Research Article

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

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Abstract

Although several in vitro assays that predict the sensitizing potential of chemicals have been developed, none can distinguish between chemical respiratory and skin sensizers. 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 typical skin sensizers, typical respiratory sensizers 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 naive CD4+ T cells to the DCs stimulated in the DC coculture system. In this DC/T cell coculture system, typical respiratory sensizers but not skin sensizers enhanced mRNA expression of the predominant Th2 marker interleukin-4 (IL-4) and its transcription factor GATA-binding protein 3. To improve the versatility, in place of peripheral monocytes, monocyte-derived proliferating cells called CD14-ML were also used in the DC coculture system. Similar to peripheral monocytes, enhanced mRNA expression of OX40L was observed by typical respiratory sensizers compared to skin sensizers. When these cell lines were applied to the DC/T cell coculture system with peripheral allogeneic naive CD4+ T cells, typical respiratory sensizers but not skin sensizers enhanced the mRNA expression of IL-4. Thus, this DC/T cell coculture system might be useful for discriminating between respiratory and skin sensizers by differential mRNA upregulation of IL-4 in T cells.

1 Introduction

There are two main types of allergic responses: 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 has been 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 (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 (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 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). Respiratory and skin sensitizers induce different immune responses, predominantly T helper 2 (Th2) responses versus Th1-oriented responses mixed with Th2 and Th17 responses (Adenuga et al., 2012; Arts et al., 2008; De Jong et al., 2009; Dearman et al., 1995; Goutet et al., 2012; Roggen, 2014; VandeVeld et al., 2000). Discrimination between respiratory and skin sensitizers is 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). The respiratory sensitizers induced higher expression of molecules critical for the induction of Th2 immune responses, such as IL-4 and IL-4 receptor alpha (IR-4Rα), compared to the 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, which are most precisely detected by upregulation of IL-4 in T cells as a 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, thereby becoming the ultimate in vitro assay.

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 scaffolds (Mizoguchi et al., 2017). This system successfully discriminates typical chemical respiratory sensitizers from typical skin sensitizers by measuring the critical molecule for Th2 differentiation in DCs, namely OX40 ligand (OX40L) (Furne and Furne, 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 further adding peripheral allogeneic naïve CD4+ T cells to the DCs stimulated in the DC coculture system. In this DC/T cell coculture system, the mRNA upregulation of IL-4 in T cells representing KE4 was successfully used to discriminate respiratory sensitizers from skin sensitizers. To improve the versatility, peripheral monocyte-derived immature DCs were similarly replaced with immature DCs derived from monocyte-derived proliferating cells called CD14-ML (Haruta et al., 2013; Imanura et al., 2016) in DC and DC/T cell coculture systems.

2 Materials and Methods

2.1 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% CO2/95% air in Eagle's minimum essential medium (MEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). Human peripheral blood monocytes and naïve CD4+ T cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. CD14-ML cells were cultured in uMEM medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 20% FBS, granulocyte macrophage colony-stimulating factor (GM-CSF, 50 ng/mL), macrophage colony-stimulating factor (M-CSF, 50 ng/mL), 100 U/mL penicillin, and 100 μg/mL streptomycin. BEAS-2B, MRC-5, and CD14-ML cells were passaged twice a week, and BEAS-2B and MRC-5 cells were detached from plates with trypsin for the passage. We will consider using alternative materials to replace animal-derived components such as FBS and antibiotics that are/may be derived from ascites fluid, whose production is association with pain and suffering, in the future once the reproducibility and effectiveness of those alternative materials are fully validated.

2.2 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 three respiratory sensitizers, ortho-phthalaldehyde (OPA, purity ≥ 97%, P1378), and trimellitic anhydride (TMA, purity 97%, B4600), were purchased from Sigma-Aldrich (Tab. S1). Hexamethylene-1,6-diisocyanate (HDI, purity > 98%, H0324) was purchased from Tokyo Chemical Industry Co. Ltd. A magnetic bead conjugated with monoclonal antibody (mAb) against cluster of differentiation 14 (CD14) was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). The mAbs against human CD14 (HCD14) and CD11c (clone 3.9) were obtained from BioLegend (San Diego, CA, USA), and eBioscience (La Jolla, CA, USA), respectively. Human recombinant GM-CSF, M-CSF, IL-4, and thymic stromal lymphopoietin (TSLP) were purchased from BioLegend. Human recombinant IL-2 was provided from Sionogi & Co., Ltd. (Osaka, Japan). OK-432 (penicillin-killed Streptococcus pyogenes) was provided from Chugai Pharmaceutical Co., Ltd. (Osaka, Japan), and lipopolysaccharide (LPS) was purchased from Sigma-Aldrich.

