Research Article

A Novel Coculture System for Assessing Respiratory Sensitizing Potential by IL-4 in T Cells

Izuru Mizoguchi[1], Yasuhiro Katahira[1], Shinya Inoue[1], Eri Sakamoto[1], Aruma Watanabe[1], Yuma Furusaka[1], Atsushi Irie[2], Satoru Senju[2], Yasuharu Nishimura[2,5], Shusaku Mizukami[3], Kenji Hirayama[3], Sou Nakamura[4], Koji Eto[4], Hideaki Hasegawa[1] and Takayuki Yoshimoto[1]

[1]Department of Immunoregulation, Institute of Medical Science, Tokyo Medical University, Tokyo, Japan; [2]Department of Immunogenetics, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan; [3]Department of Immunogenetics, Institute of Tropical Medicine (NEKKEN), Nagasaki University, Nagasaki, Japan; [4]Department of Clinical Application, Center for iPS Cell Research and Application (CiRA), Kyoto University, Kyoto, Japan; [5]present address: Reiwa Health Sciences University Higashi-ku, Fukuoka, Japan

Abstract

Although several *in vitro* assays that predict the sensitizing potential of chemicals have been developed, none can distinguish between chemical respiratory and skin sensitizers. Recently, we established a new three-dimensional dendritic cell (DC) coculture system consisting of a human airway epithelial cell line, immature DCs derived from human peripheral monocytes, and a human lung fibroblast cell line. In this coculture system, compared to skin sensitizers, respiratory sensitizers showed enhanced mRNA expression in DCs of the key costimulatory molecule OX40 ligand (OX40L), which is important for T helper 2 (Th2) cell differentiation. Herein, we established a new two-step DC/T cell coculture system by adding peripheral allogeneic naïve $CD4^+$ T cells to the DCs stimulated in the DC coculture system. In this DC/T cell coculture system, model respiratory sensitizers, but not skin sensitizers, enhanced mRNA expression of the predominant Th2 marker interleukin-4 (IL-4). To improve the versatility, in place of peripheral monocytes, monocyte-derived proliferating cells called CD14-ML were used in the DC coculture system. As in peripheral monocytes, enhanced mRNA expression of OX40L was induced in CD14-ML by respiratory sensitizers compared to skin sensitizers. When these cell lines were applied to the DC/T cell coculture system with peripheral allogeneic naïve $CD4^+$ T cells, respiratory sensitizers but not skin sensitizers enhanced the mRNA expression of IL-4. Thus, this DC/T cell coculture system may be useful for discriminating between respiratory and skin sensitizers by differential mRNA upregulation of IL-4 in T cells.

1 Introduction

There are two main types of allergic response: skin sensitization, which is an allergic response in the skin following skin contact such as allergic contact dermatitis, and respiratory sensitization, which is an allergic response in the airways caused by inhalation, mostly asthma. Because there is a worldwide movement to limit the use of animal models in the safety testing of chemicals, several *in vitro* assays that predict the skin sensitizing potential of chemicals have been developed including the direct peptide reactivity assay (DPRA, Gerberick et al., 2004), KeratinoSens (Natsch and Emter, 2016), interleukin-8 luciferase (IL-8 Luc) assay (Kimura et al., 2015), and the human cell line activation test (hCLAT, Ashikaga et al., 2006). Respiratory sensitizers often have adverse health effects that are quite severe and long-lasting and therefore are considered substances of very high concern (Tarlo and Lemiere, 2014). Thus, the risk management systems are quite different between chemical respiratory and skin sensitizers; however, none of the aforementioned assays can distinguish between them (Arts, 2020; North et al., 2016).

The adverse outcome pathway (AOP) for skin and respiratory sensitization pathways was established to accurately develop alternative methods for their evaluation (Kimber et al., 2018; MacKay et al., 2013; Sullivan et al., 2017). The AOP contains four key events (KEs): KE1 is covalent binding to skin proteins, KE2 is activation of keratinocytes, KE3 is activation of dendritic

Received November 18, 2021; Accepted January 31, 2022;
Epub February 24, 2022; © The Authors, 2023.

ALTEX 40(2), 204-216. doi: 10.14573/altex.2111181

Correspondence: Takayuki Yoshimoto, PhD
Department of Immunoregulation
Institute of Medical Science
Tokyo Medical University
6-1-1 Shinjuku, Shinjuku-ku
Tokyo, Japan 160-8402
(yoshimot@tokyo-med.ac.jp)

This is an Open Access article distributed under the terms of the Creative Commons Attribution 4.0 International license (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution and reproduction in any medium, provided the original work is appropriately cited.

cells (DCs), and KE4 is activation of T cells. All of the currently developed and validated *in vitro* assays are based on KEs 1-3; there is no validated *in vitro* assay based on KE4 to date (van Vliet et al., 2018), although several types of T cell-based assays have been reported including the human T cell priming assay (hTCPA, Richter et al., 2013; Vocanson et al., 2014) and a novel assay for skin sensitization using the human T lymphocyte cell line Jurkat Clone E6-1 (Hou et al., 2020).

Discrimination between respiratory and skin sensitizers is currently only achieved by the assessment of cytokine profiles in the mouse local lymph node assay, which is the gold standard assay for evaluating the sensitization potential of chemicals (Kimber et al., 1994). Respiratory and skin sensitizers induce different immune responses, predominantly T helper 2 (Th2) responses versus Th1-oriented responses mixed with Th2 and Th17 responses, respectively (Adenuga et al., 2012; Arts et al., 2008; De Jong et al., 2009; Dearman et al., 1995; Goutet et al., 2012; Roggen, 2014; Vandebriel et al., 2000). Specifically, respiratory sensitizers induce a higher expression of IL-4 and IL-4 receptor alpha (IL-4Rα), compared to skin sensitizers (Adenuga et al., 2012; De Jong et al., 2009; Goutet et al., 2012). IL-4 induces the differentiation of naïve $CD4^+$ T cells into Th2 cells and is also a critical effector cytokine produced by Th2 cells (Paul, 2015; Zhu, 2015). Therefore, the difference in molecular mechanisms between respiratory and skin sensitization is considered to highly depend on whether the chemical induces Th2 immune responses, for which IL-4 upregulation in T cells is the key marker.

Thus, we postulated that the establishment of an *in vitro* T cell-based assay that recapitulates the physiological spatiotemporal flow of chemical sensitization processes *in vivo*, including the exposure of DCs to chemicals through the upper airway epithelium and migration of the antigen-presenting DCs to the draining lymph nodes to stimulate naïve $CD4^+$ T cells (Banchereau and Steinman, 1998), could discriminate respiratory sensitizers from skin sensitizers by differential upregulation of IL-4 in T cells. Oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (OXA), formaldehyde (FA), and 2,4-dinitrochlorobenzene (DNCB) were chosen as respiratory sensitizers, and ortho-phthaldialdehyde (OPA), hexamethylene-1,6-diisocyanate (HDI), and trimellitic anhydride (TMA) were chosen as skin sensitizers. These sensitizers are classified moderate to extreme according to the local lymph node assay (Chary et al., 2018; Hou et al., 2020; Sadekar et al., 2021; Tha et al., 2021).

To mimic the human airway upper epithelium, we recently developed a novel three-dimensional (3D) DC coculture system consisting of upper airway epithelial cells, immature DCs, and lung fibroblast cells cultured in individual, stacked scaffolds (Mizoguchi et al., 2017). This system successfully discriminates model chemical respiratory sensitizers from skin sensitizers by measuring the critical molecule for Th2 differentiation in DCs, namely OX40 ligand (OX40L) (Furue and Furue, 2021; Ito et al., 2005). To further improve the system and more precisely recapitulate the *in vivo* activation of naïve $CD4^+$ T cells by DCs that are stimulated with chemical sensitizers in the upper airway epithelium and then migrate into the draining lymph nodes, we herein established a new two-step DC/T cell coculture system by adding peripheral allogeneic naïve $CD4^+$ T cells to the DCs stimulated in the DC coculture system. In this DC/T cell coculture system, the upregulation of IL-4 mRNA in T cells, representing KE4, was successfully used to discriminate respiratory sensitizers from skin sensitizers. To improve the versatility, it was investigated whether peripheral monocyte-derived immature DCs could be replaced with immature DCs derived from monocyte-derived proliferating cells called CD14-ML (Haruta et al., 2013; Imamura et al., 2016) in DC and DC/T cell coculture systems.

2 Materials and methods

Reagents

Three chemical skin sensitizers, oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; OXA, purity ≥ 90%, E0753), formaldehyde (FA, purity 36.5-38%, F8775), and 2,4-dinitrochlorobenzene (DNCB, purity 97%, 138630); and two respiratory sensitizers, ortho-phthaldialdehyde (OPA, purity ≥ 97%, P1378), and trimellitic anhydride (TMA, purity 97%, B4600), were purchased from Sigma-Aldrich (Tab. S1[1]). Hexamethylene-1,6-diisocyanate (HDI, purity > 98%, H0324), a third respiratory sensitizer, was purchased from Tokyo Chemical Industry Co., Ltd. Human recombinant GM-CSF, M-CSF, IL-4, and thymic stromal lymphopoietin (TSLP) were purchased from BioLegend. Human recombinant IL-2 was obtained from Sionogi & Co., Ltd. (Osaka, Japan). OK-432 (penicillin-killed Gram-positive *Streptococcus pyogenes*) was provided by Chugai Pharmaceutical Co., Ltd. (Osaka, Japan), and lipopolysaccharide (LPS from *Escherichia coli* O55:B5), RPMI 1640, and fetal bovine serum (FBS) were purchased from Sigma-Aldrich. αMEM was purchased from Gibco, Grand Island, NY, USA. Penicillin and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA).

