Enhancement and regulation effect of myrcene on antibody response in immunization with ovalbumin and Ag85B in mice

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Abstract

Background: MF59, which is an adjuvant belonging to C_{30} member of the terpene family, is a T helper type-2 (Th2)-biased immune enhancer. Our previous studies showed that pyriproxyfen, a member of the terpene family with fewer carbon atoms (C_{20}) than MF59, enhanced active T helper type-1 (Th1)-biased immune responses.

Objective: This study was performed to investigate the enhancement of antigen-specific immune responses by myrcene, a member of the terpene family with fewer carbon atoms (C_{10}) than pyriproxyfen.

Method: Ovalbumin (OVA) was used as an antigen to determine the effects of myrcene on the immune response. The IgG subtypes and cytokines induced by immunization of OVA with or without myrcene were monitored. Thereafter, we determined the effects of myrcene in the immune response against Ag85B, which is a dominant protective antigen for tuberculosis.

Results: The results showed that 0.8 mg/dose of myrcene enhanced antigen-specific total IgG immune response to OVA. Direct mixing of the antigen with myrcene was required for the enhancement of antibody production. Myrcene increased OVA-specific IgG2a titer, suggesting induction of Th1-immune response. The level of Th1 cytokines, IFN-γ was increased at 8 weeks after immunization, although IL-13 was also increased at the same time point. However, finally myrcene was found to increase Ag85B-specific total IgG titers at 5 weeks and specific IgG2a titer was increased at both 5 and 8 weeks. The results suggested that myrcene could enhance Th1 immune response.

Conclusions: Myrcene enhanced specific immune responses against OVA and Ag85B. This study suggested the tendency of the enhancement of Th1 immune response by myrcene.

Keywords: Ag85B, adjuvant, antigen-specific immune response, myrcene, ovalbumin

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Introduction

Adjuvants are the essential component of acellular or subunit vaccines to make the vaccines efficacious. Many biological and chemical substances have been used as adjuvants for experimental use. However, just a few adjuvants have been approved for clinical use. Only alum, AS04, and MF59 are licensed for human use. All of them are T helper type-2 (Th2)-biased immune enhancers[1] and, therefore, there is a great deal of interest in developing adjuvants for Th1 immune responses. Previously, we reported that pyriproxyfen, which belongs to the terpene family, but has fewer carbon atoms (C_{20}) than MF59 (C_{30}), significantly enhanced antigen-specific IgG responses with high titers of IgG2a and TNF-α and IFN-γ secretion. It suggested that pyriprorxyfen enhanced T helper type-1 (Th1)-biased immune responses.[2] Based on these observations, we postulated that members of

the terpene family with small numbers of carbon atoms would enhance Th1 immune responses.

The helper T cell-mediated immune response plays an important role in vaccination, and is classified into two subsets, i.e., Th1 and Th2 immune responses, which are characterized by the production of Th1-biased IgG subtype (i.e., IgG2a) and Th2-biased IgG subtype (i.e., IgG1), respectively. Induction of the Th1 or Th2 immune response can be distinguished based on the secretion of different cytokines. For example, the Th1 immune response is associated with secretion of interferon-γ (IFN-γ or interleukin-2 (IL-2) from Th1 cells and activates macrophages to secrete tumor necrosis factor-α (TNF-α), thus leading to cell-mediated immunity.[3] On the other hand, the Th2 immune response is associated with secretion of cytokines, such as IL-10, IL-13, IL-4, or IL-5, from Th2 cells, leading to humoral immunity.3 Generally, Th1 type cell-mediated immune responses elicit protection against intracellular pathogens, especially bacteria and viruses,[4,5] whereas Th2 type humoral immune responses elicit protection against extracellular pathogens.

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*Mtb*) is one of the most communicable diseases in tropical and subtropical regions. At present, the only vaccine available is the attenuated live bacillus Calmette–Guérin (BCG) vaccine. BCG confers reliable protection against *Mtb* in childhood, but fails to offer protection in adults.[6] Furthermore, although the vaccine obtained through 230 *in vitro* passages over a 13 year-period, it carries a serious risk of reverting to the pathogenic form and causing serious illness. Therefore, it is a matter of some urgency to replace BCG vaccine with safer and more efficacious vaccines. Ag85B is a 30-kDa mycolyl transferase antigen belonging to the Ag85 complex. The protein is secreted most abundantly from *Mtb*[7] and used as an immunogenic antigen for TB. B and T cell in some animal models, healthy adults, and TB patients were activated after stimulation with Ag85B,[8-10] and Ag85B is also known as a protective antigen of *Mtb*.[9,11] Immunization with Ag85B was shown to confer protection against aerosol challenge with *Mtb* in cattle.[11] Therefore, Ag85B is a leading candidate subunit vaccine antigen for TB.

