that targeting the TSLP-basophil axis may offer new opportunities for the clinical management of EoE in patients.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.N., E.D.T.W., B.S.K., M.C.S., P.R.G., M.G.N., A.B.M., A.A., C.Z. and D.A. designed and performed experiments. A.J.B., K.R.R., P.M.-K., A.C., G.W.F., M.-L.W. and J.M.S. obtained human pediatric and adult esophageal biopsies and peripheral blood samples, K.R.C. analyzed pediatric esophageal biopsy histology, and D.A.H. and T.B.-W. coordinated patient care and clinical studies. A.E.M. and Q.J.S. provided CPE, M.K. provided Baso-DTR mice, K.O.-N. and H.K. provided CD200R3-specific mAb, M.R.C. provided TSLPR-deficient mice and TSLP reagents, J.H.Y. and R.d.W.M. performed staining for human TSLP, and P.M.S. and H.H. provided genotype information on pediatric patients with EoE. M.N., E.D.T.W., B.S.K., M.C.S., P.R.G., A.A., C.Z., M.-L.W., J.M.S. and D.A. analyzed the data. M.N., E.D.T.W., M.C.S. and D.A. wrote the manuscript, and all authors critically reviewed the manuscript.

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The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Mice. Male and female BALB/c and C57BL/6 mice were purchased from the Jackson Laboratories. BALB/c *Tslpr*$^{+/+}$ and BALB/c *Tslpr*$^{-/-}$ mice were provided by Amgen, through Charles River Laboratories. BALB/c *Igh-7*$^{-/-}$ mice and C57BL/6 Baso–DTR mice were bred at the University of Pennsylvania. All mice were used at 8–12 weeks of age, and all experiments employed age-, gender- and genetic strain-matched controls to account for any variations in data sets compared across experiments. Mice were bred and housed in specific pathogen–free conditions at the University of Pennsylvania. Mice requiring medical attention were provided with appropriate veterinary care by a licensed veterinarian and were excluded from the experiments described. No other exclusion criteria existed. All experiments were performed under the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) approved protocols and in accordance with its guidelines.

Reagents and treatments. Mice were treated daily with 2 nmol MC903 (calcipotriol, Tocris Bioscience) in 20 μl of 100% EtOH applied to the ears in the presence of 100 μg OVA for 14 d. As a vehicle control, the same volume of EtOH and OVA was applied. For tape-stripping, mice were shaved on the back, tape-stripped six times with scotch sealing tape and sensitized with 100 μg OVA or saline as control daily for 14 d. For TSLP injections, mice were subcutaneously injected with 5 μg rTSLP in the presence of 100 μg OVA on days 0, 3, 6, 9 and 12. For controls, mice were injected subcutaneously with PBS or rTSLP alone. For CPE sensitization, CPE was made from whole roasted peanuts (Sainsbury's Ltd.) sterilized by gamma irradiation (Lillico Biotech) that were ground in an airflow cabinet using a mortar and pestle. The resulting paste was solubilized in pH 7.4 PBS (Gibco) and sonicated for two 20-min periods, with mixing in between. The solution was then filtered through a 75-μm tissue filter (BD Biosciences) to remove large particles of debris. Lipopolysaccharide content was tested (Lonza) and reported less than 0.006 ng mL^{-1}. Mice were treated daily with 2 nmol MC903 in 20 μl of 100% EtOH on ears in the presence of 100 μg CPE for 14 d. As a vehicle control, the same volume of EtOH and CPE was applied. Mice were challenged i.g. with 50 mg OVA or 10 mg CPE on days 14 and 17.5 and killed on day 18. Upon first i.g. OVA or CPE challenge, mice were continuously fed water containing 1.5 g L^{-1} OVA or given continuous access to whole roasted peanut. Mice subjected to repeated challenge with OVA to induce prolonged inflammation in the esophagus were challenged i.g. with 50 mg OVA on days 14, 17.5, 18, 20, 22, 24 and 26 and killed on day 27. For depletion with TSLP-specific mAb[17], mice were injected with 500 μg of control IgG or TSLP-specific mAb commercially produced by Amgen intraperitoneally every 3 d during the course of the experiment starting at day −1 or every other day starting at day 18. For basophil depletion by diphtheria toxin treatment, Baso-DTR^{+} or Baso-DTR^{-} littermate control mice were treated with 500 ng diphtheria toxin (Sigma) intraperitoneally on days −1, 3, 7 and 12. For depletion with CD200R3-specific mAb (Ba103)[46], mice were injected with 100 μg of control IgG or CD200R3-specific mAb (clone Ba103, provided by H. Karasuyama) intravenously every 4 d during the course of the experiment starting at day −1 or every other day starting at day 18. To assess food impaction in the esophagus, mice exposed to prolonged esophageal inflammation were fasted for at least 30 min and up to 2 h. Mice were then killed, and their esophagi were examined for the presence of impacted food.

Cohort of human subjects with eosinophilic esophagitis. Pediatric participants from a cohort of control subjects or subjects with EoE at the University of Pennsylvania Penn-Children's Hospital of Philadelphia (CHOP) Joint Center for Digestive, Liver and Pancreatic Medicine or the Center for Pediatric Eosinophilic Disorders at CHOP were analyzed and were provided under a CHOP IRB to M.-L.W. and A.J.B. Adult participants from a cohort of control subjects or subjects with EoE being treated at the Hospital of the University of Pennsylvania Division of Gastroenterology were also assessed and were provided under a University of Pennsylvania IRB to G.W.F. and P.M.-K. Written consent was obtained from all participants or their parents or legal guardians, and for pediatric participants, verbal assent from the child was additionally obtained. Subjects defined as having EoE had no other chronic condition except asthma, allergic rhinitis, food allergy, urticaria or atopic dermatitis. Control subjects presented with epigastric abdominal pain but had normal endoscopic and microscopic results. Pediatric subjects with EoE were on proton pump inhibitor therapy, but subjects on systemic corticosteroid treatment or antibiotics were excluded. Subjects with active EoE had an esophageal eosinophil count of ≥15 per HPF after 8 weeks of treatment with a proton pump inhibitor. Subjects with inactive EoE had previously been diagnosed with active EoE but had an esophageal eosinophil count of < 15 per HPF at the time of sample collection. During routine endoscopy, three esophageal biopsies were collected for histological analysis of esophageal eosinophil counts. During the same procedure, two esophageal tissue biopsies were collected for research purposes, for either real-time PCR, immunohistochemistry or flow cytometry. For flow cytometry, single-cell suspensions were made by filtering the mechanically disrupted tissue through a 70-μm filter (BD Biosciences) for flow cytometry. Peripheral blood from pediatric subjects from a cohort of control subjects or subjects with active or inactive EoE that were genotyped for a gain-of-function *TSLP* polymorphism at the University of Pennsylvania Penn-CHOP Joint Center for Digestive, Liver and Pancreatic Medicine or the Center for Pediatric Eosinophilic Disorders at CHOP was analyzed and was provided under a CHOP IRB to J.M.S. and K.R.R. Written consent was obtained from all participants or their parents or legal guardians, and for pediatric participants, verbal assent from the child was additionally obtained. Peripheral blood was collected by venipuncture, and serum was isolated. PBMCs were isolated by Ficoll gradient as previously described[17], and cells were analyzed by flow cytometry. For genotyping of pediatric subjects with EoE, all samples were genotyped on either the Illumina HumanHap 550 or 610 BeadChips according to the manufacturer's protocols. Data normalization and canonical genotype clustering were carried out using the Illumina Genome Studio package. Samples with call rate <98% were excluded from further analysis.

