated anti-CD4, PE/Cy7-conjugated anti-TCR DO11.10, FITC-conjugated anti-CCR9, APC-conjugated anti-α4β7 and APC/Cy7-conjugated anti-CD11c. Intracellular Foxp3 was stained using a Foxp3 staining buffer set (eBioscience) according to the manufacturer's instructions. Fluorescent levels were measured by FACS Verse

(BD Biosciences). To evaluate RALDH activity, the ALDEFLUOR assay kit (StemCell Technologies, Vancouver, Canada) was used, and RALDH activity was confirmed by comparing with the control sample containing the ALDH inhibitor (*N*,*N*-diethylaminobenzaldehyde; DEAB). The fluorescence levels were measured by FACS Verse (BD Bioscience). All data were analyzed with FlowJo (BD Biosciences).

Quantitative PCR. Adipose tissue was homogenized using QIAzol Lysis Reagent (QIAGEN, Hilden, Germany) with Tissue Ruptor II (QIAGEN) and tissue total RNA was extracted using RNeasy Lipid Tissue Mini Kit (QIAGEN) according to the manufacturer's instructions. Total RNA from the cells was isolated using a QIAshredder (QIAGEN) and RNeasy Mini Kit (QIAGEN). Quantitative PCR was performed with QuantiTect SYBR Green PCR Kits (QIAGEN) using a CFX Connect Real-Time PCR Detection System (Bio-Rad). All relative gene expression levels were normalized to the gene expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All the primer sequences for qPCR were shown in the **Table S1**.

Statistics. All the values are given as the mean±SEM and were analyzed by either two-tailed paired Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparisons. A p-value < 0.05 was considered a significant difference.

Reference

Lumeng, C.N., Bodzin, J.L., and Saltiel, A.R. (2007). Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J. Clin. Invest. *117*, 175-184.