2.3 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 was collected from healthy volunteers, and mononuclear cells were immediately purified by 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 a magnetic bead conjugated with mAb against CD14 (Miltenyi Biotec). The purity was analyzed by flow cytometry with Brilliant Violet 421 anti-CD14 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%.

1 doi:10.14573/altex.2111181s
2.4 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 were infected with lentivirus expressing c-MYC, BIM1, and B-cell lymphoma 2 (BCL-2) and then were cultured in αMEM medium containing GM-CSF (50 ng/mL) and M-CSF (50 ng/mL) in 20% FBS according to a published method (Haruta et al., 2013; Imamura et al., 2016). Outgrowing cells appeared around 3 weeks and were further expanded to prepare frozen stocks or use for experiments. Mycoplasma testing was carried out using e-Mycova54 VALid Mycoplasma PCR Detection Kit (Nitron Biotechnology, Inc., Gyeonggi-do, Korea) when the cell lines were established. Cells of passages 7 to 15 were routinely used.

2.5 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 M-CSF (50 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 for 3 days. The purity of the resultant immature DCs was analyzed after staining with anti-CD11c and was routinely approximately 90%.

2.6 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 medium was gently added to the scaffold and incubated for 3 days. A 50-μL aliquot of suspension of immature DCs (0.5–1.0 × 10^6 cells) was gently seeded directly onto the center of the scaffold in the 24-well plate containing GM-CSF (50 ng/mL), and IL-4 (10 ng/mL) was gently added to the scaffold and incubated for 24 h. After incubation, the individual scaffolds were gently detached from the 12-well insert or 24-well plate and stacked in the order of MRC-5 cells (bottom), immature DCs (middle), and BEAS-2B cells (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, and MEM was gently added and incubated for another 4 h. Then the medium was gently removed and a 5-μL aliquot of a chemical sensitizer, which was initially dissolved in dimethyl sulfoxide (DMSO) and then diluted with MEM, was gently added at six places on the top scaffold and left for 30 min followed by the addition of 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, the DC scaffold membrane was equally cut into 3 or 4 pieces, and RNA was extracted from each of them for real-time RT-PCR analysis. The concentrations of chemical sensitizers were titrated in advance and used as high as possible. Samples were not used whose hypoxanthine phosphoribosyltransferase (HPRT) mRNA expression level was less than one-tenth that of the untreated sample (Mizoguchi et al., 2017).

2.7 Preparation of the DC/T cell coculture system
The DC/T cell coculture was prepared by further adding peripheral allogeneic naïve CD4+ T cells to the DCs stimulated in the DC coculture system. After 6 to 12 h, the stacked scaffolds stimulated with a chemical sensitizer in the DC coculture were disassembled, and the DC scaffold was taken out and placed on the bottom of a new 24-well plate. Then, 50 μL allogeneic naïve CD4+ T cells (1.5 × 10^6 cells) were stimulated in a 24-well plate containing IL-2 (100 U/mL) and further incubated by adding 100 μL RPMI 1640 medium every 2 days for the indicated times followed by real-time RT-PCR analysis.

2.8 Real-time RT-PCR
Total RNA was prepared using an RNasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was prepared using oligo(dT) primer and SuperScript VI RT (Thermo Fisher Scientific). 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). 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. The primers used in this study are listed in Table S2.

