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Lipopolysaccharide from Yoshino cedar trees (*Cryptomeria japonica*) induces high levels of human macrophage polarization

Noriko Ouji-Sageshima, Masahiro Kitabatake, Satoki Fushimi, Satoshi Suzuki, Yuzu Fukui, Ryutaro Furukawa and Toshihiro Ito*

Abstract

Japanese cedar, *Cryptomeria japonica,* is an evergreen conifer native to Japan and is often used as a building material. The humidity control properties of wood are known, but there have been few detailed analyses of its effects on living organisms. Therefore, we investigated the effects of cedar water-soluble components on human macrophages, which are essential for maintaining biological homeostasis and innate immunity. In this study, we prepared aqueous extracts from *Cryptomeria japonica* in Yoshino, Nara prefecture (called as Yoshino cedar). Yoshino cedar aqueous extracts stimulated macrophages toward the classically activated phenotype and inhibited the macrophage phenotype thought to mediate allergic responses. Next, we measured the lipopolysaccharide (LPS) concentration in the Yoshino cedar aqueous extract and found it was present at a high concentration. The major receptor of LPS is Toll-like receptor 4 (TLR4). To confirm whether LPS in Yoshino cedar aqueous extracts activate macrophages through the LPS/TLR4 pathway, we analyzed its effects on TLR4-deficient mouse embryonic fibroblasts (MEF) and wild-type (WT) MEF. TLR4-deficient MEF did not produce the proinflammatory cytokines seen in WT MEF. This result showed that LPS in the Yoshino cedar aqueous extracts activate macrophages via TLR4. This information will help us better understand the benefits of cedar for human health, including allergies.

Keywords: *Cryptomeria japonica*, Lipopolysaccharide, Macrophage, Allergy

Introduction

Wood has multiple effects on human health, including the immune system [1, 2]. Japanese cedar (*Cryptomeria japonica D. Don*) is an indigenous and abundant planted woody species in Japan. Cedar wood is known for its pleasant fragrance, lustrous color, and soft, pliable texture. Regarded for centuries as a wood of luxury, it has been used for the construction of houses, shrines, temples, and ships; it has a soothing color that creates a warm atmosphere [3]. In addition, woods such as cedar secrete lipid-soluble aromatic organic compounds from their leaves, which were reported to have effects on the autonomic nervous system, central nervous system, and endocrine system, resulting in physiological relaxation [1, 2]. For example, the activation of natural killer (NK) cells by forest therapy contributed to anti-tumor effects and lipid-soluble components in woods enhanced nitric oxide production by macrophages [4]. Based on these reports, lipid-soluble components of wood are expected to enhance helper T (Th)1 reactions that promote immune functions. Wood, including Yoshino cedar, also contains water-soluble substances and has the humidity control properties, which is suitable for building materials. However, the effects of the water-soluble components of these woods on the immune system have not been investigated in detail. It is important to understand the effect of the humidity control properties of cedar on biological functions, especially on immune functions such as allergy.

*Correspondence: toshi-ito@naramed-u.ac.jp

Department of Immunology, Nara Medical University, 840 Shijo-Cho, Kashihara, Nara 634-8521, Japan

Macrophages, called monocytes in the peripheral blood, are immune cells that reside in the tissues of various organs. They maintain biological homeostasis and are the first natural responders that control and orchestrate host defenses against infection [5]. Macrophages generally exist as two distinct subsets, M1 macrophages, which promote Th1 responses, and M2 macrophages, which promote Th2 responses. M1 macrophages, classically known as activated macrophages, are proinflammatory and polarized by lipopolysaccharide (LPS). M2 macrophages, known as alternatively activated macrophages, have anti-inflammatory and immunoregulatory effects, and are polarized by Th2 cytokines such as interleukin (IL)-4 and IL-13 to produce anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)-β [6]. Of note, increased M2 macrophage polarization and activation are involved in allergic responses including asthma, atopic dermatitis, and allergic rhinitis [7]. The incidence of allergic disease has increased in industrialized countries over the past decades because of increased M2 responses, and/or decreased M1 responses [8]. The main explanation for this is termed the "hygiene hypothesis" [9], which proposes that decreased exposure to microbial products is one of the main drivers of allergic disease, which induces increased M2 responses, and/or decreased M1 responses [8].

In this study, we investigated the effects of the water-soluble components of Yoshino cedar on M1/M2 polarization to verify whether the humidity control effect of Yoshino cedar affects the regulation of immune functions.