On the other hand, myrcene (7-methyl-3-methylene-1, 6-octadiene) is found in a wide variety of foods, including lemon peel oil, orange peel oil, orange, and lime juice, and has been identified in over 200 plants. It is a C_{10} member of the terpene family with fewer carbon atoms than pyriproxyfen and an acyclic monoterpene hydrocarbon, is already well known for its antioxidant and antibacterial properties.[2,12,13]

In the present study, to explore for adjuvants capable of enhancing Th1 immune responses, we examined the effects of myrcene on antigen-specific immune responses. We selected the widely used antigen ovalbumin (OVA) as a model to characterize the effects of myrcene on immune responses as previously reported with pyriproxyfen.[2] Moreover, IgG subtypes and cytokines were also monitored. This study also examined the use of myrcene with Ag85B, the leading TB antigen, for enhancement of specific immune responses in mice with a view toward clinical application. The IgG subtypes were monitored as well.

Methods

Ethics statement

Animal care and experiments were performed according to the guidelines approved by the Laboratory Animal Care and Use Committee of Fukuoka University (Certificate No. 1104474).

Animals

Four-week-old female BALB/c mice were purchased from Kyudo Co. Ltd. (Saga, Japan) and housed in a controlled specific pathogen-free environment under a 12-hour light/dark cycle (lights on from 07:00 to 19:00) with temperature and humidity controlled to 23°C±2°C and 55%±5%, respectively. Feed (CE-2; Clea Japan, Tokyo, Japan) and water were provided *ad libitum*.

Immunization

For immunization, OVA (Sigma-Aldrich, St. Louis, MO) and Ag85B (abcam, Cambridge, MA) were separately dissolved and diluted in phosphate-buffered saline (PBS) at concentrations of 50 μg/ml and 5 μg/ml, respectively. Thereafter, 7 μl of 800 μg/μl of myrcene (Wako Pure Chemical Industries Ltd., Osaka, Japan) was diluted in 14 μl of 99% dimethyl sulfoxide (DMSO) and made up to 0.7 ml with PBS for adjustment to 8.0 mg/ml. This myrcene solution was subsequently diluted with an equivalent volume of OVA or Ag85B solution to bring to 4.0 mg/ml of myrcene. The OVA alone or Ag85B alone (control sample) was made up to 0.7 ml of PBS with 21 μl of 99% DMSO and then diluted with an equivalent volume of OVA or Ag85B solution. Myrcene alone was made to dilute 8.0 mg/ml of myrcene solution with an equivalent volume of PBS. Alum solution (Imject Alum; Thermo Scientific, Rockford, IL) was prepared by mixing 1 μl of alum (40 μg/μl) in 100 μl of OVA or Ag85B solution according to the manufacturer's protocol, and finally adjusted 200 μg/ml. All immunizations were performed by intraperitoneal injection in a volume of 200 μl.

To examine the effects of myrcene on the immune response, groups of 6 mice were immunized at 3-week intervals on weeks 0, 3, and 6 with OVA alone, myrcene alone, OVA containing alum, or myrcene. Blood samples were collected from each mouse via the tail vein at 3, 5 and 7 weeks.

To characterize the effects of myrcene on the immune response, groups of 6 mice were immunized at 3-week intervals according to the above schedule, and myrcene was injected mixed with OVA or injected separately at the same time (0 hours) or the OVA injection was given 24 hours after the injection of myrcene. Blood samples were collected on week 8.

To measure the levels of IgG1/IgG2a, groups of 6 mice were immunized at 3-week intervals (weeks 0, 3, and 6) and injected with OVA with or without myrcene or alum, and then blood samples were collected on weeks 5 and 8.

Finally, to confirm the effects of myrcene on Ag85B immune response, groups of 6 mice were immunized at 3-week intervals on weeks 0, 3, and 6 with myrcene alone, Ag85B in 1% DMSO, Ag85B containing alum, or myrcene. Blood samples were collected at 3, 5, and 7 weeks.

To measure the levels of IgG1/IgG2a in response to Ag85B with myrcene, groups of 6 mice were immunized at 3-week intervals on weeks 0, 3, and 6 according to the above schedule and blood samples were collected on weeks 5, and 8.

After collection, blood samples were centrifuged at 12000 rpm for 15 minutes to collect sera. The sera were heat-inactivated at 50°C for 30 minutes and then kept at −20°C until use.

Measurement of antigen-specific immune response

Sera from immunized mice were subjected to enzyme-linked immunosorbent assay (ELISA) to detect the levels of IgG and IgG subtypes, as described previously.[2] Serum samples were initially diluted 1:20 to measure the Ag85B-specific total IgG and IgG2a and, 1:300 for IgG1 immune responses. Values of end-point total IgG titers above the background cutoff level (optical density was at least 2-fold greater in OVA/Ag85B-coated wells than non-coated wells) were considered positive. Titers are shown as end-point dilutions.

Measurement of cytokine levels

Cytokine profiles were checked to confirm the Th1/Th2 bias for enhancement of immune responses by myrcene plus OVA. Two groups of 6 mice were immunized on weeks 0, 3, and 6 and injected with OVA in 1% DMSO with or without myrcene or alum prior to collection of the spleen on weeks 3 and 8. Cytokine levels were measured by sandwich ELISA as described previously.[2] Splenocytes were restimulated with 0.5 mg/ml OVA.

Statistical analyses

The statistical analyses were done with one-way ANOVA and Tukey post-hoc test for comparison of multiple groups. In all analyses, $P<0.05$ was taken to indicate statistical significance.