Human real-time PCR and immunohistochemistry. For real-time PCR analysis of gene expression in human esophageal biopsies, human subject biopsy samples were collected and placed in RNA*later* (Ambion). RNA was isolated using the mirVana miRNA Isolation Kit according to the manufacturer's recommendations (Ambion) and reverse transcribed using a high-capacity cDNA reverse transcriptase kit (Applied Biosystems). Quantitative real-time PCR was performed using the Taqman Fast Universal PCR Master Mix kit and preformulated TaqMan Gene Expression Assays for *TSLP* (Applied Biosystems). Reactions were performed in triplicate using 96-well optical plates on a StepOnePlus Real-Time PCR System (Applied Biosystems). GAPDH was used as an endogenous control to normalize the samples using the C_T method of relative quantification, where C_T is the threshold cycle. For immunohistochemical staining for human TSLP, human esophageal biopsies were embedded in paraffin and sectioned. Sections were deparaffinized and stained with a primary human TSLP-specific mAb or an isotype control antibody (validated by J.H. Yearley and R. de Waal Malefyt and commercially produced by Merck Research Laboratories), and positive staining was visualized using the DAB substrate kit (Vector Laboratories).

Flow cytometry. For mouse studies, esophageal tissues of two or three mice were pooled within each replicate experiment, opened longitudinally, digested in 1 mg mL^{-1} collagenase/DNase (Roche) for 30 min, and mashed through 70-μm nylon mesh filters. Single-cell suspensions were incubated with Aqua Live/Dead Fixable Dye (Invitrogen) for dead cell exclusion and stained with fluorochrome-conjugated mAbs purchased from eBioscience specific for mouse CD3ε (145-2C11, 1:300), CD4 (GK1.5, 1:300), CD8 (53-6.7, 1:300), NK1.1 (PK136, 1:300), CD19 (eBio1D3, 1:300), FcεR1 (MAR-1, 1:200), IgE (23G3, 1:200), CD45 (30-F11, 1:200), CD49b (DX5, 1:200,), CD117 (c-kit, 1:200), fluorochrome-conjugated mAbs purchased from Biolegend specific for mouse CD11c (N418, 1:200), CD5 (53-7.3, 1:300), B220 (RA3-6B2, 1:300) and a fluorochrome-conjugated mAb purchased from BD Bioscience specific for mouse Siglec-F (E50-2440, 1:200), or fluorochrome-conjugated mAbs purchased from eBioscience specific for human CD19 (HIB19, 1:200), CD45 (HI30, 1:100), CD49b (ebioY418, 1:200), FcεR1 (AER-37, 1:50), CD123 (6H6, 1:100) and c-kit (104D2, 1:30) or fluorochrome-conjugated mAbs purchased from BD Biosciences specific for human CD56 (B159, 1:200), CD11c (B-ly6, 1:200) and TCRαβ (IP26, 1:200). For intracellular staining, surface-stained cells were washed, fixed in 2% paraformaldehyde, permeabilized using eBioscience Permeabilization Buffer (eBioscience) according to manufacturer instructions, stained intracellularly with human 2D7-specific mAb (2D7, 1:25) (eBioscience), washed and

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resuspended in flow cytometry buffer. All cells were run on a four-laser 14-color LSR II (BD Biosciences), and FlowJo 8.7.1 (Tree Star) was used to analyze data. Mouse eosinophils were identified as live, lin$^-$ (CD3,CD5,CD19,CD11c,NK1.1), CD45$^+$Siglec-F$^+$ side-scatter (SSC)-high cells. Mouse basophils were identified as live, lin$^-$ (CD3,CD5,CD19,CD11c,NK1.1), c-kit$^-$CD49b$^+$IgE$^+$ cells (or as FcεRI$^+$ cells in *Igh-7*$^{-/-}$ mice). Human basophils in the esophageal biopsy were identified as live, lin$^-$ (CD19,CD56,CD11c,TCRαβ), CD49b$^+$FcεRI$^+$c-kit$^-$2D7$^+$ cells. Human basophils in the PBMCs were identified as live, lin$^-$ (CD19,CD56,CD11c,TCRαβ), CD123$^+$FcεRI$^+$ cells.

Optical coherence tomography. An OCT system operating at 1.3-μm center wavelength at 47 kHz axial scan rate (~30 frames per s) was developed and used for obtaining volumetric images of freshly excised mouse esophagus. The axial and transverse resolutions were 6 μm and 10 μm in tissue, respectively, and the imaging depth was approximately 2 mm, sufficient to image through the entire thickness of the mouse esophagus. Prior to OCT imaging, the esophagus was removed from the mouse, and a plastic tube with 0.75-mm outer diameter was inserted, allowing for the lumenal surface to be clearly differentiated in cross-sectional images. The esophagus was immersed in saline solution to remove light reflection from the surface. Subsequently, three-dimensional OCT images were obtained from multiple locations along the esophagus, with each data set covering 3 × 1.5 × 1.5 mm^3. The thickness values of the squamous epithelial layer were measured from cross-sectional OCT images every 200 μm along the esophagus within each data set. Average squamous epithelial thickness values from the middle of the esophagus were calculated from each mouse by an investigator blinded to group allocations and were used for comparison between different groups.

Mouse cell cultures, ELISA, real-time PCR, histology and electron microscopy. To measure spontaneous release of TSLP, whole ears were incubated for 12 h in complete culture medium (DMEM, 10% FBS), and cell-free supernatants were stored for a TSLP ELISA using a commercially available kit (eBioscience). For antigen re-stimulation, splenocytes or mesenteric lymph node cells were isolated, and single-cell suspensions were stimulated with 200 μg OVA for 72 h. Cell-free supernatants were used for standard sandwich ELISA. Antigen-specific IgE responses were measured as described previously[61]. For histological analysis, at necroscopy, the esophagus was fixed in 4% paraformaldehyde and embedded in paraffin, and 5-μm sections were cut and stained with hematoxylin and eosin (H&E). For immunofluorescence, sections were deparrafinized and stained with biotinylated Siglec-F–specific mAb from R&D Systems (BAF1706, 1:200), followed by secondary staining with Cy3-conjugated streptavidin (Jackson Laboratory) and counterstaining with DAPI (Molecular Probes). For EM, esophageal tissues were fixed with 2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, overnight at 4 °C. After buffer washes, the samples were post-fixed in 2.0% osmium tetroxide for 1 h at room temperature and rinsed in dH_2O before *en bloc* staining with 2% uranyl acetate. After dehydration through a graded ethanol series, the tissue was infiltrated and embedded in Embed-812 (Electron Microscopy Sciences). Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 1010 electron microscope fitted with a Hamamatsu digital camera and AMT Advantage image capture software. For real-time PCR analysis, RNA was isolated from esophageal tissue using an RNeasy mini kit (Qiagen) or the mirVana miRNA isolation kit (Ambion) according to the manufacturer's instructions. cDNA was generated using a SuperscriptII reverse transcription kit (Invitrogen). Real-time quantitative PCR was performed on cDNA using SYBR green master mix (Applied Biosystems) and commercially available primer sets from Qiagen (Quantitect primer assays). Samples were run on a real-time PCR system (ABI 7500; Applied Biosystems), normalized to β-actin and displayed as fold induction over controls.

Statistical analysis. Results are shown as mean ± s.e.m. To determine group sizes necessary for adequate statistical power, power analysis was performed using preliminary data sets for all analyses presented. Mice were assigned at random to treatment groups for all mouse studies. Mouse studies were not performed in a blinded fashion, except where indicated. Analyses of basophil responses in esophageal biopsy samples and peripheral blood were conducted in such a manner that the investigator was blinded to the disease state (number of eosinophils per HPF in the biopsy) and *TSLP* genotype until after flow cytometric analyses were completed. Analysis of *TSLP* expression levels in the biopsies of control subjects and those with EoE were not performed in a blinded fashion. All inclusion and exclusion criteria for mouse and human studies were pre-established. For mouse studies, statistical significance was determined using a nonparametric, two-tailed Mann-Whitney *t*-test, a nonparametric, one-way Kruskal-Wallis ANOVA test followed by Dunn's *post hoc* testing or a nonparametric, two-way ANOVA followed by Bonferroni's *post hoc* testing. For human studies, a nonparametric, two-tailed Mann-Whitney *t*-test or a nonparametric, one-way Kruskal-Wallis ANOVA followed by Dunn's *post hoc* testing were used. Correlation analysis was performed using a nonparametric Spearman correlation (sensitivity analyses were performed), and a linear regression of the data is displayed. All data meet the assumptions of the statistical tests used. Within each group there is an estimate of variation, and the variance between groups is similar. For each statistical analysis, appropriate tests were selected based on whether the data was normally distributed and whether multiple comparisons were made. Results were considered significant at $P \leq 0.05$. Statistical analyses were performed using Prism version 5.0a (GraphPad Software).