Human $CD14^+$ monocytes and naïve $CD4^+$ T cells

This study was approved by the institutional review board of Tokyo Medical University (Nos. SH3323 and T2021-0141; Tokyo, Japan). Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki. Fresh human peripheral blood, anticoagulated with heparin, was collected from seven healthy volunteers (Tab. S2[1]), and mononuclear cells were immediately purified using Lympholyte-H (Cedarlane, Burlington, Canada) density gradient centrifugation.

$CD14^+$ monocytes were further purified from peripheral blood mononuclear cells by positive selection using the AutoMACS Pro cell separator with magnetic beads conjugated with monoclonal antibodies against CD14 (Miltenyi Biotec, Bergisch Gladbach, Germany). Their purity was analyzed by flow cytometry with Brilliant Violet 421 anti-CD14 (HCD14, BioLegend,

[1] doi:10.14573/altex.2111181s

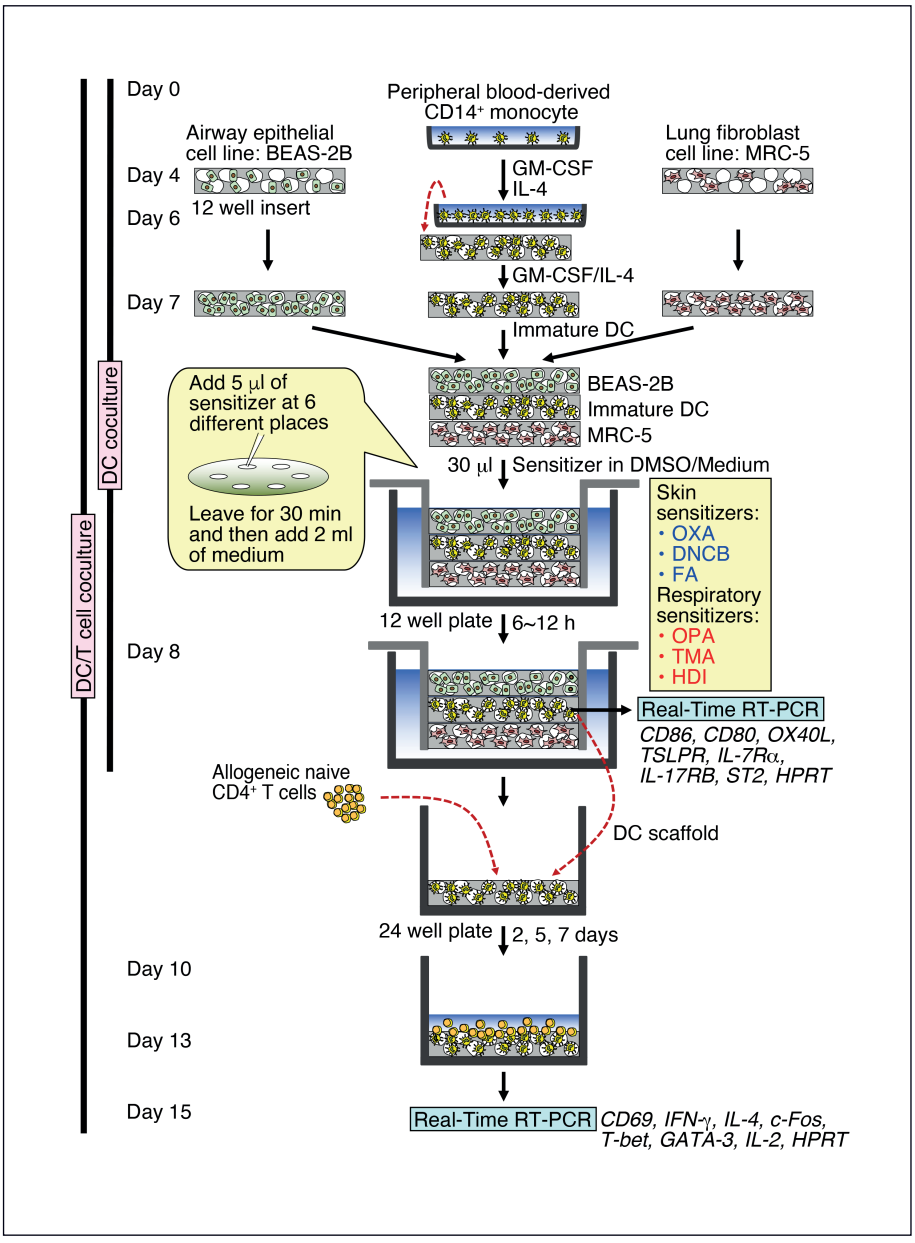

Fig. 1: Establishment of a novel DC/T cell coculture system
The DC coculture system was first established by using the airway epithelial cell line BEAS-2B, peripheral blood mononuclear cell $CD14^+$ monocyte-derived immature DCs, and the lung fibroblast cell line MRC-5, which were initially cultured in individual scaffolds and then assembled on the bottom of an insert well by stacking the scaffolds. Then the chemical sensitizer was gently added to the top scaffold and left for 30 min followed by the addition of medium. After 9 h, the stacked scaffolds were disassembled, and total RNA was extracted from the DC scaffold for real-time RT-PCR analysis. For the DC/T cell coculture system, after 6 to 12 h, only the DC scaffold was placed on the bottom of a new 24-well plate. Then allogeneic naïve $CD4^+$ T cells were added to the DC scaffold and further incubated for 2, 5 or 7 days followed by real-time RT-PCR analysis.

San Diego, CA, USA) using the FACS Canto II System (BD Biosciences, San Jose, CA, USA) followed by analysis with FlowJo Software (Tree Star, Ashland, OR, USA), and was routinely more than 99%.

Naïve $CD4^+$ T cells were purified from peripheral blood mononuclear cells using Human Naïve $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec) and the autoMACS Pro cell separator. The purity of $CD45RA^+CD4^+$ T cells was routinely more than 98% as analyzed by flow cytometry with PECy7 anti-CD45RA (HI100, BioLegend) and Pacific Blue anti-CD4 (OKT4, BioLegend).

Human peripheral blood monocytes were cultured in RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin and immediately differentiated into immature DCs, see below; fresh naïve $CD4^+$ T cells were seeded on the scaffolds as described below.

Preparation of CD14-ML cell lines

CD14-ML cell lines were established according to Dr. Senju's method (Haruta et al., 2013; Imamura et al., 2016). Briefly, $CD14^+$ monocytes from three healthy donors (Tab. S2[1]) were individually infected with lentivirus expressing c-MYC, BMI1, and B-cell lymphoma 2 (BCL-2) and then were cultured in αMEM medium containing 20% FBS, GM-CSF (50 ng/mL), M-CSF (50 ng/mL), 100 U/mL penicillin, and 100 µg/mL streptomycin.

The medium was changed twice a week. Outgrowing cells appeared at around 3 weeks and were passaged twice per week using trypsin. Mycoplasma testing was carried out using e-Myco™ VALiD Mycoplasma PCR Detection Kit (iNtRON Biotechnology, Inc., Gyeonggi-do, Korea) when the cell lines were established. Cells of passages 7 to 15 were used.

Preparation of immature DCs

To prepare immature DCs, peripheral $CD14^+$ monocytes (1×10^6 cells/mL) were stimulated in a 24-well plate with GM-CSF (50 ng/mL) and IL-4 (10 ng/mL) in RPMI 1640 medium containing 10% FBS for 6 days. CD14-ML cells (1×10^6 cells/mL) were stimulated with GM-CSF (50 ng/mL), M-CSF (50 ng/mL), and IL-4 (100 ng/mL) in αMEM medium containing 20% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin for 3 days. The purity of the resultant immature DCs was analyzed after staining with APC anti-CD11c (clone 3.9, eBioscience, La Jolla, CA, USA) and Brilliant Violet 421 anti-CD14 (HCD14, BioLegend) and was routinely approximately 90%.

Cell culture

The human upper airway epithelial cell line BEAS-2B (CRL-9609) (Reddel et al., 1988) and human lung fibroblast cell line MRC-5 (CCL-171) (Jacobs et al., 1970) were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured at 37°C under 5% CO_2/95% air in Eagle's minimum essential medium (MEM; Gibco, Grand Island, NY, USA) containing 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. BEAS-2B and MRC-5 were passaged twice a week using trypsin (Invitrogen).

Preparation of the DC coculture system

The DC coculture was prepared by using Alvetex scaffold 12-well inserts or 24-well plates (Knight et al., 2011), which were purchased from ReproCell (Glasgow, UK), as previously described (Mizoguchi et al., 2017). The scaffold membrane was initially washed sequentially with ethanol, phosphate-buffered saline, and medium according to the manufacturer's instructions.