Experimental

Materials

Thirty different pieces of heart wood from Japanese cedar were collected in August 2020 and March 2021 from Kawakami village, Yoshino, Nara ($n = 20$), or two other building material production areas, Shikoku and Kyusyu ($n = 10$) outside the Nara prefecture. Each timber was dried at the felling site and provided in a state ready for use as a building material. We analyzed products that finished the preparation required for building materials. We tested trees that were about 100 years old from Yoshino and about 30–40 years from other areas. These all-timber samples were almost the same diameter, and provided by Kawakami Suppli (Yoshino, Japan) and dried naturally. Each timber sample was chopped and sliced by four-side planing using a molding machine (GMX-5000, Tokiwa Industry, Gifu, Japan) into $50 \times 100 \times 1$–2 mm pieces, and kept at room temperature until use.

Preparation of 37 °C aqueous extracts from Japanese cedar and the measurement of endotoxin levels

The chopped and sliced samples were additionally cut into $5 \times 5 \times 1$–2 mm pieces. One gram of each chipped sample was soaked in 10 ml water and shaken at 200 rpm/min overnight at 37 °C. Each extract was filtered through a 0.45-μm filter (Merck Millipore, Burlington, MA, USA). The extract was used by extracting once from each material. The extracts were stored at − 30 °C until analyzed as aqueous extract. Endotoxin levels in cedar extracts were measured by the Toxin-Sensor™ Limulus Amebocyte Lysate chromogenic endpoint assay according to the manufacturer's protocol (GenScript, Piscataway, NJ, USA).

Monocyte isolation and macrophage culture

The study was conducted in accordance with the principles expressed in the Declaration of Helsinki and approved by the Ethics Committee of Nara Medical University (Approval No. 2108). Blood samples were obtained from healthy donors after written informed consent was obtained. Peripheral blood mononuclear cells (PBMC) were obtained by Lymphoprep™ Tube (Abbott Diagnostics Technologies, Oslo, Norway). Monocytes were isolated from PBMC using anti-CD14 magnetic beads (Miltenyi Biotec, Cologne, Germany) based on Kittan et al.'s report [9]. Isolated $CD14^+$ monocytes were cultured at a concentration of 0.5–1.0×10^6 cells/ml in complete RPMI 1640 medium (Fujifilm Wako Pure Chemical Co., Osaka, Japan) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Fujifilm Wako Pure Chemical Co.), supplemented with 20 ng/ml macrophage colony stimulating factor (M-CSF) (PeproTech, Cranbury, NJ, USA). On day 3, fresh complete RPMI 1640 media containing M-CSF (20 ng/ml) was added. On day 7, fully differentiated macrophages were obtained from $CD14^+$ monocytes and harvested and replated in RPMI 1640 medium. After resting overnight, cells were stimulated with or without 1/100 and 1/1000 dilution of extracts from Japanese cedar for 24 h. Sterile pure water was used for the control treatment. After stimulation, we collected the supernatant and cells. The supernatant was collected for the analysis of C–X–C motif chemokine ligand 10 (CXCL10) and IL-12p40 proteins and stored at − 80 °C until used. The cells were collected for mRNAs analysis by quantitative reverse transcriptase–polymerase chain reaction (qRT–PCR). We analyzed six genes, *C–C chemokine receptor 7 (CCR7), CXCL10, interleukin 12B (IL12b)* which is known as IL-12p40*, arachidonate 15-lipoxygenase*

(ALOX15), folate receptor beta (FOLR2), and *mannose receptor C-type 1* (*MRC1)*, by qRT–PCR as described below.