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Basophils are required for the induction of Th2 immunity to haptens and peptide antigens

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The relative contributions of basophils and dendritic cells in Th2 skewing to foreign antigen exposure remain unclear. Here we report the ability of basophils to induce Th2 polarization upon epicutaneous sensitization with different antigens using basophil conditionally depleted Bas TRECK transgenic mice. Basophils are responsible for Th2 skewing to haptens and peptide antigens, but not protein antigens *in vivo*. Consistent with this, basophils cannot take up or process ovalbumin protein in significant quantities, but present ovalbumin peptide to T cells for Th2 differentiation via major histocompatibility complex class II. Intriguingly, basophils promote Th2 skewing upon ovalbumin protein exposure in the presence of dendritic cells. Taken together, our results suggest that basophils alone are able to induce Th2 skewing with haptens and peptide antigens but require dendritic cells for the induction of Th2 for protein antigens upon epicutaneous immunization.

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The induction of adaptive cellular immunity in the skin is initiated by antigen-presenting cells (APCs) such as dendritic cells (DCs), which provide signals through the peptide-major histocompatibility complex (MHC), costimulatory molecules, and instructive cytokines to naive T cells upon antigen encounter[1–3]. Distinct subsets of $CD4^+$ T helper (Th) cells are then generated to control different types of protective immunity[4]. Th2 cells are crucial for the clearance of parasites, such as helminths, via expansion and activation of innate effector cells, including eosinophils and basophils[5]. The induction of Th2 immune responses was previously considered to depend mainly on DCs[6]. However, this dogma has recently been challenged as basophils might also have a pivotal role in this process[7–10].

It has been demonstrated that $CD49b^+$ $Fc\varepsilon RI^+$ $c\text{-}Kit^-$ basophils migrate into the draining lymph nodes (LNs) from the site of helminth infection or papain injection and act as APCs by taking up and processing antigens[7,9,10]. In addition, basophils express MHC class II and costimulatory molecules and secrete interleukin (IL)-4 and thymic stromal lymphopoietin (TSLP), which are critical for Th2 development. Thus, basophils alone are considered to induce Th2 polarization from naive T cells without requiring DCs in certain conditions. On the contrary, another group has found that IL-4-producing basophils were recruited to the mediastinal LNs upon first house dust mite exposure and contributed to the strength of the Th2 response in the lung, but that basophils could not present antigens or express the chaperones involved in antigen presentation[11]. These authors, therefore, proposed that DCs are necessary and sufficient for the induction of Th2 immunity to house dust mites in the lung without the requirement of basophils. Consistent with this, it has been reported that Th2 responses were severely impaired either after *Schistosoma mansoni* egg injection or during active *S. mansoni* infection by depletion of $CD11c^+$ cells, but not by depletion of basophils using anti-$Fc\varepsilon RI\alpha$ antibody[12]. Therefore, it is of great importance to determine whether or not, and under which conditions, basophils induce Th2 skewing to foreign antigen exposure.

Recently, we demonstrated that basophils use a specific 4 kb enhancer fragment containing the 3′-untranslated region and HS4 elements to regulate *Il4* gene expression[13]. Utilizing this system, we have generated mice that express human diphtheria toxin receptor under the control of HS4, which are named basophil-specific enhancer-mediated toxin receptor-mediated conditional cell knock-out (Bas TRECK) transgenic (Tg) mice[14–16]. In Bas TRECK Tg mice, basophils are specifically and conditionally depleted by diphtheria toxin (DT) treatment. Using these mice, we examined whether basophils induce Th2 polarization upon stimulation by different antigens such as haptens, peptides and protein antigens. We herein demonstrate that basophils are necessary for the induction of cutaneous Th2 immunity against haptens and peptide antigens but are dispensable for protein antigens.

Results

Impaired induction of IgG1 to peptide antigens. We first confirmed that basophils in the bone marrow (BM) were completely depleted without affecting T cell, mast cell or DC population in Bas TRECK Tg mice after an intraperitoneal injection of DT (Fig. 1a, and see Supplementary Figs S1A–D,S2). To test whether basophils are involved in the induction of cutaneous Th2 responses against protein antigens, we pretreated wild-type C57BL/6 (B6) and Bas TRECK Tg mice with DT and immunized them with ovalbumin (OVA) protein via a cutaneous patch to induce a Th2-type cutaneous immune response[17,18].

In this model, DT-treated Bas TRECK Tg (basophil-depleted) mice exhibited similar clinical manifestations to B6 mice (Fig. 1b,c). Basophils were accumulated in the skin lesion of B6 mice but absent in that of basophil-depleted mice (see Supplementary Fig. S2). In addition, the levels of serum OVA-specific IgG1 in basophil-depleted mice were comparable to those in B6 mice (Fig. 1d), whereas the production of Th1-dependent serum IgG2a was not induced by cutaneous application of OVA proteins (Fig. 1d, right panel). To evaluate the T-cell stimulatory capacity of basophils upon protein antigen exposure, the skin draining LN cells from B6 and basophil-depleted mice after cutaneous OVA protein application were challenged with OVA protein *in vitro*. The incorporation of ^3H-thymidine in the presence of OVA protein was comparable between basophil-depleted mice and B6 mice (see Supplementary Fig. S3). The above results suggest that basophils were not essential to Th2-type immune responses induced by cutaneous application of protein antigens.

Previous studies have reported that basophils, but not DCs, functioned as APC for peptide antigen-induced Th2 *in vitro*[9]. As such, we next compared the *in vivo* Th2-induction capacity of basophils upon intraperitoneal exposure to OVA protein or peptide (amino acids 323–339) mixed with alum as a strong adjuvant of Th2 responses. In this model, a significantly decreased OVA-specific IgG1 level was observed in basophil-depleted mice with OVA peptide immunization, but not with OVA protein immunization (Fig. 1e). At the same time point, antigen-specific IgE was undetectable in both groups due to the genetic background of these mice as B6 (refs 19,20); therefore, OVA-specific IgG1 has been used as a marker of Th2-dependent immunoglobulin in this model[19]. In addition, we examined that IgG1 production by anti-CD40 is enhanced by IL-4 (Supplementary Fig. S4), which also supports the rationale that IgG1 can be used as a marker of Th2 induction. We also found that the frequency of $IL\text{-}4^+$ cells in spleen $CD4^+$ T cells from basophil-depleted mice was significantly lower than that from B6 mice upon OVA peptide intraperitoneal immunization, but not upon OVA protein immunization (Fig. 1f,g). The frequency of $IL\text{-}4^+$ cells in non-T cells was also comparable between these two groups (Supplementary Fig. S5).

In addition, after immunized with OVA peptide, the numbers of eosinophils, $CD4^+$, $CD4^+CD44^+CD62L^+$ central memory, and $CD4^+CD44^+CD62L^-$ effector memory T cells in splenocytes from basophil-depleted mice were significantly decreased compared with those from B6 mice (Supplementary Fig. S6A). Consistently, upon immunization with OVA peptide, the messenger RNA (mRNA) levels of *IL-4* and *IL-13* in the mesenteric LNs in basophil-depleted mice were significantly decreased compared with those in B6 mice (Supplementary Fig. S6B).

To further evaluate the role of basophils on T-cell differentiation after immunization with OVA protein or OVA peptide, splenocytes from B6 and basophil-depleted mice were re-challenged in the presence or absence of each antigen *in vitro*. Markedly decreased incorporation of ^3H-thymidine and the levels of IL-4, IL-5 and IL-13 in the culture supernatant were examined in basophil-depleted mice upon OVA peptide immunization, but not upon OVA protein immunization (Supplementary Fig. S7A,B).