A 75-µL aliquot of suspension of BEAS-2B or MRC-5 cells (1.5×10^6 cells) was gently seeded directly onto the center of the scaffold in the 12-well insert and left for 4 h to allow cell attachment. Then MEM medium was gently added to the scaffold and incubated for 3 days. A 50-µL aliquot of suspension of immature DCs ($0.5\text{-}1.0 \times 10^6$ cells) was gently seeded directly onto the center of the scaffold in the 24-well plate and medium containing GM-CSF (50 ng/mL) and IL-4 (10 ng/mL) was gently added and incubated for 24 h.

After incubation, the individual scaffolds were gently detached from the 12-well insert or 24-well plate and stacked with MRC-5 cells at the base, immature DCs in the middle, and BEAS-2B cells on top. Then they were attached to the bottom of a new sterile 12-well insert (Fig. 1). Subsequently, the 12-well insert was placed in a 12-well plate, MEM was gently added, and the system was incubated for another 4 h.

Then the medium was gently removed and 5 µL of an aliquot of chemical sensitizer, which was initially dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and then diluted with MEM, was gently added at six places onto the top scaffold. The chemical sensitizers were used up to the highest possible concentrations based on toxicity and solubility. The concentrations of DMSO in the aliquots were 10% (OVA, OPA, FA, and HDI) or 16% or 18% (DNCB and TMA, respectively), and the same DMSO concentrations were used in the respective controls.

After 30 min incubation, 2 mL medium was added, either an equal mixture of MEM and RPMI 1640 medium (2 mL) when peripheral monocyte-derived immature DCs were used or αMEM medium alone (2 mL) when CD14-ML-derived immature DCs were used. After stimulation for 9 h, the stacked scaffold was disassembled, and RNA was extracted from the DC scaffold membrane for real-time RT-PCR analysis. In other experiments, the DC scaffold membrane was transferred to the DC/T coculture system.

Preparation of the DC/T cell coculture system

The DC/T cell coculture was prepared by adding peripheral allogeneic naïve $CD4^+$ T cells prepared from five healthy donors (Tab. S2[1]) to the DCs that had been stimulated in the DC coculture system as follows. After 6 to 12 h stimulation with the chemical sensitizers, the stacked scaffolds in the DC coculture were disassembled, and the DC scaffold was placed on the bottom of a new 24-well plate. Then, 50 µL allogeneic naïve $CD4^+$ T cells (1.5×10^6 cells) in RPMI 1640 medium containing IL-2 (100 U/mL) were added to the DC scaffold and further incubated by adding fresh 100 µL RPMI 1640 medium every 2 days for the indicated times. Cells were then collected by centrifugation and combined within each scaffold, followed by RNA extraction and subsequent real-time RT-PCR analysis.

Real-time RT-PCR

Total RNA was prepared using an RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was prepared using oligo(dT) primer and SuperScript VI RT (Thermo Fisher Scientific). The quality of RNA was checked by measuring the ratio of absorbance at 260 nm and 280 nm. Real-time PCR was performed using the SYBR Premix Ex Taq II and Thermal Cycler Dice Real Time System according to the manufacturer's instructions (Takara, Otsu, Shiga, Japan). Hypoxanthine phosphoribosyltransferase (HPRT) was used as the housekeeping gene to normalize mRNA. The relative expression of PCR products was determined by using the ΔΔCt method to compare target gene and HPRT mRNA expression. Samples whose (HPRT) mRNA expression level was less than one-tenth that of the untreated sample were not used (Mizoguchi et al., 2017). The primers used in this study are listed in Table S3[1].

Statistical analyses

Each mRNA expression was normalized to HPRT mRNA expression, and relative mRNA fold change to control vehicle was calculated for each concentration of sensitizer. However,

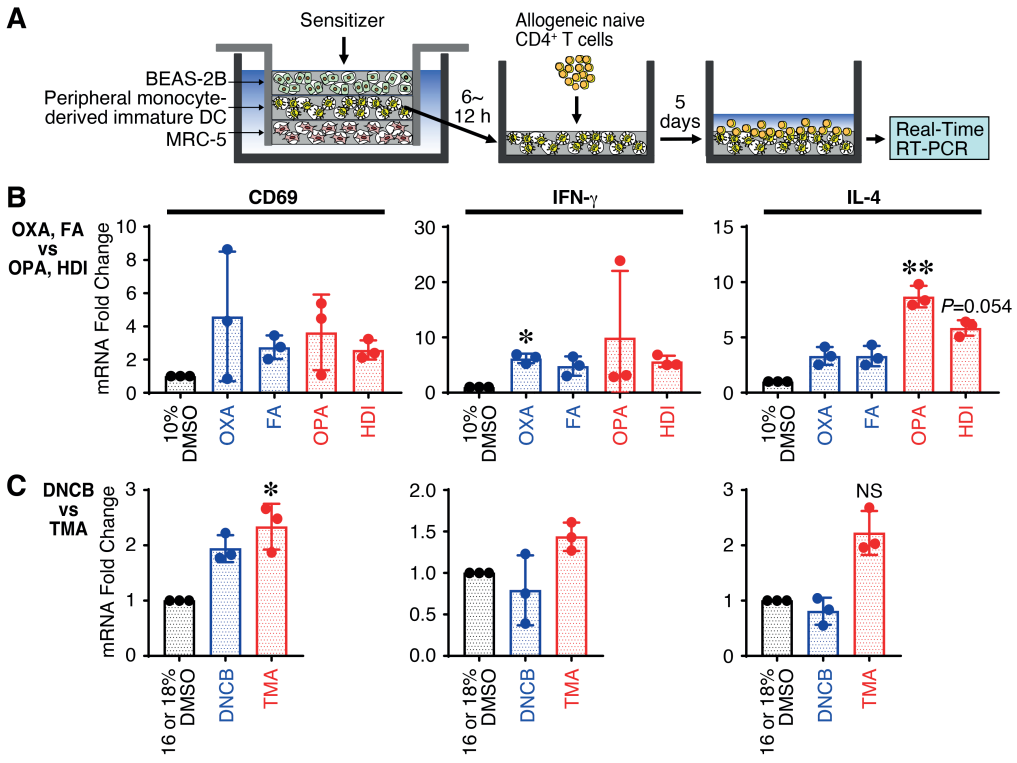

Fig. 2: Selective mRNA upregulation of IL-4 by three typical respiratory sensitizers in the DC/T coculture system with peripheral monocyte-derived immature DCs and allogeneic naïve CD4$^+$ T cells

Three sets of typical skin sensitizers OXA, FA (B), and DNCB (C) and respiratory sensitizers OPA, HDI (B), and TMA (C) were applied to the DC/T co-culture system with peripheral monocyte-derived immature DCs and allogeneic naïve CD4$^+$ T cells (A). The concentration of DMSO in the aliquot was 10% (OVA, OPA, FA, and HDI) or 16% or 18% (DNCB, and TMA) when sensitizers were added onto the top of the scaffold, and the same DMSO concentrations were used in the control. After incubation for 5 days, total RNA was extracted and subjected to real-time RT-PCR analysis to examine the expression of CD69, IFN-γ, and IL-4 together with HPRT. mRNA expression was normalized to HPRT mRNA expression, and relative mRNA fold change to control vehicle (DMSO solution) was calculated. The highest relative mRNA fold change (column filled in blue or red) among the concentrations of chemicals in each experiment was statistically analyzed among three independent experiments via the Kruskal-Wallis test with Dunn's multiple comparisons and is shown as the mean ± SD. Each independent experiment is shown in Fig. S2[1]. *$P < 0.05$; **$P < 0.01$. NS; not significant.

the dose-dependent responses to sensitizers were slightly varied among different DC donors or different combinations of donors (DC donors and T cell donors) as we could not always obtain primary cells from the same donor or the same donor combination. Therefore, we used the highest relative mRNA fold change among the tested concentrations of sensitizers in each experiment, and statistically analyzed the highest relative mRNA fold change among three independent experiments. Statistical analyses were performed by the Kruskal-Wallis test with Dunn's multiple comparisons using GraphPad Prism v7 (GraphPad Software Inc., La Jolla, CA, USA). The difference in the highest relative mRNA fold change to control vehicle (DMSO solution) between skin and respiratory sensitizers in each pair set was statistically analyzed by using the unpaired two-tailed Student's t-test. Data are presented as the mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant.

3 Results

3.1 Establishment of a novel two-step DC/T cell coculture system

To mimic the human upper airway epithelium, we recently established a 3D DC coculture system consisting of the airway epithelial cell line BEAS-2B, peripheral blood mononuclear cell-derived immature DCs, and the lung fibroblast cell line

MRC-5 using Alvetex scaffolds made of porous polystyrene (Knight et al., 2011; Mizoguchi et al., 2017) (Fig. 1). Immunohistochemical analysis with anti-CD11c revealed that no vigorous migration of DCs into the other scaffolds occurred during the initial 24 h after stimulation with chemical sensitizers (Mizoguchi et al., 2017). The stacked scaffolds were disassembled after stimulation, the DC scaffold was transferred to the bottom of a new 24-well plate, and allogeneic naïve $CD4^+$ T cells were added to the DC scaffold. We believe that this spatiotemporal flow recapitulates the *in vivo* chemical sensitization process; after immature DCs capture antigen and mature, antigen presenting DCs migrate to the draining lymph node to stimulate naïve $CD4^+$ T cells. This process is essential to initiate the adaptive immune response (Banchereau and Steinman, 1998).