Culture of mouse embryonic fibroblast (MEF) cells

Mouse embryonic fibroblast (MEF) cells, commonly used to test the immunological response through Toll-like receptors (TLRs), expressed high levels of mRNA for TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR9 [10]. To test the effect of LPS in aqueous substances isolated from Yoshino cedar on M1 skewing of macrophages, we used TLR4-expressing MEF cells (wild-type, WT) and TLR4-knockout MEF cells (KO). Animal experiments performed in this study were approved by The Animal Care and Use Committee at Nara Medical University (Approval No. 12718), and all experiments were performed following the policy of the Care and Use of Laboratory Animals, Nara Medical University. MEF cells from WT C57BL/6 mice (CLEA Japan, Tokyo, Japan) were harvested as previously described [11]. Briefly, a pregnant female mouse was euthanized at Embryo E14.5 or 15.5, the abdomen was swabbed with 70% ethanol and cut open, the uterus was removed, and embryos were placed in a 10-cm petri dish (Corning, Corning, NY, USA) containing 10 ml sterile PBS on ice. The embryos were minced with fresh, sterile razor blades into small fragments. Then, 5 ml of Trypsin–EDTA (0.25%) (Fujifilm Wako Pure Chemical Co.) was added and transferred to a 50-ml conical tube (Corning) after pipetting up and down several times with a 5-ml pipette to disaggregate the tissue. The tissue was placed into the tube in a 37 °C water bath with shaking for 1 h. Next, 5 ml of Dulbecco's modified Eagle medium (DMEM) (Fujifilm Wako Pure Chemical Co.) was added, and spun down at 1200 ×*g* for 5 min at room temperature. MEF cells were expanded in a 100-mm dish containing 10 ml DMEM complete culture medium, then stored in liquid nitrogen until used. MEF cells from TLR4 KO mice were purchased from Oriental Bio Service Co. (Kyoto, Japan). MEF cells from WT and TLR4 KO mice were cultured in complete DMEM medium overnight and then stimulated with or without 1/100 and 1/1000 dilutions of Japanese cedar extracts for 24 h. Sterile pure water was used for the control treatment. After stimulation, we collected supernatants and cells. The supernatant was collected for the analysis of CXCL10 and IL-12p40 protein and stored at − 80 °C until used. The cells were collected for mRNAs analysis by qRT–PCR, and for TLR4 signaling analysis by western blotting using cell lysates. We analyzed five genes, *Il1b, Il6, tumor necrosis factor (Tnf), Cxcl10,* and *Il12b*, by qRT–PCR and two proteins, phospho-nuclear factor-kappa B (p-NF-κB) p65, and β-actin, by western blotting using the WES system (Protein Simple Japan Co., Ltd., San Jose, CA, USA).

RNA extraction and qRT–PCR

RNAs from human macrophages and MEF were isolated using an RNeasy mini kit (Qiagen, Germantown, MD, USA). Total RNA was extracted and then 1 μg of total RNA was reverse-transcribed to cDNA with a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). qRT–PCR analysis was performed using StepOne with TaqMan PCR master mix (Applied Biosystems, Foster City, CA, USA) on the default setting. The quantification of genes of interest was normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and expressed as the fold increase over the negative control for each treatment at each time point as previously described [12].

Protein analysis

All protein analyses in supernatants were performed using a commercial ELISA kit. Human CXCL10 was analyzed using a Human CXCL10/IP-10 Immunoassay Quantikine ELISA (Cat No. DIP100, R&D Systems, Inc., Minneapolis, MN, USA). Human IL-12p40 was analyzed using a Human IL-12/IL-23(P40) ELISA MAX TM Standard set (Cat No. 430701, BioLegend, San Diego, CA, USA). Mouse CXCL10 was analyzed using a Mouse CXCL10/IP-10/CRG-2 Duoset ELISA (Cat No. DY466-05, R&D Systems Inc.). Mouse IL-12p40 analysis was performed by ELISA MAX™ Deluxe Set Mouse IL-12/IL-23(P40) (Cat No. 431604, BioLegend). Each detailed procedure was performed according to the manufacturer's instructions.

TLR4 signaling analysis by western blotting

To test the association of LPS in aqueous substances derived from Yoshino cedar on M1 skewing of macrophages through the TLR4 pathway, we detected the phosphorylation of NF-κB by western blotting using the WES system (Protein Simple Japan Co., Ltd.). Cells were lysed with RIPA buffer (50 mmol/L Tris–HCl (pH 7.6), 150 mmol/L NaCl, 1% nonidet P40 substitute, 0.5% sodium deoxycholate, 0.1% sodium deoxysulfate, ProteoGuard™ EDTA-Free Protease Inhibitor Cocktail (Takara Bio Inc., Shiga, Japan)).

Cell lysates were sonicated and centrifuged at 20,000 ×*g* for 15 min at 4 °C. The supernatants of cell lysates were collected and stored at − 80 °C until used. Protein concentrations were determined using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). We analyzed two proteins, p-NF-κB p65 (Phospho-NF-κB p65 (Ser536) (93H1) Rabbit mAb #3033, Cell Signaling Technology, Inc. Danvers, MA, USA) and β-actin

(Monoclonal Anti-β-Actin, Clone AC-15, Sigma-Aldrich Co. LLC, St. Louis, MO, USA) according to the manufacturer's instructions.

Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM) and are representative of at least two independent experiments. Statistical analyses of endotoxin levels and in vitro gene expressions were performed by the Mann–Whitney *U* test using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Differences in the in vitro gene expression of MEF were analyzed by one-way ANOVA with Tukey's multiple comparisons test using GraphPad Prism 7 (GraphPad Software). *P* values < 0.05 were considered statistically significant.

Results and discussion

The potential of extracts from Japanese cedar to polarize macrophages

Resting monocyte-derived human macrophages from a human healthy volunteer were stimulated with extracts from Japanese cedar (1/100 and 1/1000 dilution) for 24 h, and the gene expressions of representative markers for M1 and M2 macrophages were analyzed. Figure 1 shows the expressions of *CCR7*, *CXCL10*, and *IL12b* genes, representative markers of M1 macrophages, after the treatment of macrophages with cedar extracts. Gene expressions of all M1 markers investigated, including *CCR7, CXCL10,* and *IL12b*, were significantly higher in macrophages treated with extracts from Japanese cedars from the Yoshino area compared with those from other areas (Fig. 1a). Even in lower concertation (1/1000), most of Yoshino cedar aqueous extracts activated macrophages toward the M1 phenotype including *CCR7* and *IL12b* (Fig. 1a). In addition, IL-12p40 and CXCL10 protein levels were also significantly higher, consistent with qPCR results (Fig. 1b). However, the gene expressions of M2 markers, *ALOX15* and *FOLR2*, were significantly lower in macrophages treated with extracts from Japanese cedars from the Yoshino area compared with those from other areas (Fig. 2). Gene expression of the *MRC1* was decreased following stimulation with extracts from Yoshino cedar, but there was no significant difference in *MRC1* gene expression between extracts from the