We next evaluated Th2 induction under the condition where both OVA protein and OVA peptide are used at the same time with alum. The levels of serum OVA-specific IgG1 (Supplementary Fig. S8A–C), the frequency of $IL\text{-}4^+$ cells in $CD4^+$ T from basophil-depleted mice was similar to that from B6 mice in all *mol* ratio (protein:peptide = 2:1, 1:1, and 1:2) (Supplementary Fig. S8D,E).

Attenuation of inflammation by repeated hapten application. Haptens are one of external antigens via cutaneous penetration. A previous report demonstrated that repeated elicitation with

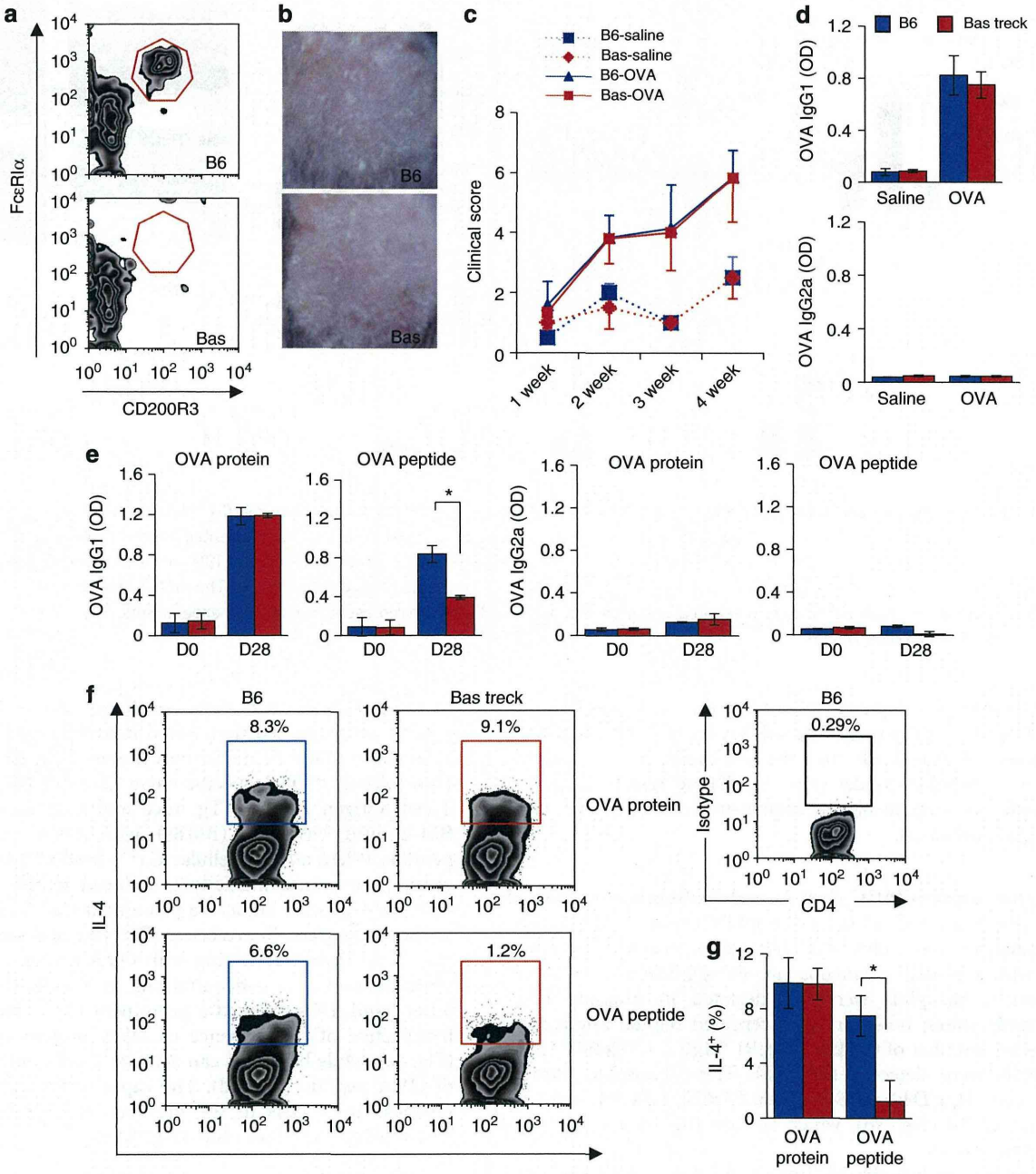

Figure 1 | Impaired induction of IgG1 to peptide antigens in the absence of basophils. (**a**) Basophils in BM in B6 and Bas TRECK mice after an intraperitoneal injection of diphtheria toxin. (**b**,**c**) Clinical manifestations (**b**) and clinical scores (**c**) of Th2-type cutaneous immune response model in DT-treated B6 and DT-treated Bas TRECK mice ($n=10$ per group). (**d**) Serum OVA-specific IgG1 levels and IgG2a in DT-treated Bas TRECK mice and DT-treated B6. (**e**) OVA-specific IgG1 and IgG2a responses in DT-treated B6 mice and DT-treated Bas TRECK mice in the model of immunization with OVA protein or peptide (amino acids 323–339) on day 0 and 28 mixed with alum, a strong promoter of Th2 responses. (**f**,**g**) Intracellular cytokine staining for IL-4 (**f**) and the frequency of IL-4$^+$ cells (**g**) in CD4$^+$ T cells. All data are presented as the mean ± s.d. and are representative of three experiments. *$P<0.05$, Wilcoxon signed-rank test versus corresponding groups.

hapten results in a shift from Th1- to Th2-mediated cutaneous inflammation, which mimics atopic dermatitis[21]. Therefore, to examine the role of basophils in hapten-induced Th2-type inflammation in the skin, we performed repeated cutaneous application of oxazolone to B6 mice and basophil-depleted mice.

Although Th1-mediated delayed-type hypersensitivity as manifested by the ear swelling response to a single elicitation of oxazolone was similar between B6 and basophil-depleted mice (Fig. 2a), the repeated application-induced immune reaction in basophil-depleted mice was much less than that in B6 mice (Fig. 2b). Immediate-type hypersensitivity and late phase reaction, as manifested by the ear swelling responses 1 and 6 h after the last hapten application in basophil-depleted mice was significantly attenuated compared with those in B6 mice (Fig. 2c). Histological examination revealed attenuated epidermal thickening and cell infiltrations in the dermis of basophil-depleted mice (Fig. 2d,e, Supplementary Table S1). In addition, serum oxazolone-specific IgG1 levels in basophil-depleted mice were significantly decreased compared with those in B6 mice (Fig. 2f, left panel). Furthermore, the oxazolone-specific IgG2a