We assumed that allogeneic naïve $CD4^+$ T cells would lead to a stronger response than syngeneic naïve $CD4^+$ T cells as it had been shown that repetitive stimulation is necessary to stimulate syngeneic naïve $CD4^+$ T cells (Richter et al., 2013; van Vliet et al., 2018; Vocanson et al., 2014), while allogeneic naïve $CD4^+$ T cells respond strongly to the first stimulation (Lakkis and Lechler, 2013; Sherman and Chattopadhyay, 1993).

3.2 Selective mRNA upregulation of IL-4 by three typical respiratory sensitizers in the DC/T coculture system with peripheral monocyte-derived immature DCs and allogeneic naïve $CD4^+$ T cells

Because the blood volume obtained from one volunteer and the resultant number of monocytes and naïve $CD4^+$ T cells are limited, we measured only one set of skin and respiratory sensitizers at 2 to 3 different concentrations at a time. First, one set of typical skin and respiratory chemical sensitizers, OXA and OPA, was applied to the DC/T cell coculture system (Fig. S1A). The expression of the T-cell activation marker CD69, Th1 differentiation marker IFN-γ, and Th2 differentiation marker IL-4 was performed after 2, 5, and 7 days. Preliminary data suggested that after stimulation of allogeneic naïve $CD4^+$ T cells with untreated DCs for 2 days, the mRNA expression of CD69 and IFN-γ but not IL-4 was increased due to the allogeneic response (Fig. S1B). The skin sensitizer OXA but not respiratory sensitizer OPA further upregulated IFN-γ mRNA expression, while OPA but not OXA upregulated IL-4 mRNA expression slightly. Both sensitizers did not upregulate CD69 mRNA expression further. On day 5, OPA but not OXA upregulated IL-4 mRNA expression (Fig. S1C). On day 7, no difference in the upregulation of IFN-γ and IL-4 mRNA was observed (data not shown). These results indicated that the DC/T cell coculture system with peripheral monocyte-derived immature DCs and allogeneic naïve $CD4^+$ T cells can differentially detect the early mRNA upregulation of IFN-γ by OXA and later mRNA upregulation of IL-4 by OPA.

Two further sets of typical skin and respiratory sensitizers, FA and HDI, and DNCB and TMA, as well as OXA and OPA, were applied to the DC/T cell coculture system (Fig. 2A). After stimulation of allogeneic naïve $CD4^+$ T cells with sensitizer-treated DCs for 5 days, the mRNA expression of CD69, IFN-γ, and IL-4 was analyzed. We selected the highest mRNA expression induced by any concentration of a chemical in each experiment for further analysis (Fig. S2A-D). When three sets of typical skin and respiratory sensitizers were compared all together, no significant difference was observed (data not shown). When OXA, FA, OPA, and HDI, whose DMSO concentration was the same, 10%, were compared, OPA significantly upregulated IL-4 mRNA expression, and HDI tended towards significant upregulation (Fig. 2B). When DNCB and TMA, whose DMSO percentage was higher, 16% or 18%, were compared, TMA also tended to upregulated IL-4 mRNA expression. In contrast, the three skin sensitizers failed to upregulate IL-4 expression (Fig. 2B,C). Moreover, when skin and respiratory sensitizers were compared in each pair set only, all three respiratory sensitizers significantly increased IL-4 mRNA expression more than the respective skin sensitizers (Fig. S2E-G). Thus, this DC/T cell coculture system may be useful to differentiate skin and respiratory sensitizers based on IL-4 mRNA upregulation.

To support the kinetics of the upregulation of IL-4 mRNA by OPA, we further analyzed the mRNA expression of the transcription factors c-Fos (Bendfeldt et al., 2012), T-bet (Szabo et al., 2000), and GATA-binding protein 3 (GATA-3) (Zhou and Ouyang, 2003), which are essential for induction of their respective target cytokines IL-2, IFN-γ, and IL-4 after 2 days stimulation. Preliminary data suggest that OPA but not OXA upregulated mRNA expression of GATA-3, which is in line with the subsequent upregulation of IL-4 (Fig. S3). No mRNA upregulation of c-Fos and T-bet was observed at this time point. Thus, this DC/T cell coculture system indicates the selective mRNA upregulation of GATA-3 by OPA, further supporting the induction of Th2 immune responses in response to a respiratory sensitizer.

3.3 Selective mRNA upregulation of OX40L in response to three typical respiratory sensitizers in the DC coculture system with CD14-ML-derived immature DCs

CD14-ML cell lines were established as an alternative to fresh peripheral $CD14^+$ monocytes by transducing c-MYC, BIM1, and BCL-2, which are related to cell survival and cell cycle, into peripheral $CD14^+$ monocytes from three donors; the resultant CD14-ML cells proliferated vigorously as previously reported (Haruta et al., 2013; Imamura et al., 2016) (Fig. S4A). Further addition of IL-4 caused CD14-ML cells to differentiate into immature DCs. They could then be matured by stimulation with bacterial toll-like receptor (TLR) ligands such as 5 µg/mL OK-432 or 100 ng/mL LPS for 1 day.

FACS analysis revealed that CD14-ML cells were largely positive for the DC marker CD11c as well as the monocyte marker CD14 (Fig. S4B, top left panel). Upon differentiation into immature DCs with GM-CSF, M-CSF, and IL-4, CD14 expression decreased and CD11c expression greatly increased. After stimulation with TLR ligands, the surface expression of costimulatory molecules CD86 and CD80 and to a lesser extent major histocompatibility class (MHC) class II human leukocyte antigen (HLA)-DR was upregulated (Fig. S4B, panels to right).

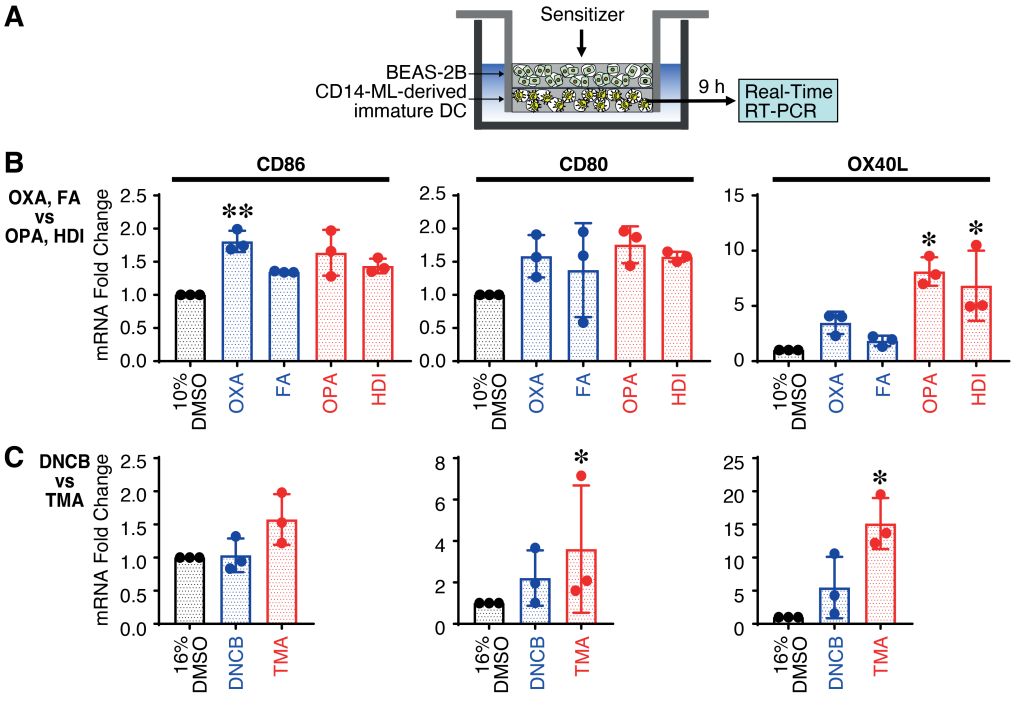

Fig. 3: Selective mRNA upregulation of OX40L by three typical respiratory sensitizers in the DC coculture system with CD14-ML-derived immature DCs

Three sets of typical skin sensitizers OXA, FA (B), and DNCB (C) and respiratory sensitizers OPA, HDI (B), and TMA (C) were applied to the DC coculture system with CD14-ML-derived immature DCs (A). The concentration of DMSO in the aliquot was 10% (OVA, OPA, FA, and HDI) or 16% (DNCB, and TMA) when sensitizers were added onto the top of the scaffold, and the same DMSO concentrations were used in the control. After incubation for 9 h, total RNA was extracted from the DC scaffold stimulated with chemical sensitizers and subjected to real-time RT-PCR analysis to examine the expression of CD86, CD80, and OX40L together with HPRT. mRNA expression was normalized to HPRT mRNA expression and relative mRNA fold change to control vehicle (DMSO solution) was calculated. The highest relative mRNA fold change (column filled in blue or red) among the concentrations of chemicals in each experiment was statistically analyzed among three independent experiments via the Kruskal-Wallis test with Dunn's multiple comparisons and is shown as the mean ± SD. Each independent experiment is shown in Fig. S5[1]. $*P < 0.05$; $**P < 0.01$.