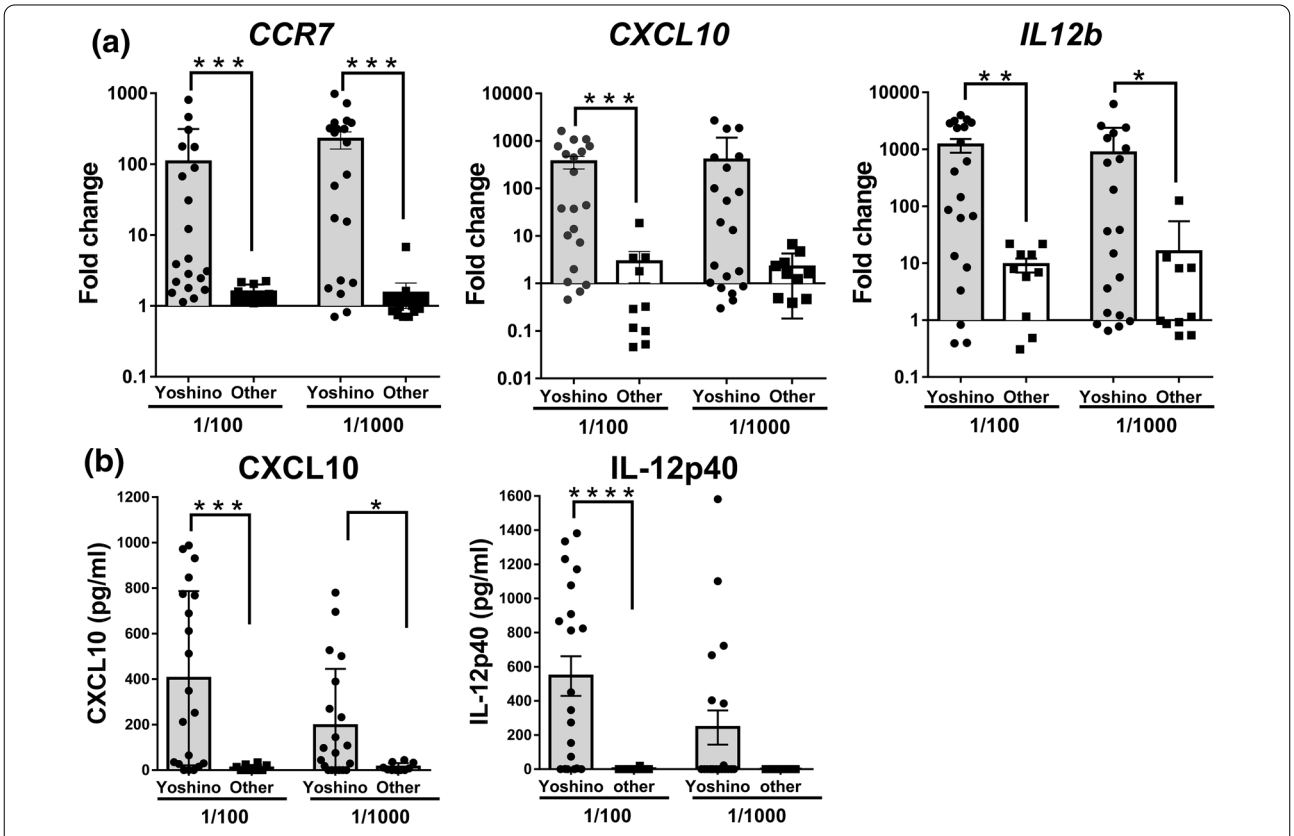

Fig. 1 Effect of *Cryptomeria japonica* on M1 macrophage polarization. **a** Gene expressions of the M1 macrophage markers *CCR7, CXCL10,* and *IL12b* in human-derived macrophages stimulated by *Cryptomeria japonica* from Yoshino ($n = 20$) and other areas ($n = 10$). **b** Protein levels of the inflammatory cytokines CXCL10, and IL-12p40 from human-derived macrophages stimulated by *Cryptomeria japonica* from Yoshino ($n = 20$) and other areas ($n = 10$). Data are the mean ± standard error of the mean. *$P < 0.05$, ***$P < 0.001$ (Mann–Whitney *U* test)

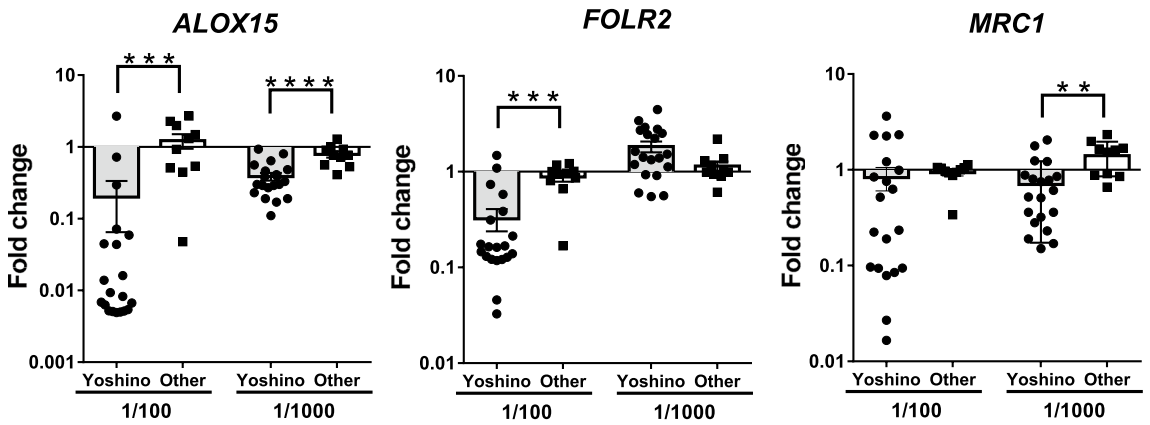

Fig. 2 Effect of *Cryptomeria japonica* on M2 macrophage polarization. Gene expressions of the M2 macrophage markers *ALOX15*, *FOLR2* and *MRC1* in human-derived macrophages stimulated by *Cryptomeria japonica* from Yoshino ($n=20$) and other areas ($n=10$). Data are the mean ± standard error of the mean. ***$P<0.001$, ****$P<0.0001$ (Mann–Whitney *U* test)