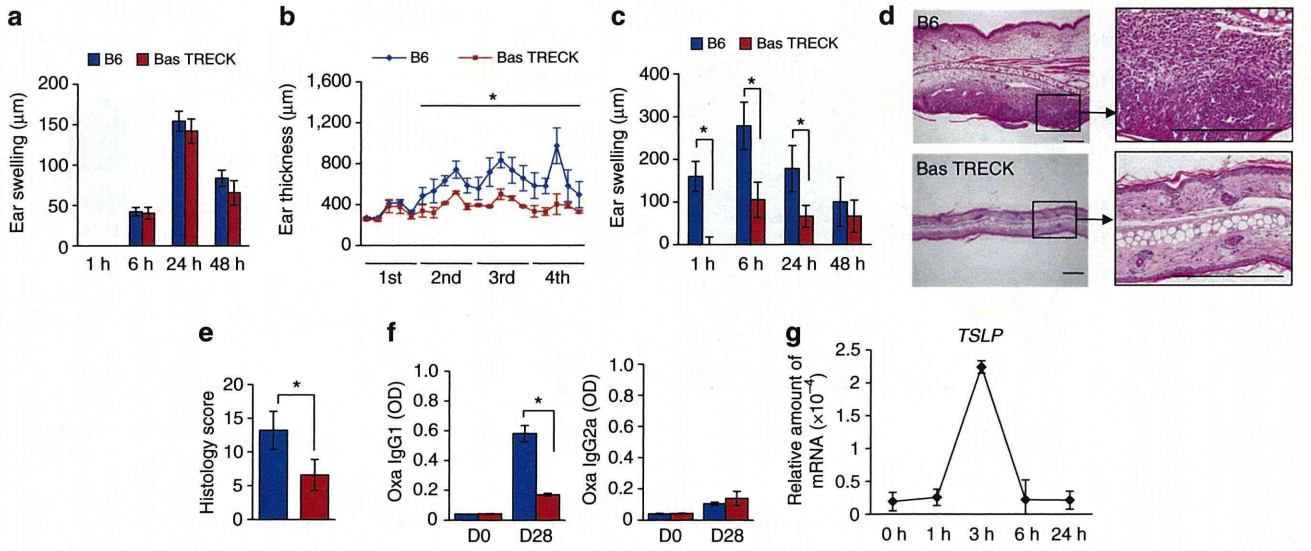

Figure 2 | Attenuation of ear inflammation by repeated hapten application in the DT-treated Bas TRECK mice. (**a**) Th1 immune response to single application of oxazolone of DT-treated B6 and DT-treated Bas TRECK (Bas -) mice. (**b**) Time course of repeated hapten application in these mice, (**c**) On fourth application, the ear swelling of these mice in the acute phase. (**d**) Histological examination (Scale bar, 100 μm), and scores (**e**). (**f**) Serum oxazolone-specific IgG1 and IgG2a levels in DT-treated B6 or DT-treated Bas TRECK (Bas -) mice on day 0 and 28. (**g**) The mRNA level of *TSLP* in the skin lesion after repeated hapten application. All data are presented as the mean ± s.d. ($n > 3$) and are representative of three experiments. *$P < 0.05$ (Wilcoxon signed-rank test) versus corresponding groups.

levels in basophil-depleted mice were the same as those in B6 mice (Fig. 2f, right panel). The mRNA level of *TSLP* in the skin lesion was increased 3 h after the last challenge in the repeated hapten application model (Fig. 2g). These results suggest that basophils are responsible for hapten-induced Th2 skin reaction and IgG1 induction.

Basophils express MHC class II and costimulatory molecules. Next, we examined whether basophils express MHC class II, costimulatory molecules and chemokine receptors in draining LNs with repeated cutaneous hapten application. As reported previously, basophils were not detected in draining LNs in the steady states; however, after repeated hapten application, a significant number of $CD49b^+$ $Fc\varepsilon RI^+$ IgE^+ $CD200R^+$ c-Kit^- basophils were detected (Fig. 3a). These basophils expressed MHC class II, CD40, CD80, CD86, CD62L, CXCR4, and CCR7, but not CCR4 (Fig. 3b), which suggest their potential to enter lymphoid tissues.

Basophils produce the Th2-inducing cytokine IL-4 and express TSLP after stimulation with papain *in vitro* and *in vivo*[10]. However, it remains unclear whether basophils express IL-4 in the repeated hapten application model. After repeated hapten application, a fraction of basophils produced IL-4 in draining LNs using an intracellular staining assay without any stimulation *in vitro* (Fig. 3c). As TSLP is known as a critical inducer and activator of basophils, the induction of basophils in the draining LNs (Fig. 3a) and their activation (Fig. 3b,c) may depend on the induction of TSLP in the draining LNs (Fig. 2h). In addition, basophils were found in the vicinity of T cells in the T-cell zone of the draining LNs after the hapten application model (Fig. 3d). Therefore, basophils accumulated in the draining LNs may be a source of IL-4 for the induction of Th2 response in the repeated hapten application model.

Basophils stimulate T cells with OVA peptide. Next, we examined whether basophils activate T cells *in vitro*. To address this issue, we used Fluo-8 for intracellular Ca^{2+} staining and monitored the influx of Ca^{2+} as an indicator of T-cell activation. Firstly, we confirmed that sorted basophils exhibited their characteristic multilobed nuclei by cytology (Fig. 4a). We incubated Fluo-8-labelled OVA-specific naive $CD4^+$ T cells from DO11.10 T-cell antigen receptor Tg mice and CellTracker Red-labelled BM-derived basophils (BMBs) with OVA protein or OVA peptide. When an intracellular Ca^{2+} level of T cells is increased, green fluorescence intensity is induced in this system. Mixing with OVA peptide induced a prominent Ca^{2+} increase in T cells as shown in green fluorescence (Fig. 4b,c and see Supplementary Movie 1). However, mixing with OVA protein did not increase intracellular Ca^{2+} concentrations in T cells (Fig. 4d). On the other hand, DCs induced a prominent Ca^{2+} increase in T cells irrespective of the presence of OVA protein or OVA peptide (Fig. 4e), while basophils can activate T cells only in the presence of OVA peptide (Fig. 4d). The capacity for inducing a calcium response in T cells in the presence of peptide is comparable between DCs and basophils (Fig. 4d,e).

Basophils are unable to process protein antigens. Our studies demonstrated that basophils have a role in Th2 induction with peptide antigens, but not with protein antigens. One possible reason for this is that basophils are unable to take up and process protein antigens. To explore this, we examined whether basophils take up and process OVA protein into OVA peptide. To address this issue, we used dye quenched (DQ) ovalbumin (DQ-OVA), a fluorogenic substrate for proteases, which becomes fluorescent upon hydrolysis of the DQ-OVA to single peptide by proteases. Using this system, we tested BMBs for their ability to take up and process OVA and found that $CD49b^+$ $Fc\varepsilon RI^+$ $CD11c^-$ BMBs could hardly take up and process OVA protein compared with bone marrow-derived dendritic cells (BMDCs) (Fig. 5a).

Next, we used *in vitro* systems for Th2 differentiation and T-cell stimulatory capacity with purified OVA-specific $CD4^+$ T cells from 4get × DO11.10 double Tg mice or from CFSE-labelled DO11.10 mice. Co-culture of BMBs and $CD4^+$ T cells together in the presence of antigens led to IL-4-enhanced green fluorescence