To demonstrate functionality of CD14-ML cells, we stimulated them and CD14-ML-derived immature DCs with TSLP, a critical upstream cytokine inducing type 2 inflammation in various diseases including asthma and atopic dermatitis (Ziegler et al., 2013). TSLP directly stimulates DCs and enhances the expression of OX40L, which is in turn important for the induction of Th2 cells (Ito et al., 2005). TSLP increased the mRNA expression of OX40L in CD14-ML-derived immature DCs but not CD14-ML cells, and the differentiation of CD14-ML cells into immature DCs at 20% FBS resulted in higher OX40L levels than differentiation at 10% FBS (Fig. S4C). OX40L expression was further enhanced by the addition of LPS (Fig. S4D). Thus, CD14-ML cells seem to be a suitable source of DCs, because they proliferate vigorously and show similar phenotypes to peripheral monocytes (Haruta et al., 2013; Imamura et al., 2016).

To determine whether peripheral monocyte-derived DCs can be replaced with CD14-derived immature DCs in the coculture system, the three skin and three respiratory chemical sensitizers were applied to the DC coculture system with CD14-ML-derived immature DCs (Fig. 3A). Here, to further increase the versatility, the DC coculture comprised only the epithelial cell line BEAS-2B and CD14-derived immature DCs; the lung fibroblast cell line MRC-5 was omitted. We selected the highest mRNA expression induced by any concentration of a chemical in each experiment for further analysis (Fig. S5A-D). When the

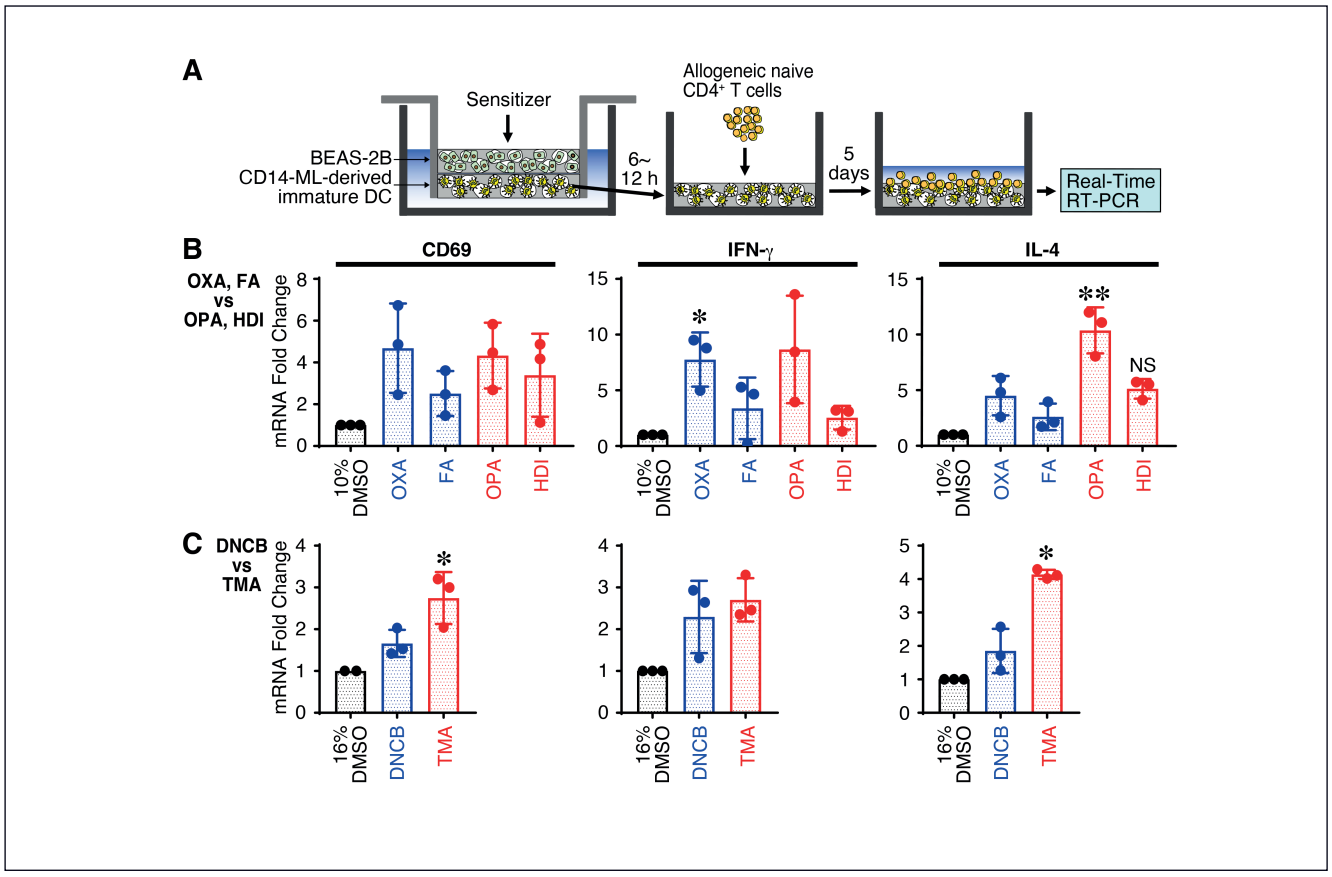

Fig. 4: Selective mRNA upregulation of IL-4 by three typical respiratory sensitizers in the DC/T coculture system with CD14-ML-derived immature DCs and allogeneic naïve CD4$^+$ T cells

Three sets of typical skin sensitizers OXA, FA (B), and DNCB (C) and respiratory sensitizers OPA, HDI (B), and TMA (C) were applied to the DC/T cell coculture system with CD14-ML-derived immature DCs and allogeneic naïve CD4$^+$ T cells (A). The concentration of DMSO in the aliquot was 10% (OVA, OPA, FA, and HDI) or 16% (DNCB, and TMA) when sensitizers were added onto the top of the scaffold, and the same DMSO concentrations were used in the control. After incubation for 5 days, total RNA was extracted from CD4$^+$ T cells stimulated with chemical sensitizer-treated DCs and subjected to real-time RT-PCR analysis to examine the expression of CD69, IFN-γ, and IL-4 together with HPRT. mRNA expression was normalized to HPRT mRNA expression and relative mRNA fold change to control vehicle (DMSO solution) was calculated. The highest relative mRNA fold change (column filled in blue or red) among varied concentrations of chemicals in each experiment was statistically analyzed among three independent experiments via the Kruskal-Wallis test with Dunn's multiple comparisons and is shown as the mean ± SD. Each independent experiment is shown in Fig. S7[1]. *$P < 0.05$; **$P < 0.01$. NS; not significant.

three sets of typical skin and respiratory sensitizers were compared all together, no significant difference was observed (data not shown). When OXA, FA, OPA, and HDI, whose DMSO concentration was the same, 10%, were compared, OPA and HDI significantly upregulated OX40L mRNA expression (Fig. 3B). When DNCB and TMA, whose DMSO concentration was the same, 16%, were compared, TMA significantly upregulated OX40L mRNA expression. In contrast, the three skin sensitizers failed to upregulate OX40L (Fig. 3B,C). Moreover, when skin and respiratory sensitizers were compared in each pair set only, all three respiratory sensitizers increased OX40L mRNA expression, although not significantly in all cases (Fig. S5E-G). These results suggest that differential OX40L mRNA upregulation by respiratory versus skin sensitizers was achieved in the DC coculture system with CD14-ML-derived immature DCs, also in the absence of fibroblasts. Therefore, the DC coculture system consisting of BEAS-2B cells and CD14-ML-derived immature DCs was used in subsequent studies.

We also examined the effects of skin and respiratory sensitizers on the mRNA expression of cytokine receptors for epithelial cell-derived cytokines, i.e. receptors for TSLP, IL-25, and IL-33, which are also critically important for the induction of Th2 immune responses (Roan et al., 2019) (Fig. S6A). The receptor complexes for TSLP, IL-25, and IL-33 are composed of

TSLPR and IL-7Rα (Pandey et al., 2000) and IL-17RB (Rickel et al., 2008), and ST2 and IL-1R accessory protein IL-1RAcP (Chackerian et al., 2007), respectively. Preliminary data suggest that OPA induced differential mRNA upregulation of ST2 (Fig. S6B), and TSLPR and IL-7Rα upregulation was observed in response to HDI and TMA (Fig. S6C,D). These results did not offer an additional endpoint for the discrimination between respiratory and skin sensitizers.