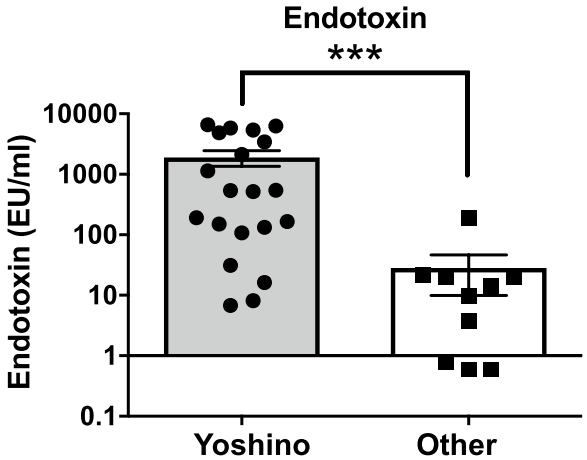

Fig. 3 Concentration of endotoxin (LPS) in *Cryptomeria japonica*. Concentrations of endotoxin (LPS) from aqueous extracts of *Cryptomeria japonica* from Yoshino ($n=20$) and other areas ($n=10$) were measured by ELISA. Data are the mean ± standard error of the mean. ***$P<0.001$ (Mann–Whitney *U*-test)

Yoshino and other areas. These data suggest that extracts from Yoshino cedar might be involved in macrophage polarization, especially promoting M1 and inhibiting M2 polarization.

Endotoxin (LPS) concentration in extracts from Japanese cedar

To investigate why the extracts from Yoshino cedars have a high potential to polarize macrophages toward an M1 phenotype, we measured the concentration of endotoxin (LPS) in 1 ml of aqueous extract from Japanese cedars, because LPS is commonly used in vitro to induce an M1 phenotype and is widely present in the environment. The endotoxin concentration was significantly higher in Japanese cedars from the Yoshino area compared with those from other areas (Fig. 3). Bacterial concentrations in soil are approximately 10^8 per gram, with most bacteria being Gram negative [13], which can be a source of high concentrations of endotoxin. There are a few aqueous extracts from Yoshino cedars with low concentration of endotoxin, which suggests not all the Yoshino cedar increase *CXCL10* and *IL12b* gene expression (Fig. 1). Japanese cedars secrete biogenic volatile organic compounds (BVOCs) that vary by area. BVOCs from Yoshino cedar included monoterpene and diterpene at a ratio of approximately 1:1, whereas Shikoku and Kyushu cedars have a ratio of 3:1 [14, 15]. This difference is thought to be related to the differences in soil composition, which includes different fungi [14] and compositions of the bedrock in the area [15]. Low-density planting is a technique that has been promoted in Japan in recent years, whereas low-density planting has been continuous in Yoshino for 500 years since the sixteenth century; thus, the soil components and soil bacteria and fungi in the Yoshino area may differ from other areas. Yoshino cedars are older than other local cedars and has had a longer soil contact time than other local products. We speculate that these differences might be the reason for the high concentration of LPS and high potential to M1 polarization in aqueous extracts from Yoshino cedar. However, the cause and mechanism of the high amounts of endotoxin in Yoshino cedars compared with cedars from other areas and countries are unclear.

Environmental exposure to microbial products, as measured by endotoxin levels, is associated with a significant decrease in the risk of allergy in childhood. In