Results

The enhancement of OVA-induced IgG immune responses based on myrcene

To evaluate the effects of myrcene, we first examined the total IgG immune response to 0.8 mg/dose of myrcene plus OVA-immunized mice at different time points. Mice were immunized on weeks 0, 3, and 6 with OVA alone, myrcene alone or with alum, or myrcene. As shown in **Figure 1**, administration of myrcene alone has no significant effect on OVA-specific total IgG response at any time points examined. However, mice immunized with OVA together with myrcene rapidly reached a significantly greater OVA-specific total IgG titer by week 3 after initial administration compared with control (8.3-fold increase, $P=0.009$), which increased further by week 5 (13.5-fold increase, $P=0.02$) and remained significant and stable until week 7 ($P=0.05$) compared with the controls. Positive control mice immunized with OVA plus alum showed a slow but gradual response, which reached significant titers only by week 7 (4.8-fold increase compared with controls, $P=0.02$).
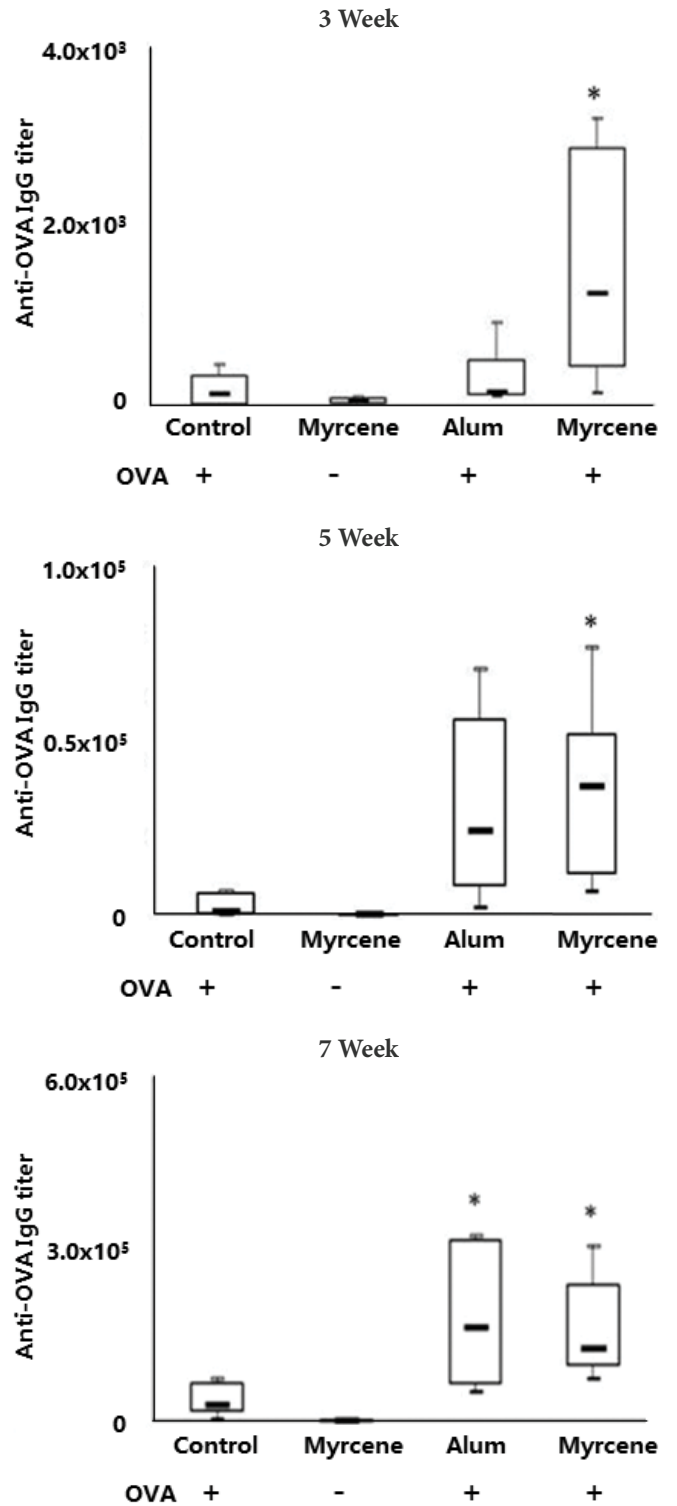