2.9 Statistical analyses
Data are presented as the mean ± standard deviation (SD). Only one representative data of multiple independent experiments is shown due to space constraints. Statistical analyses were performed by using one-way analysis of variance (ANOVA) and the Dunnett’s or Tukey–Kramer multiple comparison test for comparing more than three groups using GraphPad Prism v7 (GraphPad Software Inc., La Jolla, CA, USA). 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 the AlveteX scaffold 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 other scaffolds was observed during the initial 24 h after stimulation with chemical sensitizers (Mizoguchi et al., 2017). Therefore, after 6 to 12 h, the stacked
scaffolds were disassembled. The DC scaffold was removed and placed on the bottom of a new 24-well plate, and allogeneic naive CD4⁺ T cells were added to the DC scaffold. We believe that this spatiotemporal flow well recapitulates the in vivo chemical sensitization process; after immature DCs capture antigen and become mature, the antigen-presenting DCs migrate to the draining lymph node to stimulate naive CD4⁺ T cells. This process is essential to initiate the adaptive immune response (Banchereau and Steinman, 1998). In addition, we made another assumption that in place of syngeneic naive CD4⁺ T cells, allogeneic naive CD4⁺ T cells would be well applicable. In the case of syngeneic naive CD4⁺ T cells, the response is too weak and therefore repetitive stimulation is necessary to detect the response (Richter et al., 2013; van Vliet et al., 2018; Vocanson et al., 2014). However, the response of allogeneic naive CD4⁺ T cells is strong and even after the first stimulation the response can be easily detected (Lakkis and Lechler, 2013; Sherman and Chattopadhyay, 1993).

3.2 Early mRNA upregulation of IFN-γ by OXA and late mRNA upregulation of IL-4 by OPA in the DC/T coculture system with peripheral monocyte-derived immature DCs and allogeneic naive CD4⁺ T cells

First, one set of typical skin and respiratory chemical sensitizers, OXA and OPA, was applied to the DC/T cell coculture system (Fig. 2A). Time-kinetic analysis of the mRNA expression of the T-cell activation marker CD69, Th1 differentiation marker IFN-γ, and Th2 differentiation marker IL-4 was performed. After stimulation of allogeneic naive 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. 2B). However, the skin sensitizer OXA but not respiratory sensitizer OPA further upregulated IFN-γ mRNA expression, while OPA but not OXA tended to up-regulate IL-4 mRNA expression. Both sensitizers did not further upregulate CD69 mRNA expression. On day 5, OPA but not OXA significantly upregulated IL-4 mRNA expression (Fig. 2C). On day 7, no difference in the upregulation of IFN-γ and IL-4 mRNA was observed (Fig. 2D). These results suggest that the DC/T cell coculture system with peripheral monocyte-derived immature DCs and allogeneic naive CD4⁺ T cells can differentially detect the early mRNA upregulation of IFN-γ by OXA and late mRNA upregulation of IL-4 by OPA.
Fig. 2: Early mRNA upregulation of IFN-γ by OXA and late mRNA upregulation of IL-4 by OPA in the DC/T cell coculture system with peripheral monocyte-derived immature DCs and allogeneic naive CD4+ T cells.

Typical skin and respiratory chemical sensitizers, OXA and OPA, were applied to the DC/T cell coculture system with peripheral monocyte-derived immature DCs and allogeneic naive CD4+ T cells (A). After incubation for 2 (B), 5 (C), and 7 (D) days, total RNA was prepared 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. Data were analyzed via ANOVA with Dunnett’s test to compare differences relative to a culture without chemical sensitizer (−) (B) or CD4+ T cell (C, D) and with the Tukey-Kramer test to compare differences between skin and respiratory sensitizers. Data are shown as the mean ± SD (n = 3) and are representative of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant. Replicate experiments #1 and #2 (B-D) were shown in the Fig. S1 and S2, respectively.
3.3 Selective mRNA upregulation of GATA-binding protein 3 by OPA in the DC/T cell coculture system with peripheral monocyte-derived immature DCs and allogeneic naive CD4⁺ T cells

To confirm the late 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 critically important for induction of their respective target cytokines IL-2, IFN-γ, and IL-4, respectively (Fig. 3A). OPA but not OXA significantly upregulated the mRNA expression of GATA-3, which is critically important for IL-4 induction (Fig. 3B). No significant mRNA upregulation of c-Fos and T-bet was observed at this time point, although it might have been too late to detect their upregulation on day 2. Thus, this DC/T cell coculture system can detect the selective mRNA upregulation of GATA-3 by OPA, further supporting the induction of Th2 immune responses.