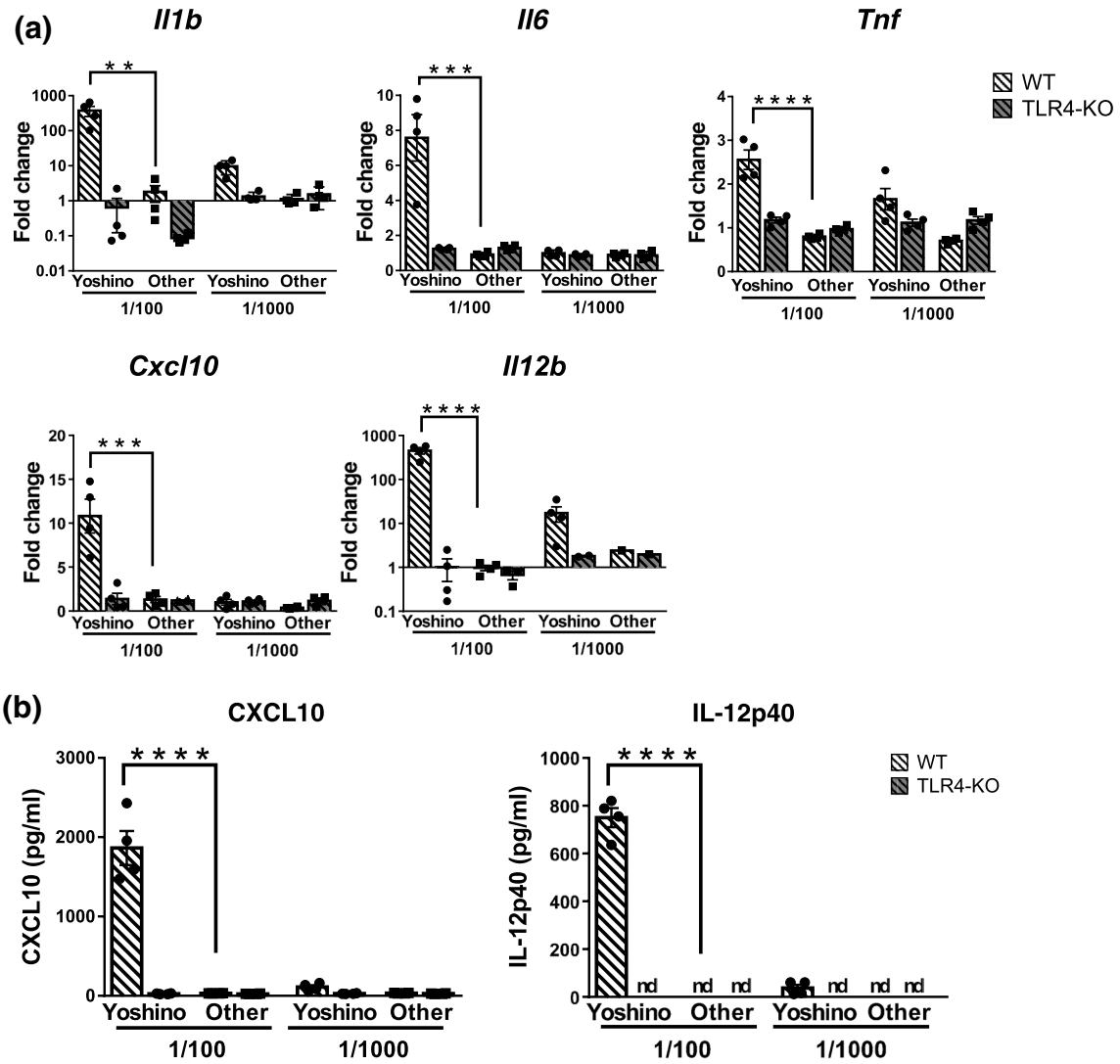

Fig. 4 Effect of *Cryptomeria japonica* on MEF cells. **a** Gene expressions of the inflammatory cytokines *Il1b, Il6, Tnf, Cxcl10* and *IL12b* from WT and TLR4 KO MEF cells stimulated by *Cryptomeria japonica* from Yoshino ($n=4$) and other areas ($n=4$). **b** Protein levels of the inflammatory cytokines CXCL10, and IL-12p40 from WT and TLR4 KO MEF cells stimulated by *Cryptomeria japonica* from Yoshino ($n=4$) and other areas ($n=4$). Data are the mean ± standard error of the mean. $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$ (one-way ANOVA with Tukey's multiple comparisons test). nd; not detected

addition, infants sensitized to common circulating allergens in a "clean" environment are at a higher risk of developing asthma later in life than infants exposed to high levels of environmental endotoxin, which suggests a requirement for exposure to high levels of endotoxin for protection against allergy during infancy [16]. Other epidemiological studies claimed that endotoxin exposure during childhood might protect against the development of allergic asthma later in life [17, 18]. Our data suggest that Yoshino cedar might help to reduce allergic responses after exposure to high levels of endotoxin by M1 macrophage polarization. In this study, we investigated aqueous solutions extracted from cedars, whereas other studies investigated the direct effects of wood-derived volatile components on human health including the immune system [1, 2, 19]. For example, Li et al. showed that a forest bathing trip, called "Shinrinyoku" in Japan, increased NK cell activity, which might be at least

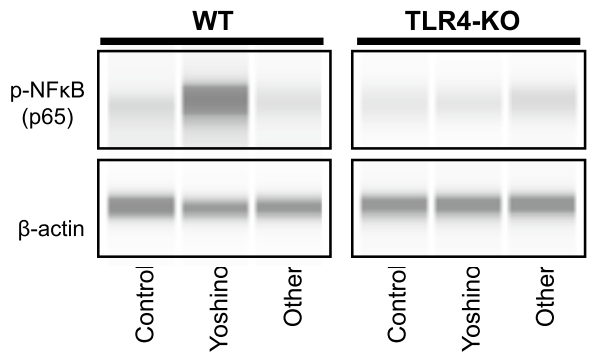

Fig. 5 Yoshino cedar stimulatled MEF cells on the LPS–TLR4 pathway. Protein levels of phosphorylated nuclear factor-kappa B (p-NF-κB) were tested by Western blotting using WES system. Each samples were standalized by β-actin

partially mediated by increasing the number of NK cells [19]. Future studies are essential to investigate the immunological mechanisms involved and epidemiological surveys of allergy in humans using Yoshino cedar itself and examining the environment in the Yoshino area.