Figure 1. Levels of OVA-specific total IgG immune responses Mice were immunized intraperitoneally on weeks 0, 3, and 6. Control mice received OVA alone. Other groups received 0.8 mg/dose of myrcene alone or OVA mixed with alum or 0.8 mg/dose of myrcene. Serum was collected on weeks 3, 5, and 7, and relative end-point titers of anti-OVA total IgG were examined by ELISA. Values above the cut-off level were considered positive. Data are expressed as medians±standard error of the mean (SEM). *$P<0.05$, **$P<0.01$ *vs.* control, assessed by one-way ANOVA and Tukey post-hoc test. ($n=6$/group).
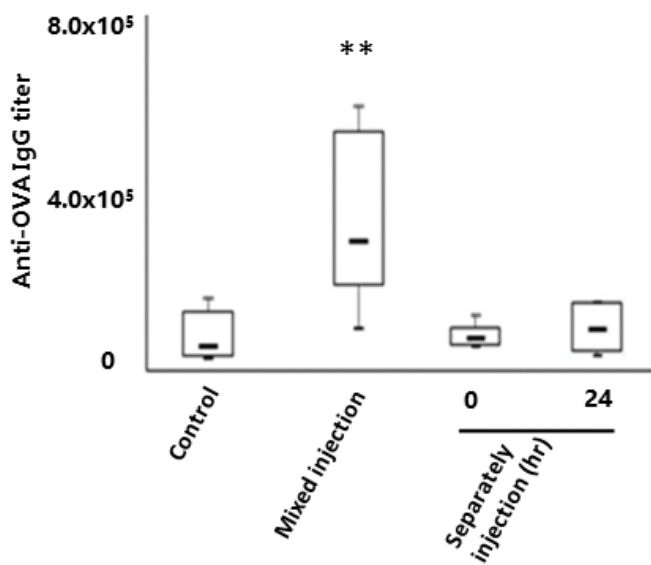
Figure 2. Mixing-dependent OVA-specific total IgG immune responses

Mice were immunized intraperitoneally on weeks 0, 3, and 6. Control mice received OVA alone. Other groups received OVA containing 0.8 mg/dose of myrcene (mixed injection) or 0.8 mg/dose of myrcene injection followed by OVA injection separately at the same time (0 hr) or 24 hours after the injection of myrcene. Serum was collected on week 8 and relative end-point titers of anti-OVA total IgG were examined by ELISA. Values above the cut-off level were considered positive. Data are expressed as medians±standard error of the mean (SEM). **$P<0.01$ *vs.* control, assessed by one-way ANOVA and Tukey post-hoc test. (n=6/group).
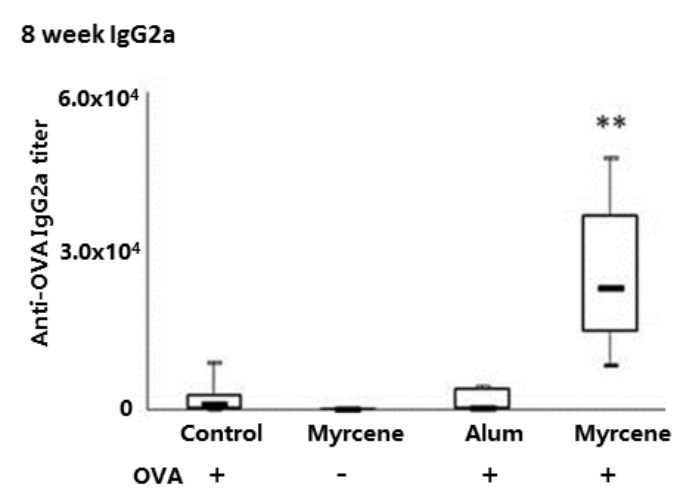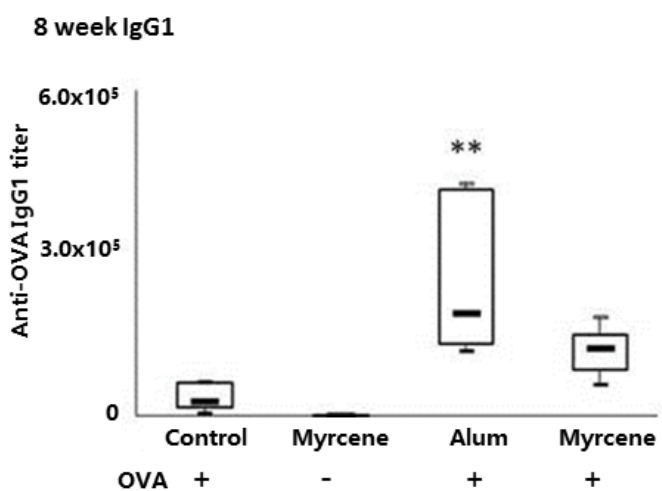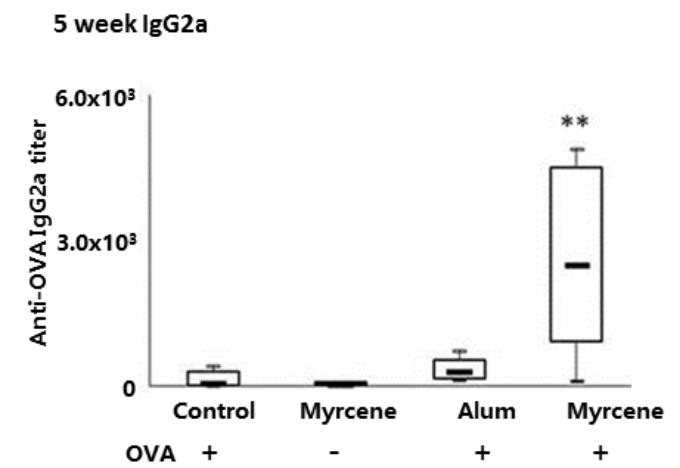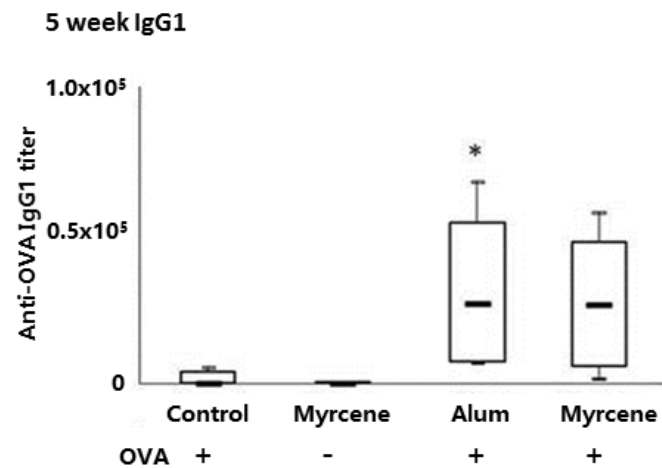