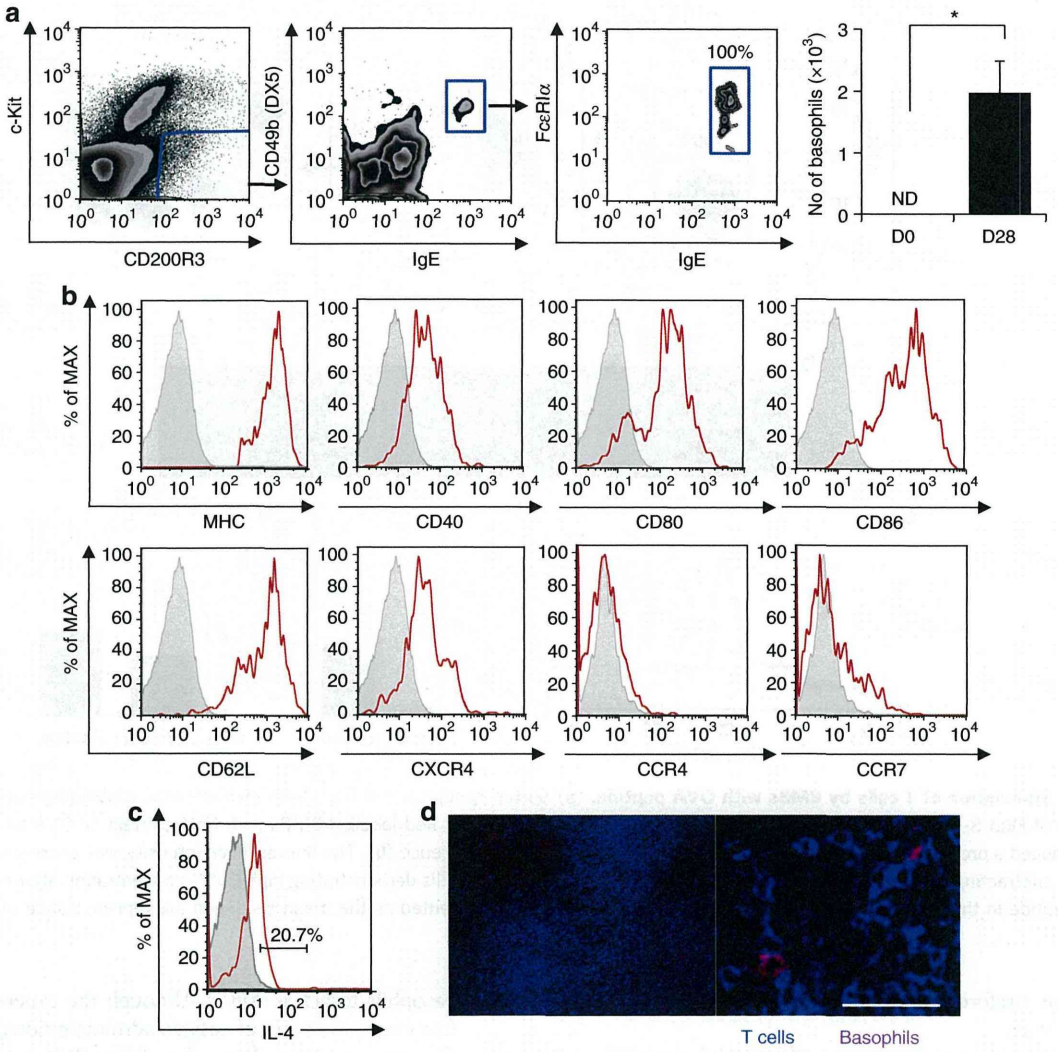

Figure 3 | Basophils expressed MHC class II and costimulatory molecules in the repeated hapten application model. (**a**) The number of CD49b$^+$ FcεRI$^+$ IgE$^+$ CD200R$^+$ c-Kit$^-$ basophils as gated (left panels), before (D0) and 28 days after repeated hapten application. (**b**) Expression of MHC class II, CD40, CD80, and CD86, CD62L, CCR4, CXCR4 and CCR7, on basophils (shaded histogram represents isotype-matched control antibody). (**c**) IL-4 production in basophils in draining LNs by means of an *in vivo* intracellular staining assay after repeated hapten application. (**d**) Basophils were found in the vicinity of T cells in the T-cell zone of the draining LNs after the hapten application model. All data are presented as the mean ± s.d. and are representative of three experiments. Scale bar, 50 μm.

protein (eGFP) expression in CD4$^+$ T cells as reported previously[8]. Although OVA peptide induced IL-4 induction in CD4$^+$ T cells in a dose-dependent manner, OVA protein did not (Fig. 5b,d). We also performed intracellular cytokine staining for IL-4 to measure IL-4 production at the protein level. Although OVA peptide induced IL-4 production in CD4$^+$ T cells, OVA protein did not (Supplementary Fig. S9A,B). Moreover, induction of Th2 responses by basophils was IL-4 dependent using neutralizing anti-IL-4 antibody (Supplementary Fig. S9C). T-cell stimulatory capacity was measured by a CFSE-diluted assay after incubation for 1 (Fig. 5c) or 3 days (Supplementary Fig. S10). T-cell proliferation was enhanced by basophils in the presence of OVA peptide but not in the presence of OVA protein (Fig. 5c,e, and Supplementary Fig. S10).

As basophils cannot take up or process protein antigens (Fig. 5a), other cells are required to prepare peptide to be presentable. One of the candidates is DCs as it has been reported that the Th2 response to cysteine proteases requires the cooperation of DCs and basophils via reactive oxygen species-mediated signalling[22]. Therefore, we examined whether DCs support basophil-dependent Th2 induction upon exposure to protein antigens. To assess this issue, we evaluated Th2 differentiation by culturing BMBs with OVA protein in the presence or absence of BMDCs. Basophils significantly increased the frequency of IL-4–eGFP-positive CD4$^+$ T cells upon OVA protein exposure in the presence of DCs (Fig. 5f). In addition, we demonstrated that the addition of a blocking antibody to MHC class II abrogated the basophil-induced Th2 responses, which rules out the possibility that the antigen has a direct effect on the T cells (Fig. 5g).

It has recently been shown that basophils are a heterogeneous population that can be driven by either IL-3 or TSLP[15]. The number of BMBs driven by IL-3 after a repeated hapten application model was high and that after OVA cutaneous patch was rather low when compared with control mice (Fig. 6b, left panel). Intriguingly, the numbers of BMBs driven by TSLP after both repeated hapten application and OVA cutaneous patch models were high when compared with control mice (Fig. 6b, right panel). These results suggest that delivery of haptens promotes both IL-3 and TSLP responses, while delivery

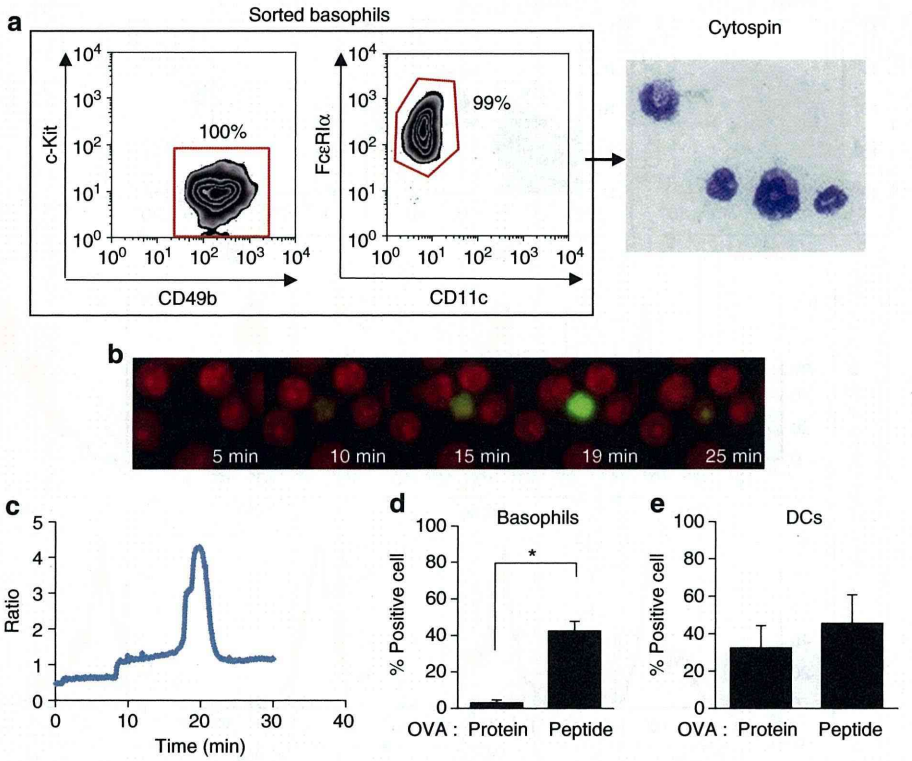

Figure 4 | Stimulation of T cells by BMBs with OVA peptide. (**a**) Sorted basophils exhibited their characteristic multilobed nuclei by cytology. (**b**,**c**) Incubation of Fluo-8-labelled naive CD4$^+$ T cells from DO11.10 and CellTracker Red-labelled BMBs with OVA protein or OVA peptide. Mixing with OVA peptide induced a prominent Ca^{2+} increase in T cells as shown in green fluorescence (**b**). The fluorescence intensity was expressed as a ratio to the initial value after subtracting background fluorescence (**c**). (**d**,**e**) The percentage of T cells demonstrating high Ca^{2+} concentration after mixing with OVA protein or OVA peptide in the presence of basophils (**d**) or DCs (**e**). All data are presented as the mean ± s.d. and are representative of three experiments.

of proteins preferentially promotes TSLP responses and impairs IL-3 response.