3.4 Selective mRNA upregulation of IL-4 by three typical respiratory sensitizers in the DC/T cell coculture system with CD14-ML-derived immature DCs and allogeneic naïve CD4+ T cells

Finally, we used CD14-ML-derived immature DCs in place of monocyte-derived immature DCs in the DC/T cell coculture system without the lung fibroblast cell line MRC-5 (Fig. 4A). We selected the highest mRNA expression induced by any concentration of a chemical in each experiment for further analysis (Fig. S7A-D). When three sets of typical skin and respiratory sensitizers were compared all together, no significant difference was observed among them (data not shown). When OXA, FA, OPA, and HDI, whose DMSO concentration was the same, 10%, were compared, OPA significantly upregulated IL-4 mRNA expression, and HDI tended towards upregulation (Fig. 4B). When DNCB and TMA, whose DMSO concentration was the same, 16%, were compared, TMA significantly upregulated IL-4 mRNA expression. In contrast, the three skin sensitizers failed to upregulate IL-4 expression (Fig. 4B,C). When skin and respiratory sensitizers were compared in each pair set, all three respiratory sensitizers significantly increased IL-4 mRNA expression more than the skin sensitizers (Fig. S7E-G).

These results suggest that the differential mRNA upregulation of IL-4 by respiratory and skin sensitizers can also be observed in the DC/T cell coculture system using CD14-ML-derived immature DCs, but without the lung fibroblast cell line MRC-5.

4 Discussion

To mimic the upper airway epithelium, we recently established a 3D DC coculture system consisting of the human airway epithelial cell line BEAS-2B, immature DCs derived from human peripheral monocytes, and the lung fibroblast cell line MCR-5 using scaffolds made of porous polystyrene, and added sensitizers to the top of the epithelial scaffold (Mizoguchi et al., 2017). In this coculture system, compared to skin sensitizers, respiratory sensitizers induced increased mRNA expression of the key costimulatory molecule OX40L, which is important for Th2 differentiation, in DCs (Furue and Furue, 2021; Ito et al., 2005). To further apply this system to targeting KE4 in the AOP of sensitization, the DC scaffold was removed and placed in a new plate together with allogeneic naïve $CD4^+$ T cells after the stimulation with sensitizers, mimicking the migration of antigen-presenting DCs to the draining lymph nodes (Banchereau and Steinman, 1998). This DC/T cell coculture system allows the sensitive measurement of IL-4, which is a potent effector cytokine that induces Th2 differentiation (Paul, 2015; Zhu, 2015), as an endpoint to discriminate between respiratory and skin sensitizers as exemplified using three respiratory and three skin sensitizers. To further improve the versatility of the system, we generated 3 monocyte-derived proliferating cell CD14-ML cell lines from different donors by introducing the cell survival and cell cycle-related genes, c-MYC, BMI1, and BCL-2, into peripheral $CD14^+$ monocytes (Haruta et al., 2013; Imamura et al., 2016). CD14-ML cells proliferate well and display DC maturation, antigen-presenting activity, and potent antitumor activity similar to peripheral monocytes (Haruta et al., 2013; Imamura et al., 2016). Differential upregulation of both OX40L and IL-4 by respiratory sensitizers was observed when CD14-ML-derived immature DCs were used in place of peripheral monocyte-derived immature DCs in the DC and DC/T coculture systems.

In the present study, primary blood cells from donor volunteers were used, which have several restrictions related to ethical issues. The volume of blood provided from one donor and the frequency with which blood can be obtained from the same donor are limited. The cell number obtained from one preparation was usually only sufficient for an experiment using only one pair of sensitizers in three different concentrations, e.g., DNCB and TMA is a typical and frequently used pair of skin and respiratory sensitizers (Dearman et al., 2008; Vandebriel et al., 2007; Vanoirbeek et al., 2006). When the data from the three sets of typical skin and respiratory sensitizers were compared all together using the raw data or data relative to control vehicle, no significant difference was observed (data not shown). This is because the absolute values of the data obtained using primary cells from different donors and different combinations of donors greatly varied in individual experiments. In addition, the concentrations of DMSO used were different depending on the sensitizers, 10% and 16% or 18%; the solubility of TMA in DMSO was relatively low, and the percentage of the DMSO solution needed to be relatively high (16% or 18%). Therefore, we separated the sensitizers into two groups depending on the DMSO concentration, 10% (OXA, FA, OPA, and HDI), and 16% or 18% (DNCB and TMA), and performed a statistical analysis in each group (Fig. 2-4). When skin and respiratory sensitizers were compared in each pair set only, the three respiratory sensitizers increased mRNA expression of OX40L and IL-4 in DCs and $CD4^+$ T cells, respectively, compared to skin sensitizers (Fig. S2E-G, S5E-G, S7E-G). Instead of measuring IL-4 mRNA, the detection of IL-4 in the culture supernatant at the protein level by ELISA could be a good alternative. However, our preliminary experiments revealed that the levels of IL-4 produced by the primary $CD4^+$ T cells stimulated by respiratory sensitizers were below the detection limit. We are currently trying to establish an allogenic Th2 cell line or clone cells after repetitive stimulation by allogenic DCs to use as candidate cells that produce large amounts of IL-4 in response to respiratory sensitizers.

Control $CD4^+$ T cells in the DC/T coculture system tended to express higher levels of CD69, IFN-γ, and IL-4 than $CD4^+$ T cells and DCs (Fig. 2, 4, S2, S7). This is presumably because

the cell numbers and contents between $CD4^+$ T cells alone and the mixture of $CD4^+$ T cells and DCs were different and these molecules are markers of T cells that are preferentially expressed in $CD4^+$ T cells compared to DCs. The mRNA expression of CD69 and IFN-γ was not consistently induced by the six sensitizers (Fig. 2, 4, S2, S7). This is probably due to different kinetics for the induction of CD69 and IFN-γ mRNA versus IL-4 mRNA and also their different responsiveness to allogenic reaction. As shown in Fig. S1, upregulation of IFN-γ mRNA was observed as early as on day 2, while upregulation of IL-4 mRNA was observed on day 5. In addition, induction of CD69 and IFN-γ mRNA might be more dependent on the donor combination between DCs and $CD4^+$ T cells than that of IL-4 mRNA. This is because CD69 is a marker of activated T cells and IFN-γ is the cytokine produced by Th1 cells, which are preferentially activated by an allogenic reaction, while IL-4 is produced by Th2 cells. The reason why mRNA expression of CD80 and CD86 was not consistently induced by the six sensitizers (Fig. 3, S5) could be that the mRNA expression of CD80 and CD86 may be more dependent on DC donors compared to that of OX40L. Almost all the relative induction of CD80 and CD86 by OXA and OPA in DCs derived from CD14-ML of donor A and B was over 1.5 (Fig. 3B, S5B, and Table S2), while that by FA, HDI, DNCB, and TMA in DCs derived from CD14-ML of donor C was below 1.5 (Fig. S5C, D and Table S2).

Under physiological conditions, the sensitization process proceeds as follows (Kimber et al., 2018; MacKay et al., 2013; Sullivan et al., 2017): Chemical sensitizers first attach to the surface of the epithelium and penetrate it. During this process, chemical sensitizers covalently bind to serum or cellular proteins, making them immunogenic, which is called haptenization (Chipinda et al., 2011). Generally, the proteins that bind chemical sensitizers are present abundantly in the serum and cells, such as albumin, actin, myosin, and heat shock proteins (Chipinda et al., 2011; Parkinson et al., 2018), and protein specificity has little to do with which type of immune response is subsequently activated by the sensitizer. Immature DCs capture the haptenized proteins, process them, and present their peptides on MHC class II with concomitant upregulation of costimulatory molecules such as CD86 and CD80 and chemokine receptor CCR7. Subsequently, the DCs enter the high endothelial venules and migrate to the draining lymph node via attraction to CCL19/21 (Weninger and von Andrian, 2003). There, the antigen-presenting DCs stimulate naïve $CD4^+$ T cells to differentiate into effectors including Th1, Th2, and Th17 cells in order to initiate adaptive immunity.

Several types of T cell-based assays have been reported including a novel assay for skin sensitization using the human T lymphocyte cell line Jurkat Clone E6-1 (Hou et al., 2020) and the hTCPA (Richter et al., 2013; Vocanson et al., 2014). The hTCPA utilizes peripheral monocyte-derived immature DCs and syngeneic naïve T cells from healthy blood donors. To facilitate the maturation of DCs, immature DCs are first stimulated with a non-cytotoxic concentration of the test substance together with LPS (Richter et al., 2013; van Vliet et al., 2018; Vocanson et al., 2014). Then DCs are cocultured with syngeneic naïve T cells. After priming, the T cells are re-stimulated with mature DCs treated with the same substance, and the induction of test substance-specific T-cell responses is assessed by detection of T-cell proliferation, cytokine production, or cell surface marker expression. The Cosmetics Europe Skin Tolerance Task Force organized a workshop to discuss how the information provided by T-cell assays could be used for the safety assessment of cosmetic ingredients. The participants agreed on the importance of developing and applying T cell-based assays, because the induction of a T-cell response is one of the requirements for skin sensitization and the only downstream test method able to detect immunogenic potential resulting from all possible mechanisms of skin sensitization, including those not yet covered by the current AOP (van Vliet et al., 2018). However, the workshop pointed out that the current T cell-based assays are laborious and time-consuming, and also have donor-to-donor variability, leading to difficulties in transferability for future ring studies to evaluate accuracy and reproducibility (van Vliet et al., 2018). Therefore, the workshop concluded that the current hTCPA needs further refinement and simplification.