3.4 Selective mRNA upregulation of IL-4 by 3 typical respiratory sensitizers in the DC/T cell coculture system with peripheral monocyte-derived immature DCs and allogeneic naive CD4⁺ T cells

To further examine the accuracy, in addition to the set of typical sensitizers OXA and OPA, two more sets of typical sensitizers DNCB and TMA, and FA and HDI were applied to the DC/T cell coculture system (Fig. 4A). After stimulation of allogeneic naive CD4⁺ T cells with sensitizer-treated DCs for 5 days, the mRNA expression of CD69, IFN-γ, and IL-4 was analyzed. Among three sets of typical sensitizers, respiratory sensitizers significantly upregulated IL-4 mRNA expression compared to skin sensitizers (Fig. 4B, C, D). Thus, this DC/T cell coculture system is useful to evaluate skin and respiratory sensitizers by the differential mRNA upregulation of IL-4.

Fig. 3: Selective mRNA upregulation of GATA-3 by OPA in the DC/T cell coculture system with CD14-ML-derived immature DCs and allogeneic naive CD4⁺ T cells

Typical skin and respiratory chemical sensitizers, OXA and OPA, were applied to the DC/T cell coculture system with CD14-ML-derived immature DCs and allogeneic naive CD4⁺ T cells (A). After incubation for 2 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 expression of the transcription factors c-Fos, T-bet, and GATA-3 together with their respective target cytokines IL-2, IFN-γ, and IL-4, respectively, and HPRT (B). Data were analyzed via ANOVA with Dunnett’s test to compare differences relative to a culture without chemical sensitizer (−) and with Tukey-Kramer test to compare differences between skin sensitizer and respiratory sensitizer. Data are shown as mean ± SD (n = 3) and are representative of two independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant. Replicate experiments #1 (B) were shown in the Fig. S3.
Fig. 4: Selective mRNA up-regulation of IL-4 by three typical respiratory sensitizers in the DC/T coculture system with CD14-ML-derived immature DCs and allogeneic naive CD4+ T cells

Three sets of typical skin and respiratory chemical sensitizers, OXA and OPA (B), DNCB and TMA (C), and FA and HDI (D) were applied to the DC/T co-culture system with CD14-ML-derived immature DCs and allogeneic naive CD4+ T cells (A) 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. Data were analyzed via ANOVA with Dunnett’s test to compare differences relative to CD4+ T cell (B) or a culture without chemical sensitizer (−) (C, D) and with the Tukey-Kramer test to compare differences between skin and respiratory sensitizers. Data are shown as the mean ± SD (B, C, n = 3; D, n = 4) and are representative of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant. Replicate experiments #1 and #2 (B-D) were shown in the Fig. S4 and S5, respectively.
3.5 Cell surface expression of CD14, CD11c, CD86, CD80, and human leukocyte antigen-DR and mRNA expression of OX40L in CD14-ML cells after differentiation into DCs and their maturation

CD14-ML cell lines were established by transducing genes c-MYC, BIM1, and BCL-2, which are related to cell survival and cell cycle, into peripheral CD14+ monocytes; the resultant CD14-ML cells proliferated vigorously as previously reported (Haruta et al., 2013; Imamura et al., 2016) (Fig. 5A). By further addition of IL-4, CD14-ML cells differentiated into immature DCs and then matured by stimulation with toll-like receptor (TLR) ligands such as OK-432 or LPS, which are potent maturation inducers for human monocyte-derived DCs. Cell surface analysis by fluorescence-activated cell sorting revealed that CD14-ML cells were largely positive for the DC marker CD11c as well as the monocyte marker CD14 (Fig. 5B). By differentiation into immature DCs with GM-CSF, M-CSF, and IL-4, and then stimulated with OX40L (100 ng/mL) in the presence or absence of varying concentrations of LPS as indicated (C, D), mRNA expression of OX40L and HPRT was determined by real-time RT-PCR analysis. Data are representative of more than two independent experiments. Replicate experiments #1 (B-D) were shown in the Fig. S6.
stimulated CD14-ML cells 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 seems to be better for the TSLP-mediated OX40L upregulation (Fig. 5C), which was further enhanced by the addition of LPS (Fig. 5D). Thus, CD14-ML cells seem to be the most suitable cell line as a source of DCs, because they proliferate vigorously and show similar phenotypes as peripheral monocytes.