We next evaluated the nature of basophil populations using a repeated hapten application model in wild-type and TSLP receptor-deficient mice. The numbers of basophils in the skin and draining LNs in TSLP receptor-deficient mice was significantly decreased compared with those in wild-type mice (Fig. 6c,d). These results suggest that the nature of the basophil populations recruited to the skin and the draining LNs to be determined as TSLP-dependent at least in part in this model.

Discussion

In this study, we have demonstrated that basophils are responsible for Th2 skewing to haptens and peptide antigens but not protein antigens *in vivo*. We also found that basophils expressed MHC class II, CD40, CD80, CD86 and IL-4 in the hapten-induced Th2 model, but that basophils cannot take up or process OVA protein sufficiently using the DQ-OVA system. Basophils directly stimulated T cells via MHC class II/peptide complex and promoted IL-4 production in CD4$^+$ T cells upon exposure to peptide antigens but not to protein antigens *in vitro*. Intriguingly, basophils sufficiently promoted Th2 skewing upon OVA protein exposure in the presence of DCs. These results suggest that basophils alone are able to induce Th2 skewing with haptens and peptide antigens but require DCs for the induction of Th2 for protein antigens.

Despite recent reports that basophils act as APCs for helminth antigens or skin-injected papain[7,9,10], another group reported that basophils do not function as APCs for inhaled house dust mite allergens[11]. Therefore, there remained a discrepancy in the ability of basophils to act as APCs. Although the experimental models differ (for example, routes of antigen administrations), we assume that this discrepancy stems from the different type of antigens, such as protein, peptide and hapten. Hapten antigens may bind to MHC class II on the surface of basophils directly, and peptide can be acquired and presented by basophils, while protein antigens are not presented efficiently by basophils because they are hardly digested by these cells. In fact, previous reports demonstrated that basophils promote Th2 induction using OVA peptide but not OVA protein *in vitro*[7,9]. The protease allergen papain reaches the LNs after its cutaneous immunization and induces MHC class II expression on basophils in accord with preparing OVA peptide antigens from OVA protein *in vivo*[9]. Another group has reported that basophils pulsed with anti-2, 4-dinitrophenyl (DNP IgE) enhance Th2 skewing upon exposure to DNP-conjugated OVA by taking up DNP-OVA–IgE anti-DNP immune complexes[7]. Although house dust mites also contain cysteine protease activity, they are not sufficient for Th2 induction as they do not upregulate MHC class II on basophils in this model, even though cysteine proteases may function to generate peptide antigens from protein antigens *in vivo*[11].

Herein, we have shown that DCs support basophils to increase the frequency of IL-4–eGFP-positive CD4$^+$ T cells. As basophils cannot take up or process protein antigens efficiently, DCs may prepare peptide to be presented by basophils or may promote basophils to produce IL-4 to skew Th2. In line with this, our recent study has demonstrated that Langerhans cells, an epidermal DC subset, mediate epicutaneous sensitization with OVA protein antigens to induce Th2-type immune responses[23]. In addition, Th2 reactions are reduced upon sensitization with protein antigens or Schistosome infections using a CD11c-depletion model[11,12]; therefore, DCs seem to be necessary for Th2

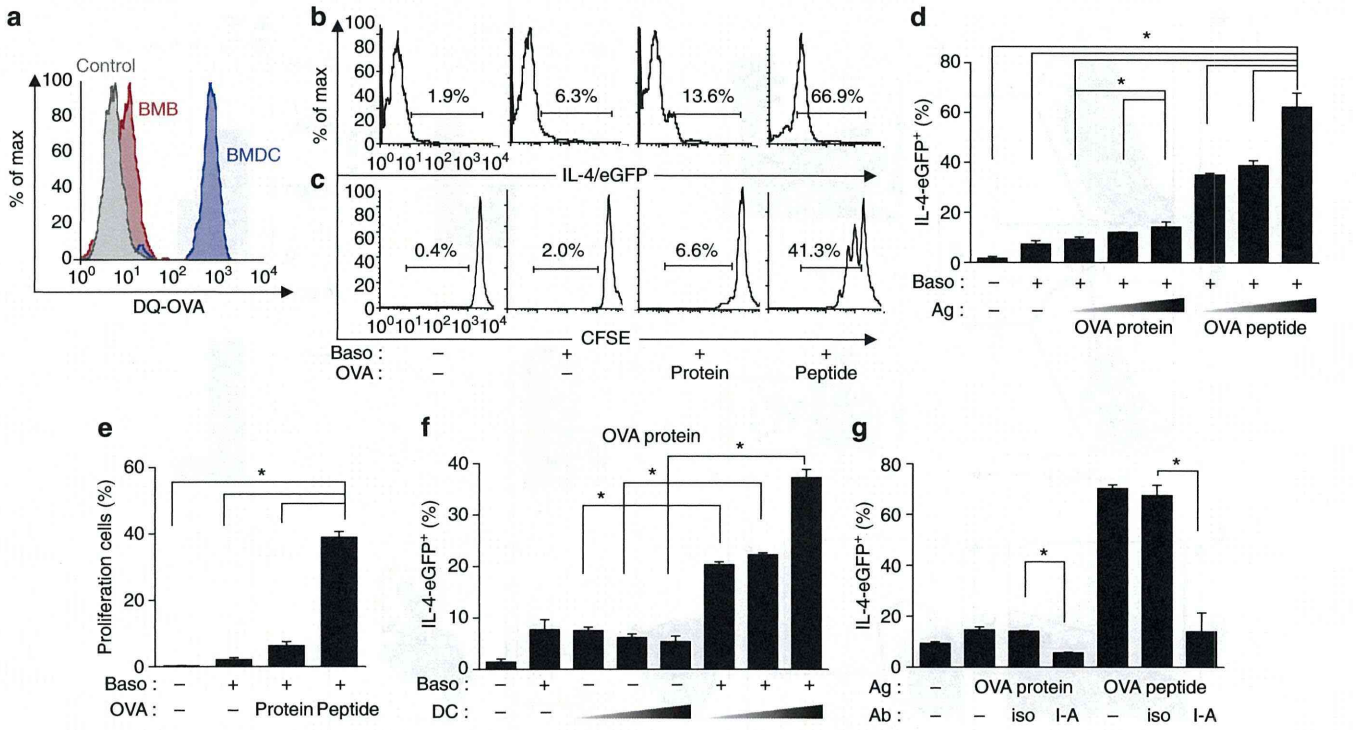

Figure 5 | Basophils were unable to take up and process protein antigens but induce Th2 differentiation with peptide antigens *in vitro*. (**a**) Using DQ-OVA that becomes fluorescent upon hydrolysis by phagocyte, we tested the ability to take up and process OVA by BMBs and DCs. (**b**–**d**) *In vitro* system of Th2 differentiation and T-cell stimulatory capacity. OVA-specific CD4$^+$ T cells from 4get × DO11.10 double transgenic mice (**b**,**d**) or from DO11.10 mice (**c**,**e**) were cocultured with basophils (Baso) in the presence of OVA protein or peptide. The frequency of IL-4/eGFP-positive cells, an indicator for Th2 differentiation, is shown (**b**,**d**). The populations of CFSE-diluted OVA-specific CD4$^+$ T cells after incubation for 1 day, as an indication for T-cell proliferation, were shown (**c**,**e**). (**f**) OVA-specific CD4$^+$ T cells were co-cultured with basophils (Baso) and/or DCs in the presence of OVA protein, and the frequency of IL-4-eGFP-positive CD4$^+$ T cells was shown. (**g**) The frequency of IL-4-eGFP$^+$ cells in the presence or absence of neutralizing anti-MHC class II antibody (iso: isotype-matched control, I-A: anti-MHC class II antibody). All data are presented as the mean ± s.d. and are representative of three experiments.

induction both *in vivo* and *in vitro* upon protein antigen exposure. As basophils were found in the vicinity of T cells in the T-cell zone of the draining LNs, basophils, T cells and DCs may act towards Th2 induction in a coordinated way. In future, it will be intriguing to further evaluate whether DCs present peptide to basophils directly or by trogocytosis.