M1 macrophage polarization by extracts from Yoshino cedar is dependent on the LPS–TLR4 pathway

LPS is a prototypic cell wall component of Gram-negative bacteria that activates immune cells via transmembrane TLR4 on immune cells and non-immune cells [20]. Intracellular signaling through TLR4 leads to the activation of nuclear factors and production of inflammatory cytokines [21]. Thus, to demonstrate whether cytokine/chemokine induction related to M1 macrophage polarization induced by extracts from Yoshino cedar is dependent on LPS–TLR4, we stimulated MEF cells from WT and TLR4 KO mice with extracts from Japanese cedars for 24 h (Fig. 4). MEF cells expressed high levels of mRNA for TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR9 [10]. MEF cells are a good tool to investigate the association between TLRs and target molecules, because it is easier to understand which TLRs associates with target molecule using cells knocked out the target TLR. In the experimental group stimulated with Yoshino cedar, gene expressions of the inflammatory cytokines, *Il1b, Il6, Tnf, Cxcl10* and *IL12b,* were increased in WT-derived MEF cells. Although these genes were activated in TLR4 KO-derived MEF cells, there were significant differences with WT-derived MEF cells. In addition, IL-12p40 and CXCL10 protein levels were also significantly higher, consistent with the qPCR results (Fig. 4b). However, extracts from other areas did not induce these inflammatory cytokines and there was no significant difference in inflammatory cytokine induction between WT and TLR4 KO-derived MEF cells. Moreover, protein levels of p-NF-κB, a transcription factor with critical roles in activating inflammation through the TLR4 signaling pathway, were increased in WT-derived MEF cells by stimulation with Yoshino cedar extracts, whereas p-NF-κB in TLR4 KO-derived MEF cells was not increased by stimulation with cedar extracts from other areas (Fig. 5). These data suggest that endotoxin in Yoshino cedar directly activates and polarizes M1 macrophages through the LPS–TLR4 pathway. On the basis of this finding, other immune and non-immune cells might be influenced by Yoshino cedar. To demonstrate the effect of Yoshino cedars further, it is necessary to investigate its effects in an in vivo allergy model.

Conclusions

The effects of Yoshino cedar on human macrophages were investigated to aid our understanding of the effects of wood on immune cells. The following conclusions were obtained:

1. Yoshino cedar contains higher amounts of LPS (endotoxin) compared with cedars from other areas in Japan. This might depend on the unique cultivation methods and soil in the Yoshino area; however, further studies are warranted.
2. When compared with Japanese cedars from other areas, those in the Yoshino area strongly promoted macrophage polarization toward an M1 phenotype and inhibited macrophage polarization toward an M2 phenotype.
3. The effects of Yoshino cedars on macrophage polarization toward an M1 phenotype are dependent on the LPS–TLR4 pathway.
4. The effects of Yoshino cedars on M1/M2 macrophage polarization compared with that from other areas in Japan were discussed with references. However, the immunological mechanisms involved in allergy are complex and there are still many unknown effects of *Cryptomeria japonica*, especially Yoshino cedars, on human health.

Abbreviations

ALOX15: Arachidonate 15-lipoxygenase; CCR: C–C chemokine receptor; CXCL: C–X–C motif chemokine ligand; FOLR2: Folate receptor beta; IL: Interleukin; KO: Knockout; LPS: Lipopolysaccharide; M-CSF: Macrophage colony stimulating factor; MEF: Mouse embryonic fibroblast; MRC: Mannose receptor C; TLR: Toll-like receptor; TNF: Tumor necrosis factor; WT: Wild-type.

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Author contributions
The experiments and data analyses were conducted by all authors, and the manuscript was written by NO and TI. All authors read and approved the final manuscript.

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Availability of data and materials
The data sets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
All healthy volunteers accepted the collection of blood samples based on written informed consent under approval by the Ethics Committee of Nara Medical University (Approval No. 2108).

Competing interests
The authors declare that they have no competing interests.

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