To overcome these problems and, more precisely, recapitulate the physiological spatiotemporal flow of the sensitization process, we have come up with the following improvements and assumptions to enable the discrimination between respiratory and skin sensitizers. First, to mimic the *in vivo* situation of the upper airway epithelium being sensitized with chemical sensitizers, relatively high concentrations of the sensitizer solution were added onto the 3D coculture system consisting of the epithelial cell line BEAS-2B on top, peripheral monocyte-derived immature DCs in the middle, and the lung fibroblast cell line MRC-5 at the base, incubated for a short period of time, 30 min, and subsequently diluted with medium. As controls, the same high concentrations of DMSO (10 or 16%) alone in the aliquot were added, because they might affect the viability of cells. In later experiments, the MRC-5 cells were omitted from the DC and DC/T cell coculture systems without any apparent effect. We believe that this directional penetration of sensitizers from epithelial cells to immature DCs is important for the activation of innate immune responses releasing damage-associated molecular patterns (DAMPs) and inflammatory cytokines from epithelial cells, which affect the subsequent induction of DC maturation and are necessary to determine which type of adaptive immune response will be activated by the sensitizer (Sullivan et al., 2017). Within 12 h after stimulation, the DC scaffold was removed and placed in a new plate together with allogeneic naïve $CD4^+$ T cells. This process recapitulates the migration of antigen-captured and antigen-presenting DCs to the draining lymph node to stimulate naïve $CD4^+$ T cells *in vivo*. The allogeneic response is strong enough because it is generally a polyclonal response and alloantigen-specific T cells constitute ~10% of the total various antigen-specific T-cell population (Lakkis and Lechler, 2013; Sherman and Chattopadhyay, 1993). Therefore, a strong stimulatory signal can be induced by allogeneic $CD4^+$ T cells even

as a primary response (Lakkis and Lechler, 2013; Sherman and Chattopadhyay, 1993) and multiple re-stimulations, as in the case of the hTCPA (Richter et al., 2013; van Vliet et al., 2018; Vocanson et al., 2014), are not necessary. Therefore, in this study, we took advantage of the strong allogeneic response of $CD4^+$ T cells stimulated with DCs.

While models of the human airway that are composed of primary airway epithelial cells cultured at the air-liquid interphase are now commercially available under trade names such as MucilAir and EpiAirway (Balharry et al., 2008; Huang et al., 2013) and are more physiologically relevant for assessing human responses following exposure to a chemical, we chose to use the BEAS-2B and CD14-ML cell lines to minimize laborious and time-consuming processes, improve the versatility, and reduce donor-to-donor variability. The system has the limitation that it still requires primary naïve $CD4^+$ T cells from a human donor, which not every laboratory will have access to. We are currently trying to apply an allogeneic Th2 clone cell line to the DC/T cell coculture system in place of peripheral naïve $CD4^+$ T cells. A further limitation is that the system uses FBS and antibodies that are or may be derived from ascites fluid, whose production is association with pain and suffering. We will consider using alternative materials to replace these components once their reproducibility and effectiveness are fully validated.

In this study, a novel two-step DC/T cell coculture system using scaffolds was established to recapitulate the physiological spatiotemporal flow of chemical sensitization processes *in vivo*, including the exposure of DCs to chemicals through the upper airway epithelium and migration of the antigen-presenting DCs to the draining lymph nodes to stimulate naïve $CD4^+$ T cells. This system may be successfully applied to discriminate chemical respiratory sensitizers from skin sensitizers by measuring the critical molecule of Th2 differentiation and its effector function, IL-4, in $CD4^+$ T cells. There is a benefit in using primary human cells as they are from the species of interest and reflect the natural variability of the population in contrast to a cell line. However, because the number of primary cells obtained freshly from one donor and the frequency to obtain blood from the same donor are highly limited, we cannot systematically expand the coculture assay system by increasing the dose and number of chemicals, which is a limitation of the present study.

Collectively, to increase the consistency and reproducibility, the same donor, the same combination of CD14-ML donor and $CD4^+$ T cell donor, and the same concentration of DMSO solution should be used. It would be ideal to further establish the DC/T cell coculture consisting of CD14-ML-derived immature DCs and a Th2 cell line or clone cells that are allogenic to the CD14-ML cells. Thereafter, using such a more versatile DC/T coculture system, the concentration of a sensitizer can be titrated, each mRNA expression will be normalized to HPRT mRNA expression, and relative IL-4 mRNA fold change to control vehicle (DMSO solution) can be calculated in the respective concentrations of a sensitizer. Then, using the values of the highest relative IL-4 mRNA fold change of several typical skin and respiratory sensitizers, a cut-off value will be defined to discriminate between skin and respiratory sensitizers. Although further verification of the present assay system with a greater number of potential chemical sensitizers as well as improvements in the versatility as mentioned above are warranted, this two-step DC/T coculture system could be a useful tool for differentiating between respiratory and skin sensitizers *in vitro*.

References

Adenuga, D., Woolhiser, M. R., Gollapudi, B. B. et al. (2012). Differential gene expression responses distinguish contact and respiratory sensitizers and nonsensitizing irritants in the local lymph node assay. *Toxicol Sci 126*, 413-425. doi:10.1093/toxsci/kfs071

Arts, J. H., de Jong, W. H., van Triel, J. J. et al. (2008). The respiratory local lymph node assay as a tool to study respiratory sensitizers. *Toxicol Sci 106*, 423-434. doi:10.1093/toxsci/kfn199

Arts, J. (2020). How to assess respiratory sensitization of low molecular weight chemicals? *Int J Hyg Environ Health 225*, 113469. doi:10.1016/j.ijheh.2020.113469 Ashikaga, T., Yoshida, Y., Hirota, M. et al. (2006). Development of an in vitro skin sensitization test using human cell lines: the human Cell Line Activation Test (h-CLAT). I. Optimization of the h-CLAT protocol. *Toxicol In Vitro 20*, 767-773. doi:10.1016/j.tiv.2005.10.012

Balharry, D., Sexton, K., BeruBe, K. A. (2008). An in vitro approach to assess the toxicity of inhaled tobacco smoke components: nicotine, cadmium, formaldehyde and urethane. *Toxicology 244*, 66-76. doi:10.1016/j.tox.2007.11.001

Banchereau, J., Steinman, R. M. (1998). Dendritic cells and the control of immunity. *Nature 392*, 245-252. doi:10.1038/32588

Bendfeldt, H., Benary, M., Scheel, T. et al. (2012). Stable IL-2 decision making by endogenous c-Fos amounts in peripheral memory T-helper cells. *J Biol Chem 287*, 18386-18397. doi:10.1074/jbc.M112.358853

Chackerian, A. A., Oldham, E. R., Murphy, E. E. et al. (2007). IL-1 receptor accessory protein and ST2 comprise the IL-33 receptor complex. *J Immunol 179*, 2551-2555. doi:10.4049/jimmunol.179.4.2551

Chary, A., Hennen, J., Klein, S. G. et al. (2018). Respiratory sensitization: toxicological point of view on the available assays. *Arch Toxicol 92*, 803-822. doi:10.1007/s00204-017-2088-5

Chipinda, I., Hettick, J. M., Siegel, P. D. (2011). Haptenation: chemical reactivity and protein binding. *J Allergy (Cairo) 2011*, 839682. doi:10.1155/2011/839682

De Jong, W. H., Arts, J. H., De Klerk, A. et al. (2009). Contact and respiratory sensitizers can be identified by cytokine profiles following inhalation exposure. *Toxicology 261*, 103-111. doi:10.1016/j.tox.2009.04.057

Dearman, R. J., Basketter, D. A., Kimber, I. (1995). Differential cytokine production following chronic exposure of mice to chemical respiratory and contact allergens. *Immunology 86*, 545-550.

Dearman, R. J., Betts, C. J., Caddick, H. T. et al. (2008). Cytokine profiling of chemical allergens in mice: measurement of message versus protein. *Toxicology 252*, 17-25. doi:10.1016/j.tox.2008.07.055

Furue, M., Furue, M. (2021). OX40L-OX40 Signaling in Atopic Dermatitis. *J Clin Med 10*. doi:10.3390/jcm10122578

Gerberick, G. F., Vassallo, J. D., Bailey, R. E. et al. (2004). Development of a peptide reactivity assay for screening contact allergens. *Toxicol Sci 81*, 332-343. doi:10.1093/toxsci/kfh213

Goutet, M., Pepin, E., Langonne, I. et al. (2012). Identification of contact and respiratory sensitizers according to IL-4 receptor alpha expression and IL-2 production. *Toxicol Appl Pharmacol 260*, 95-104. doi:10.1016/j.taap.2012.02.009

Haruta, M., Tomita, Y., Imamura, Y. et al. (2013). Generation of a large number of functional dendritic cells from human monocytes expanded by forced expression of cMYC plus BMI1. *Hum Immunol 74*, 1400-1408. doi:10.1016/j.humimm.2013.05.017

Hou, F., Xing, C., Li, B. et al. (2020). Performance of a novel in vitro assay for skin sensitization based on activation of T lymphocytes. *ALTEX 37*, 451-468. doi:10.14573/altex.2001312

Huang, S., Wiszniewski, L., Constant, S. et al. (2013). Potential of in vitro reconstituted 3D human airway epithelia (MucilAir) to assess respiratory sensitizers. *Toxicol In Vitro 27*, 1151-1156. doi:10.1016/j.tiv.2012.10.010