Figure 3. OVA-specific IgG subtypes

Mice were immunized three times on weeks 0, 3, and 6 with OVA alone as a control, 0.8 mg/dose of myrcene alone, or OVA mixed with alum, or OVA with 0.8 mg/dose myrcene. Serum was collected on weeks 5 and 8 for determination of IgG subtypes. Relative end-point titers of OVA-specific IgG1 and IgG2a on weeks 5 and 8 were measured by ELISA. Values above the cut-off level were considered positive. Data are expressed as medians±standard error of the mean (SEM). *$P<0.05$, **$P<0.01$, *vs.* control, assessed by one-way ANOVA and Tukey post-hoc test. (n=6/group).
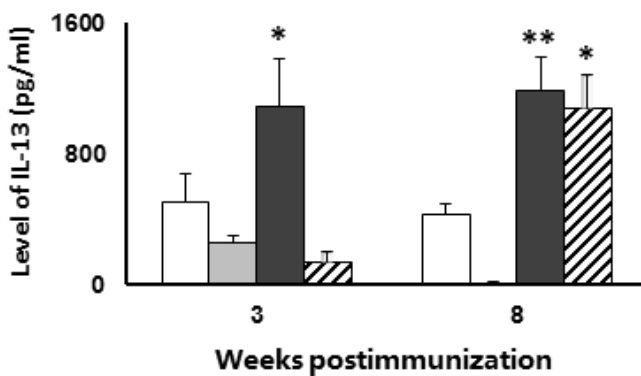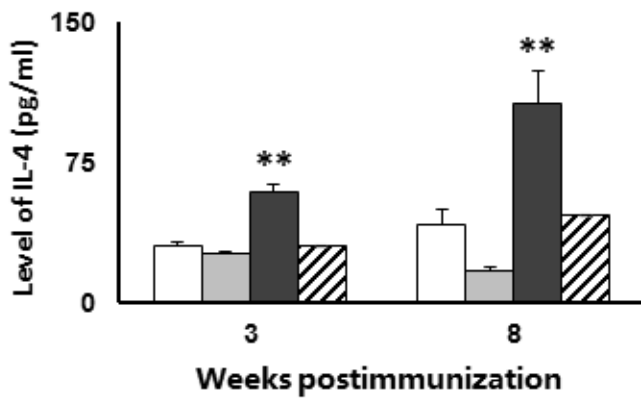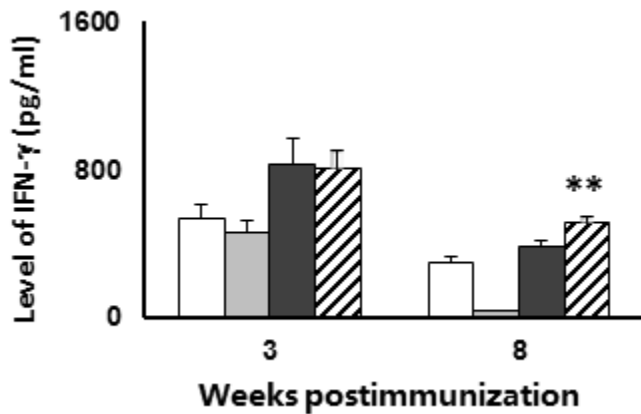
Figure 4. OVA-induced cytokine profile
Two groups of mice were immunized three times on weeks 0, 3, and 6 with OVA alone (☐) as a control, 0.8 mg/dose of myrcene alone (▨ gray), or OVA mixed with alum (■) or OVA with 0.8 mg/dose of myrcene (▨). Spleens were collected on weeks 3 and 8. Splenocytes were harvested, restimulated