Fig. 6: 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 and respiratory chemical sensitizers, OXA and OPA (B), DNCB and TMA (C), and FA and HDI (D) were applied to the DC coculture system with CD14-ML-derived immature DCs (A). 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. Data were analyzed via ANOVA with Dunnett’s test to compare differences relative to 10% or 16% DMSO and with the Tukey-Kramer test to compare differences between skin and respiratory sensitizers. Data are shown as the mean ± SD (n = 3) and are representative of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant. Replicate experiments #1 (B-D) and #2 (C, D) were shown in the Fig. S7 and S8, respectively.
3.6 Selective mRNA upregulation of OX40L by three typical respiratory sensitizers in the DC coculture system with CD14-ML-derived immature DCs

To determine whether peripheral monocyte-derived DCs can be replaced with CD14-derived immature DCs, three sets of typical skin and respiratory chemical sensitizers, OXA and OPA, DNCB and TMA, and FA and HDI were applied to the DC coculture system with CD14-ML-derived immature DCs (Fig. 6A). Here, to further increase the versatility, the DC coculture comprised only the epithelial cell line BEAS-2B and CD14-derived immature DCs, and the lung fibroblast cell line MRC-5 was omitted. Typical chemical respiratory sensitizers such as OPA (Fig. 6B), TMA (Fig. 6C), and HDI (Fig. 6D) consistently upregulated the mRNA expression of OX40L compared to typical skin sensitizers such as OXA, DNCB, and FA, respectively. Of note, these results suggest that differential OX40L mRNA upregulation by respiratory and skin sensitizers was reproducible in the DC coculture system with CD14-ML-derived immature DCs, and even without fibroblast cells. Therefore, in subsequent studies, the DC coculture system consisting of BEAS-2B cells and CD14-ML-derived immature DCs was used.

![Fig. 7: Sensitizer-dependent selective mRNA upregulation of TSLPR, IL-7Rα, and ST2 by three typical respiratory sensitizers in the DC coculture system with CD14-ML-derived immature DCs](image-url)

Three sets of typical skin and respiratory chemical sensitizers, OXA and OPA (B), DNCB and TMA (C), and FA and HDI (D) were applied to the DC co-culture system with CD14-ML-derived immature DCs (A). 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 TSLPR, IL-7Rα, IL-17RB, and ST2 together with HPRT. Data were analyzed via ANOVA with Dunnett’s test to compare differences relative to 10% or 16% DMSO and with the Tukey-Kramer test to compare differences between skin and respiratory sensitizers. Data are shown as the mean ± SD (n = 3) and are representative of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant. Replicate experiments #1 and #2 (C, D) were shown in the Fig. S9 and S10, respectively.
3.7 Sensitizer-dependent selective mRNA upregulation of TSLPR, IL-7Rα, IL-17RB, and ST2 by three typical respiratory sensitizers in the DC coculture system with CD14-ML-derived immature DCs

To further examine the usefulness of CD14-derived immature DCs, we examined the effects of skin and respiratory sensitizers on the mRNA expression of cytokine receptors for epithelial cell-derived cytokines, not only TSLP but also IL-25 and IL-33, which are also critically important for the induction of Th2 immune responses (Roan et al., 2019) (Fig. 7A). The receptor complexes for TSLP, IL-25, and IL-33 are composed of TSLPR and IL-7Rα (Pandey et al., 2000), IL-17RA and IL-17RB (Rickel et al., 2008), and ST2 and IL-1R accessory protein IL-1RAcP (Chackerian et al., 2007), respectively. Similar to OX40L upregulation, respiratory sensitizer-induced differential mRNA upregulation of ST2 was observed by OPA (Fig. 7B), and that of TSLPR and IL-7Rα was observed by TMA and HDI (Fig. 7C, D). These results suggest that in addition to selective mRNA upregulation of OX40L by three respiratory sensitizers, sensitizer-dependent selective mRNA upregulation of TSLPR, IL-7Rα, IL-17RB, and ST2 was also observed.