Imamura, Y., Haruta, M., Tomita, Y. et al. (2016). Generation of large numbers of antigen-expressing human dendritic cells using CD14-ML technology. *PLoS One 11*, e0152384. doi:10.1371/journal.pone.0152384

Ito, T., Wang, Y. H., Duramad, O. et al. (2005). TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J Exp Med 202*, 1213-1223. doi:10.1084/jem.20051135

Jacobs, J. P., Jones, C. M., Baille, J. P. (1970). Characteristics of a human diploid cell designated MRC-5. *Nature 227*, 168-170. doi:10.1038/227168a0

Kimber, I., Dearman, R. J., Scholes, E. W. et al. (1994). The local lymph node assay: developments and applications. *Toxicology 93*, 13-31. doi:10.1016/0300-483x(94)90193-7

Kimber, I., Poole, A., Basketter, D. A. (2018). Skin and respiratory chemical allergy: confluence and divergence in a hybrid adverse outcome pathway. *Toxicol Res (Camb) 7*, 586-605. doi:10.1039/c7tx00272f

Kimura, Y., Fujimura, C., Ito, Y. et al. (2015). Optimization of the IL-8 Luc assay as an in vitro test for skin sensitization. *Toxicol In Vitro 29*, 1816-1830. doi:10.1016/j.tiv.2015.07.006

Knight, E., Murray, B., Carnachan, R. et al. (2011). Alvetex(R): polystyrene scaffold technology for routine three dimensional cell culture. *Methods Mol Biol 695*, 323-340. doi:10.1007/978-1-60761-984-0_20

Lakkis, F. G., Lechler, R. I. (2013). Origin and biology of the allogeneic response. *Cold Spring Harb Perspect Med 3,* a012933. doi:10.1101/cshperspect.a014993

MacKay, C., Davies, M., Summerfield, V. et al. (2013). From pathways to people: applying the adverse outcome pathway (AOP) for skin sensitization to risk assessment. *ALTEX 30*, 473-486. doi:10.14573/altex.2013.4.473

Mizoguchi, I., Ohashi, M., Chiba, Y. et al. (2017). Prediction of chemical respiratory and contact sensitizers by OX40L expression in dendritic cells using a novel 3D coculture system. *Front Immunol 8*, 929. doi:10.3389/fimmu.2017.00929

Natsch, A., Emter, R. (2016). Nrf2 activation as a key event triggered by skin sensitisers: The development of the stable KeratinoSens reporter gene assay. *Altern Lab Anim 44*, 443-451. doi:10.1177/026119291604400513

North, C. M., Ezendam, J., Hotchkiss, J. A. et al. (2016). Developing a framework for assessing chemical respiratory sensitization: A workshop report. *Regul Toxicol Pharmacol 80*, 295-309. doi:10.1016/j.yrtph.2016.06.006

Pandey, A., Ozaki, K., Baumann, H. et al. (2000). Cloning of a receptor subunit required for signaling by thymic stromal lymphopoietin. *Nat Immunol 1*, 59-64. doi:10.1038/76923

Parkinson, E., Aleksic, M., Cubberley, R. et al. (2018). Determination of protein haptenation by chemical sensitizers within the complexity of the human skin proteome. *Toxicol Sci 162*, 429-438. doi:10.1093/toxsci/kfx265

Paul, W. E. (2015). History of interleukin-4. *Cytokine 75*, 3-7. doi:10.1016/j.cyto.2015.01.038

Reddel, R. R., Ke, Y., Gerwin, B. I. et al. (1988). Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. *Cancer Res 48*, 1904-1909.

Richter, A., Schmucker, S. S., Esser, P. R. et al. (2013). Human T cell priming assay (hTCPA) for the identification of contact allergens based on naive T cells and DC – IFN-gamma and TNF-alpha readout. *Toxicol In Vitro 27*, 1180-1185. doi:10.1016/j.tiv.2012.08.007

Rickel, E. A., Siegel, L. A., Yoon, B. R. et al. (2008). Identification of functional roles for both IL-17RB and IL-17RA in mediating IL-25-induced activities. *J Immunol 181*, 4299-4310. doi:10.4049/jimmunol.181.6.4299

Roan, F., Obata-Ninomiya, K., Ziegler, S. F. (2019). Epithelial cell-derived cytokines: more than just signaling the alarm. *J Clin Invest 129*, 1441-1451. doi:10.1172/JCI124606

Roggen, E. L. (2014). In vitro approaches for detection of chemical sensitization. *Basic Clin Pharmacol Toxicol 115*, 32-40. doi:10.1111/bcpt.12202

Sadekar, N., Boisleve, F., Dekant, W. et al. (2021). Identifying a reference list of respiratory sensitizers for the evaluation of novel approaches to study respiratory sensitization. *Crit Rev Toxicol 51*, 792-804. doi:10.1080/10408444.2021.2024142

Sherman, L. A., Chattopadhyay, S. (1993). The molecular basis of allorecognition. *Annu Rev Immunol 11*, 385-402. doi:10.1146/annurev.iy.11.040193.002125

Sullivan, K. M., Enoch, S. J., Ezendam, J. et al. (2017). An adverse outcome pathway for sensitization of the respiratory tract by low-molecular-weight chemicals: building evidence to support the utility of in vitro and in silico methods in a regulatory context. *Appl Vitro Toxicol 3*, 213-226. doi:10.1089/aivt.2017.0010

Szabo, S. J., Kim, S. T., Costa, G. L. et al. (2000). A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell 100*, 655-669. doi:10.1016/s0092-8674(00)80702-3

Tarlo, S. M., Lemiere, C. (2014). Occupational asthma. *N Engl J Med 370*, 640-649. doi:10.1056/NEJMra1301758

Tha, E. L., Canavez, A., Schuck, D. C. et al. (2021). Beyond dermal exposure: The respiratory tract as a target organ in hazard assessments of cosmetic ingredients. *Regul Toxicol Pharmacol 124*, 104976. doi:10.1016/j.yrtph.2021.104976

van Vliet, E., Kuhnl, J., Goebel, C. et al. (2018). State-of-the-art and new options to assess T cell activation by skin sensitizers: Cosmetics Europe Workshop. *ALTEX 35*, 179-192. doi:10.14573/altex.1709011

Vandebriel, R. J., De Jong, W. H., Spiekstra, S. W. et al. (2000). Assessment of preferential T-helper 1 or T-helper 2 induction by low molecular weight compounds using the local lymph node assay in conjunction with RT-PCR and ELISA for interferon-gamma and interleukin-4. *Toxicol Appl Pharmacol 162*, 77-85. doi:10.1006/taap.1999.8841

Vandebriel, R. J., Gremmer, E. R., van Hartskamp, M. et al. (2007). Effects of a diphtheria-tetanus-acellular pertussis vaccine on immune responses in murine local lymph node and lung allergy models. *Clin Vaccine Immunol 14*, 211-219. doi:10.1128/CVI.00306-06

Vanoirbeek, J. A., Tarkowski, M., Vanhooren, H. M. et al. (2006). Validation of a mouse model of chemical-induced asthma using trimellitic anhydride, a respiratory sensitizer, and dinitrochlorobenzene, a dermal sensitizer. *J Allergy Clin Immunol 117*, 1090-1097. doi:10.1016/j.jaci.2006.01.027

Vocanson, M., Achachi, A., Mutez, V. et al. (2014). Human T cell priming assay: depletion of peripheral blood lymphocytes in $CD25^+$ cells improves the in vitro detection of weak allergen-specific T cells. *Exp Suppl 104*, 89-100. doi:10.1007/978-3-0348-0726-5_7

Weninger, W., von Andrian, U. H. (2003). Chemokine regulation of naive T cell traffic in health and disease. *Semin Immunol 15*, 257-270. doi:10.1016/j.smim.2003.08.007

Zhou, M., Ouyang, W. (2003). The function role of GATA-3 in Th1 and Th2 differentiation. *Immunol Res 28*, 25-37. doi:10.1385/IR:28:1:25

Zhu, J. (2015). T helper 2 (Th2) cell differentiation, type 2 innate lymphoid cell (ILC2) development and regulation of interleukin-4 (IL-4) and IL-13 production. *Cytokine 75*, 14-24. doi:10.1016/j.cyto.2015.05.010

Ziegler, S. F., Roan, F., Bell, B. D. et al. (2013). The biology of thymic stromal lymphopoietin (TSLP). *Adv Pharmacol 66*, 129-155. doi:10.1016/B978-0-12-404717-4.00004-4

Conflict of interest

The authors declare that they have no conflicts of interests.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Acknowledgments

The authors thank Drs K. Miyazaki and J. Shimizu (MiCAN Technologies), Dr T. Yokosuka (Tokyo Medical University), healthy volunteers, Sionogi & Co., Ltd., Chugai Pharmaceutical Co., Ltd. for preparation of the CD14-ML cell lines, blood sampling, blood donation, human recombinant IL-2, and OK-432, respectively. This study was supported by a grant from the Long-Range Research Initiative of the Japan Chemical Industry Association, Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, Mandom International Research Grants on Alternatives to Animal Experiments, KOSE Cosmetology Research Foundation, Hoyu Science Foundation, and Daicel Corporation.