with OVA, and supernatants were collected and analyzed for IFN-γ, IL-4, and IL-13 by sandwich ELISA. Data are expressed as means±standard error of the mean (SEM). *P<0.05, **P<0.01 *vs.* control, assessed by one-way ANOVA and Tukey post-hoc test. (*n*=6/group).
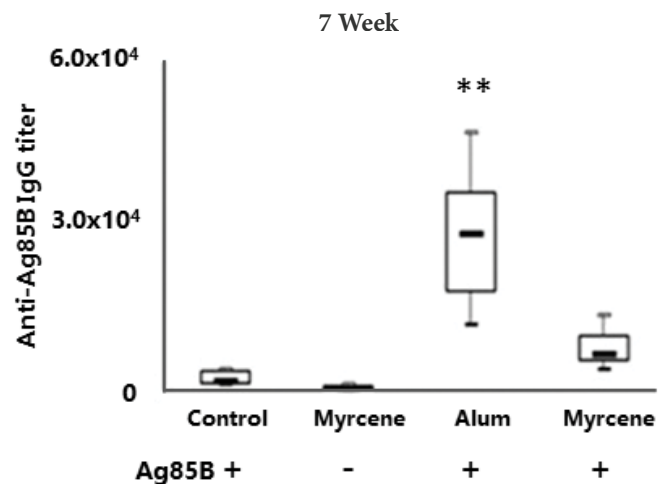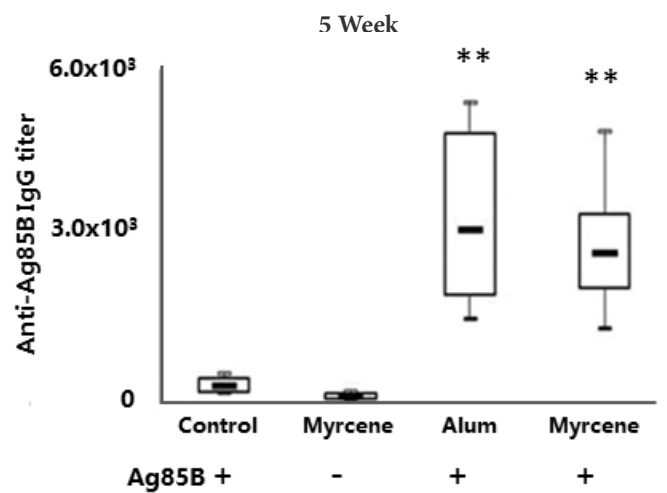
Figure 5. Levels of Ag85B-specific total IgG immune responses
Mice were immunized intraperitoneally on weeks 0, 3, and 6. Control mice received Ag85B. Other groups received 0.8 mg/dose of myrcene alone, or Ag85B mixed with alum or 0.8 mg/dose of myrcene. Serum was collected on weeks 3, 5, and 7, and relative end-point titers of anti-Ag85B total IgG were examined by ELISA. Values above the cut-off level were considered positive. Data are expressed as medians±standard error of the mean (SEM). **P<0.01, *vs.* control, assessed by one-way ANOVA and Tukey post-hoc test. (*n*=6/group).
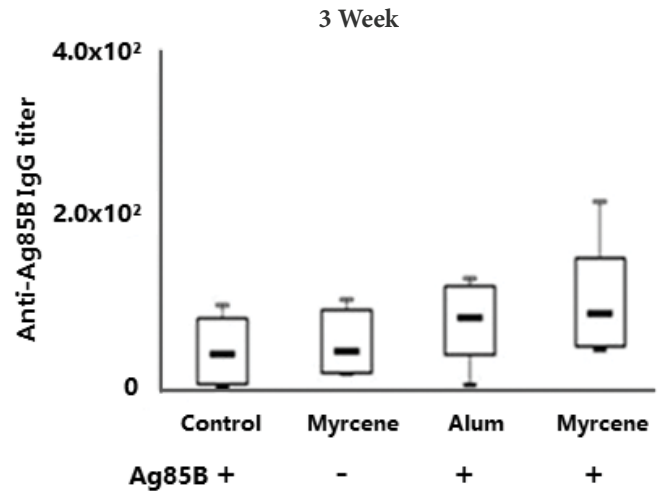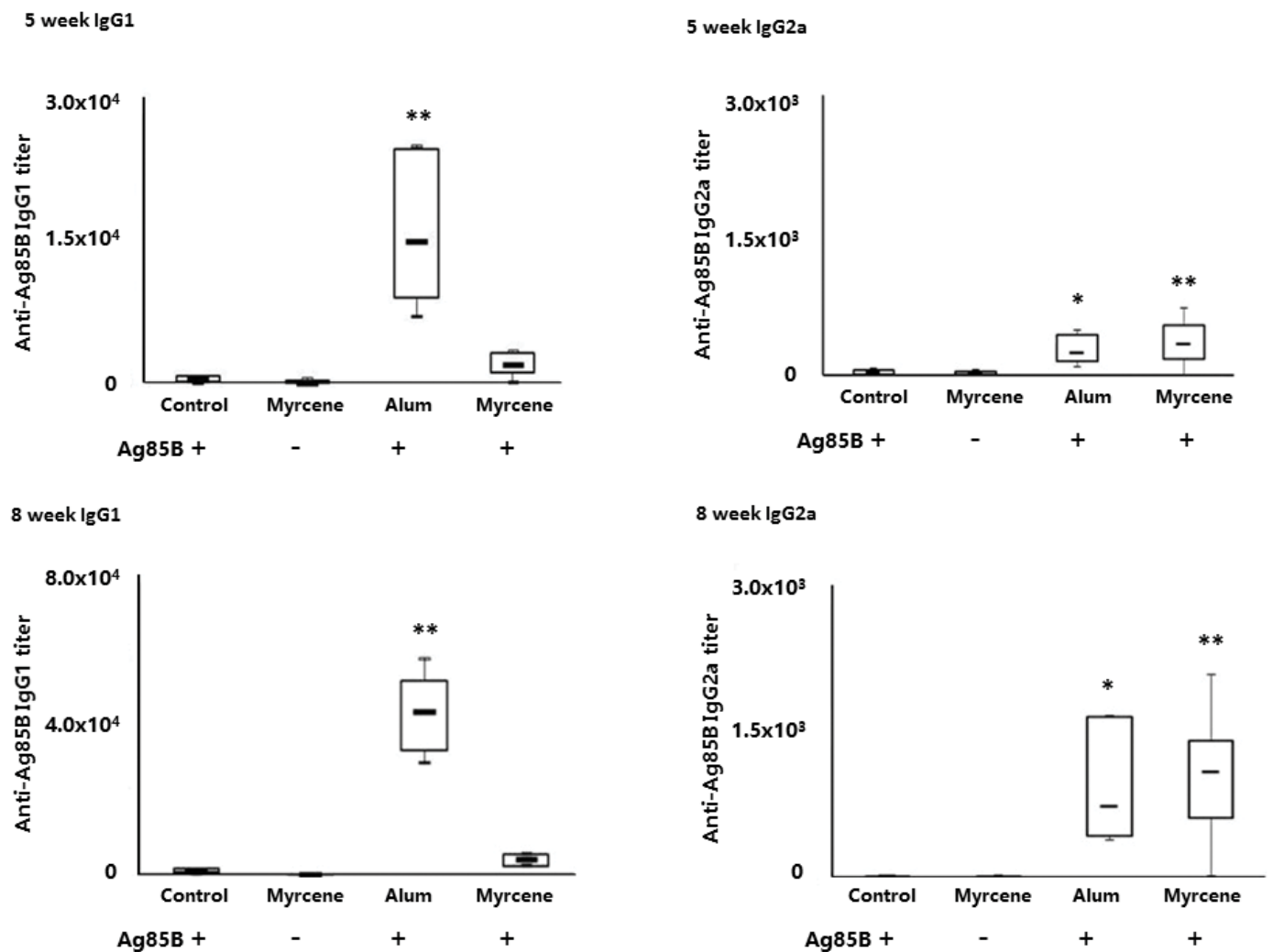
Figure 6. Ag85B-specific IgG subtypes