Fig. 8: 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 naive CD4+ T cells

Three sets of typical skin and respiratory chemical sensitizers, OXA and OPA (B), DNCB and TMA (C), and FA and HDI (D) were applied to the DC/T cell coculture system with CD14-ML-derived immature DCs and allogeneic naive CD4+ T cells (A). 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. Data were analyzed via ANOVA with Dunnett’s test to compare differences relative to a culture without chemical sensitizer (−) and with the Tukey-Kramer test to compare differences between skin and respiratory sensitizers. Data are shown as the mean ± SD (n = 3) and are representative of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant. Replicate experiments #1 (B, D) and #2 (B) were shown in the Fig. S11A, B, and C, respectively.
3.8 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 naive 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. 8A). Typical respiratory sensitizers such as OPA (Fig. 8B), TMA (Fig. 8C), and HDI (Fig. 8D) significantly increased the mRNA expression of IL-4 compared to typical skin sensitizers such as OXA, DNCB, and FA, respectively. These results suggest that the differential mRNA upregulation of IL-4 by respiratory and skin sensitizers was reproducible in the DC/T cell coculture system with CD14-ML-derived immature DCs, but without the lung fibroblast cell line MRC-5.

4 Discussion

Recently, to mimic the upper airway epithelium, we established a new 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 scaffold 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, typical respiratory sensitizers showed more enhanced mRNA expression in DCs of the key costimulatory molecule OX40L, which is important for Th2 differentiation (Furue and Furue, 2021; Ito et al., 2005). To further apply this system to targeting KE4 in the AOP of sensitization, shortly after the stimulation with sensitizers, the DC scaffold was removed and placed on the bottom of a new plate, mimicking the migration of antigen-presenting DCs to the draining lymph nodes (Banchereau and Steinman, 1998). To date, there are a lack of sensitization assays that take into account DC migration to the draining lymph node. In this study, allogeneic naive CD4+ T cells were added to the coculture system followed by real-time RT-PCR analysis. The greatest advantage of this DC/T cell coculture system is that IL-4, which induces Th2 differentiation and is also a potent effector cytokine (Paul, 2015; Zhu, 2015), can be used as a marker in T cells. Indeed, typical respiratory sensitizers but not skin sensitizers showed mRNA upregulation of IL-4 in T cells. To further improve the versatility of these systems, we generated monocyte-derived proliferating cell CD14-ML cell lines 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 have DC maturation, antigen-presenting activity, and potent antitumor activity similar to peripheral monocytes (Haruta et al., 2013; Imamura et al., 2016). Differential upregulation of not only OX40L but also IL-4 by skin and respiratory sensitizers was similarly 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, respectively.

Generally, the sensitization process proceeds under physiological situations as follows (Kimber et al., 2018; MacKay et al., 2013; Sullivan et al., 2017). Chemical sensitizers first attach to the surface of epithelium and penetrate it. During this process, chemical sensitizers covalently bind to serum and cellular proteins, making them immunogenic; this process is called haptenation (Chipinda et al., 2011). Then the immature DCs capture the haptenized proteins, process them, and present their peptides on MHC class II with 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). Then, there antigen-presenting DCs stimulate naive CD4+ T cells to differentiate them into effectors including Th1, Th2, Th17 cells, in order to initiate adaptive immunity. 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), which utilizes peripheral monocyte-derived immature DCs and syngeneic naive T cells from healthy blood donors. To facilitate the maturation of DCs, immature DCs are 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 the test substance-treated mature DCs are cocultured with syngeneic naive 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 Test Force organized a workshop and discussed how the information provided by T-cell assays could be used for the safety assessment of cosmetic ingredients. The workshop 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 methods 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 complexity in transferability for future ring studies to evaluate accuracy and reproducibility (van Vliet et al., 2018). Therefore, the workshop concluded that the current hTCPA still needs further refinement and simplification.