Mice were immunized intraperitoneally on weeks 0, 3, and 6. Control mice received Ag85B. Other groups received 0.8 mg/dose of myrcene alone, or Ag85B mixed with alum or 0.8 mg/dose of myrcene. Serum was collected on weeks 5 and 8 for determination of IgG subtypes. Relative end-point titers of Ag85B-specific IgG1 and IgG2a on weeks 5 and 8 were measured by ELISA. Values above the cut-off level were considered positive. Data are expressed as medians±standard error of the mean (SEM). *$P<0.05$, **$P<0.01$, *vs*. control, assessed by one-way ANOVA and Tukey post-hoc test. ($n = 6$/group).

Mixing-dependent effect between OVA and myrcene to enhance the immune response

Mixed or separated type injections of myrcene followed by OVA (0 hr and 24 hr) were administered to characterize its ability to enhance the immune response. **Figure 2** illustrates the time-dependent effects of myrcene plus OVA on the immune response. The results indicated that an enhanced total IgG level was found only when mice were administered OVA mixed with myrcene ($P=0.002$). The OVA-specific total IgG titers were not significantly different from controls in mice injected with myrcene followed by OVA injections separately at the same time or 24 hours after the injections of myrcene.

OVA-induced Th1/Th2 immune responses based on myrcene

To investigate the effects of myrcene on IgG subtypes to clearly delineate Th1/Th2 immune responses, mice were immunized on weeks 0, 3, and 6 with OVA alone or with myrcene or alum, and the levels of IgG1/IgG2a subtypes were measured on weeks 5 and 8. As expected, immunization with OVA containing alum resulted in significantly greater OVA-specific IgG1 titers than the controls at 5 and 8 weeks (**Figures 3**). OVA plus myrcene significantly enhanced the OVA-specific IgG2a titers at week 5 compared with controls (5-fold, respectively, $P=0.001$) and became highly significant at week 8 ($P=7.7E-05$) (**Figures 3**). However, no significant difference of OVA-specific IgG1 titers was found in OVA plus myrcene compared with controls at 5 and 8 weeks. The cytokine profiles were examined to confirm the effects of myrcene plus OVA on Th1/Th2 immune responses. Groups of 6 mice were immunized on weeks 0, 3, and 6 with OVA alone or myrcene alone or OVA with myrcene or alum. Spleen samples were collected on weeks 3 and 8 and the cytokine levels were measured by sandwich ELISA. The levels of IL-13 were measured in culture supernatants collected

after 24 hours, whereas IFN-γ and IL-4 were measured in supernatants collected after 72 hours. In mice immunized with alum along with OVA, the levels of Th2 cytokines (IL-4 and IL-13) were significantly increased compared with the controls at both time points 3 week (P=7.4E-07 and P=0.02 respectively) and 8 week (P=0.004 and P=0.005, respectively) (**Figure 4**). Although in mice immunized with OVA plus myrcene, the levels of the Th1 cytokine, IFN-γ was significantly increased only on week 8 compared with controls (P = 0.001), mice immunized with OVA plus myrcene showed increased. IL-13 level on week 8 (P = 0.02) (**Figure 4**). Administration of myrcene alone did not enhance the levels of any Th1 or Th2 cytokines (**Figure 4**)

Ag85B induced Th1/Th2 immune responses based on myrcene

To clarify the basis of Ag85B induced Th1/Th2 immune responses based on myrcene, IgG subtypes were investigated. Mice were immunized on weeks 0, 3, and 6 with Ag85B alone or with myrcene or alum, and the levels of IgG1/IgG2a subtypes were measured on weeks 5 and 8. **Figure 6** shows that immunization with Ag85B containing alum resulted in significantly greater levels of Ag85B -specific IgG1 titers than the control at both weeks 5 and 8 (P = 6.75E-04 and P = 7.79E-04, respectively). However, no significant difference of Ag85B -specific IgG1 titers was found in Ag85B plus myrcene compared with controls at 5 and 8 weeks. On the other hand, the levels of Ag85B-specific IgG2a titers were found to be significant in groups of mice immunized with Ag85B with myrcene at both time points 5 and 8 weeks (P = 2.29E-04 and P = 0.0039, respectively). Administration of myrcene alone did not enhance the levels of any IgG subtypes (**Figure 6**).

Discussion

We demonstrated previously that pyriproxyfen, which belongs to the terpene family but has fewer carbon atoms than the other terpene-based immune enhancer MF59, enhances the Th1-biased immune response.[11] These results prompted us to investigate the effects of other terpenes with fewer carbons on induction of Th1 immune responses.

In the present study, myrcene was found to rapidly enhance the OVA-specific total IgG immune response, supporting the conclusion that the observed enhancement of the response was due to myrcene. Interestingly, the enhancement of total IgG immune response by myrcene was observed only when mixed with OVA, and no enhancement of the OVA-specific immune response was observed if myrcene was injected 0 or 24 hours before the antigen. These observations suggested that direct mixing of the antigen with myrcene is required for the enhancement. Previously, the enhancement of OVA-specific total IgG immune response by pyriproxyfen was observed by injection of pyriproxyfen at 0 or 3 hours before OVA,[2] suggesting that these two agents have different mechanisms of action. Myrcene exists as two isomeric forms, α-myrcene and β-myrcene, the latter of which is known to polymerize.[14] In this study, we used myrcene including both α- and β-isomers as major ingredients. Therefore, the

polymerization of β-myrcene may have affected the enhancement of OVA-specific immune response. The mechanism of action of myrcene observed here may be similar to those of polymer adjuvants, such as carboxy vinyl polymer (CVP).[15] Mixing CVP with antigen increases the viscosity and may thereby prolong antigen presentation, thus enhancing the antigen-specific immune response.

In the case of IgG1/IgG2a immune responses, mice immunized with OVA plus alum showed higher levels of IgG1 on both weeks 5 and 8 (**Figure 3**) with increased production of Th2 cytokines (IL-4 and IL-13 at both weeks 3 and 8) (**Figure 4**), indicating that alum acts as a Th2-biased immune enhancer, thus confirming the success of these experiments. Myrcene enhanced the IgG2a titers at both weeks 5 and 8 (**Figure 3** and **Figure 6**), however, showed increased levels of IFN-γ on 8 weeks, along with change in levels of IL-13 at 8 weeks (**Figure 4**). Therefore, the results suggested that myrcene could enhance Th2 immune response against OVA as well as Th1 immune response. This phenomenon can be compared with the enhancement of balanced Th1/Th2-type immune responses with CVP,[15] where β-myrcene may act like a polymer adjuvant resulting in a mixed Th1/Th2 response. On the other hand, we could not confirm our hypothesis that members of the terpene family with a small number of carbon atoms, such as myrcene, enhance the Th1 immune response. Further experiments are needed to investigate our hypothesis using other agents, such as geraniol,[16] which is also a member of the terpene family with a small number of carbon atoms.

Finally, we confirmed the immune response with the clinically relevant TB antigen, Ag85B, and found that myrcene mixed with Ag85B enhanced specific IgG titers (**Figure 5**) as well as drastically enhanced the IgG2a titers at both weeks 5 and 8 (**Figure 6**). Therefore, these data further suggested a prominent notion towards Th1 immune response induced by the Ag85B with myrcene. Tuberculosis (TB) adjuvants containing heat-killed mycobacteria, such as complete Freund's adjuvant (CFA), also increase immune responses by activating Th1 type cell-mediated immune responses, but remain highly reactogenic and are associated with high rates of side effects.[17] For this reason, the use of CFA in human is prohibited. Therefore, there has been a great deal of interest in developing novel adjuvants for use in TB as alternatives to CFA. The enhancement of Ag85B-specific immune responses suggested that myrcene may become a useful novel adjuvant for TB.

In summary, our results suggested that myrcene is capable of enhancing antigen-specific immune responses to OVA and Ag85B. Although the mechanism underlying the shift to a Th1 immune response by members of the terpene family with a small number of carbon atoms remains unclear and the detailed underlying mechanisms of action of myrcene require further exploration.

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