To overcome these problems and more precisely recapitulate the physiological spatiotemporal flow of sensitization process, we have come up with the following improvements and assumptions. First, to mimic the in vivo situation of the upper airway epithelium sensitized with chemical sensitizers, aliquots of relatively higher concentration of the sensitizer solution were gently added to the top of the 3D coculture system consisting of the epithelial cell line BEAS-2B, peripheral monocyte-derived immature DCs, and lung fibroblast cell line MRC-5, and incubated for a while and subsequently diluted with medium. Later, the MRC-5 cells were omitted from the DC and DC/T cell coculture systems without any apparent difference. We believe that this directional penetration of sensitizers from epithelial cells to immature DCs is very important for the activation of innate immune responses releasing damage-associated molecular patterns (DAMPs) and inflammatory cytokines from epithelial cells, which greatly affect the subsequent induction of DC maturation and are necessary for determining which type of adaptive immune responses will be activated by the sensitizer (Sullivan et al., 2017). Within 12 h after stimulation, the DC scaffold was removed and placed on the bottom of a new plate, and mixed with allogeneic naive CD4+ T cells. We believe this process well recapitulates the migration of antigen-captured and antigen-presenting DCs to the draining lymph node to stimulate naive CD4+ T cells in vivo. The allogeneic response is strong enough because it is generally a polyclonal response and allogeneic T cells constitute ~10% of the total 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 in primary response (Lakkis and Lechler, 2013; Sherman and Chattopadhyay, 1993) and multiple re- stimulations as in the case of hTCPA (Richter et al., 2013; van Vliet et al., 2018; Vocanson et al., 2014) are not necessary. Generally, the proteins reactive to chemical sensitizers are proteins present abundantly in the serum and cells such as albumin, actin, myosin, and heat shock protein (Chipinda et al., 2011; Parkinson et al., 2018), and protein specificity has little to do with which type of immune response can be subsequently activated by the sensitizer. Therefore, in this study, we took advantage of the strong allogeneic response of CD4+ T cells stimulated with DCs. If a T cell-based assay is feasible, one of the best advantages is to apply it to detect IL-4 in CD4+ T cells for the evaluation of allergenicity, especially respiratory sensitization. This is because IL-4 is the best marker of Th2 immune responses, the strongest inducer of Th2 differentiation, and the most potent effector cytokine of Th2 cells (Paul, 2015; Zhu, 2015). There are nice models of the human airway that are primary airway epithelial cells cultured at air-liquid interphase and are now commercially available under trade names such as MucilAir and EpiAirway (Balharry et al., 2008; Huang et al., 2013). However, to minimize laborious and time-consuming processes and improve the versatility, BEAS-2B cell line was used instead of using these primary cells, although these models are much more physiologically relevant for assessing human responses following exposure to a chemical. Moreover, we also used monocyte-derived proliferating CD14-ML cell lines, generated by introducing c-MYC, BIM1, and BCL-2 genes into peripheral monocytes (Haruta et al., 2013; Imamura et al., 2016). The CD14-ML-derived immature DCs were successfully replaced with peripheral monocyte-derived immature DCs in the DC and DC/T cell coculture systems. If DC and DC/T cell coculture systems are thus established that comprise only cell lines, donor-to-donor variability will no longer be a concern, which limits the reliability and reproducibility.

Collectively, 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 naive 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. However, this system has the limitation that only small numbers of chemicals can be applied due to the limited ability to obtain primary naive CD4+ T cells from a human donor. To further improve the versatility, we are currently trying to apply an allogeneic Th2 clone cell line to the DC/T cell coculture system in place of peripheral naive CD4+ T cells. Although further verification of this assay system with a greater number of potential chemical sensitizers as well as improvements in the versatility are thus warranted, this two-step DC/T coculture system could be a useful tool for evaluating the sensitizing potential of chemicals in vitro.

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

Conflict of interest
The authors declare that there are no conflicts of interests.

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 Alternative to Animal Experiments, KOSE Cosmetology Research Foundation, Hoyu Science Foundation, and Daicel Corporation.