Taken together, basophils seem to be important for Th2 induction when stimulated with OVA peptide or hapten alone. Consistently, basophils hardly take up and process protein antigen compared with DCs (Fig. 5a). In line with this, DCs seem to be sufficient for Th2 induction when stimulated with OVA protein or both OVA protein and OVA peptide *in vivo*. Intriguingly, however, our *in vitro* data suggest that basophils support induction of Th2 responses upon OVA protein exposure in the presence of DCs (Fig. 5f). Therefore, DCs can to be sufficient for Th2 induction in the context of complex inflammatory environments like post-*S. mansoni* infection, where presumably various different ratios of small soluble antigens and larger proteins are present.

A fraction of basophils have the capacity to produce IL-4 in draining LNs in our model (Fig. 3c). Previously, Sullivan *et al.*[24] demonstrated that basophils produce IL-4 only in the lung tissues but not in the draining LNs when they used an *Nippostrongylus brasiliensis* infection model. In this study, we used a repeated hapten application model in which mice were immunized epicutaneously. We previously demonstrated that epidermal keratinocytes express a Th2 inducer TSLP and that TSLP-TSLP signalling is essential for IgE induction[23]. In addition, the number of basophils in the draining LNs in TSLP receptor-deficient mice was decreased compared with that in wild-type mice (Fig. 6d). At present, we still do not know how IL-4 is produced by basophils in the draining LNs in our model, but we assume that the TSLP-dependent basophils might be involved in our context.

Our results suggest that delivery of haptens and proteins differentially regulate TSLP and IL-3 response of basophils (Fig. 6b), which would provide critical insight into the reasons why some infections (which may be IL-3 or TSLP inducing) may be more DC- or basophil- dependent. It has been reported that serum IgE levels are one of the major factors that influence basophil populations[25]. Antigen-specific IgE was not detected in OVA protein or peptide sensitization models. On the other hand, TSLP is known to be elevated in the skin lesions of atopic dermatitis, upon barrier disruption, or by protease-containing antigen-exposure[26–28]. These conditions may affect heterogeneity in the basophil lineage (TSLP/IL-3), which has a role in each of these systems. It will be interesting to investigate the amounts of IL-3 and TSLP *in situ* upon stimulation with a variety of foreign antigens, including hapten, protein and bacteria, in a context-dependent manner.

Methods

Mice. Mice expressing human diphtheria toxin receptor under the control of 3′-UTR element in the *Il4* locus were generated for Bas TRECK by a transgenic strategy as reported previously[14,16]. C57BL/6 (B6) and BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). We treated C57BL/6 (B6) mice with DT as control and Bas TRECK Tg mice with DT as basophil-depleted mice. DO11.10 transgenic mice and 4get mice were purchased from Jackson Laboratory (Bar Harbor, ME). TSLP receptor-deficient mice (BALB/c) were kindly provided by Dr Steven Ziegler (University of Washington).

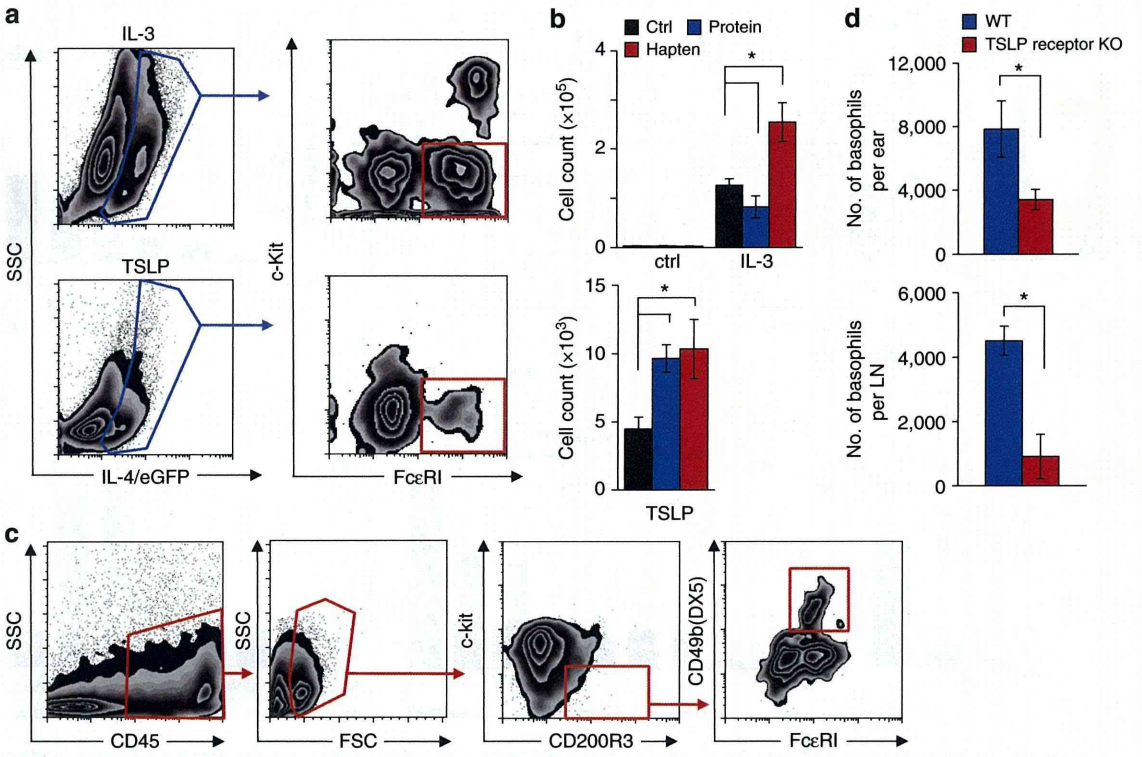

Figure 6 | Basophil development depends on the type of antigens. (**a**) BM cells were taken from 4get mice and cultured in the presence of IL-3 or TSLP. In IL-4/eGFP subsets, the FcεRI$^+$ c-Kit$^-$ basophil population is induced. (**b**) BM cells of mice with non-treatment, repeated hapten application (hapten) or OVA cutaneous patch (protein) were cultured in the absence (ctrl) or presence of IL-3 or TSLP, and basophil numbers were determined. (**c**) Basophils in the skin were detected as CD45$^+$ CD49b$^+$ FcεRI$^+$ CD200R$^+$ c-Kit$^-$ by flow cytometry. (**d**) The numbers of basophils in the skin and draining LNs of wild-type and TSLP receptor-deficient (KO) mice after repeated hapten application. All data are presented as the mean ± s.d. and are representative of two or three independent experiments.

For DT treatment, mice were injected intraperitoneally with 500 ng of DT per mouse on the first day. Then Bas-TRECK mice were treated at 3-day intervals[16]. Eight- to ten-week-old female mice were used for all of the experiments and were bred in specific pathogen-free facilities at Kyoto University. All experimental procedures were approved by the institutional animal care and use committee of the Kyoto University Graduate School of Medicine.

Reagents and antibodies. We purchased oxazolone from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), OVA from Sigma-Aldrich (St Louis, MO), OVA peptide from MBL (Nagoya, Japan), and DQ-OVA from Invitrogen (Carlsbad, CA). FITC-, PE-, PE-Cy7-, APC-, APC-7- and Pacific Blue-conjugated 145-2C11 (anti-CD3), N418 (anti-CD11c), 1C10 (anti-CD40), DX5 (anti-CD49b), 16-10A1 (anti-CD80), GL1 (anti-CD86), 2B8 (anti-CD117), M5/114.15.2 (anti-MHC class II), MEL-14 (anti-CD62L), eBioL31 (anti-CD207), 2B11 (anti-CXCR4), 4B12 (anti-CCR7), IgE (23G3), blocking antibody to IL-4 (11B11) (eBioscience, San Diego, CA), Ba103 (anti-CD200R3) (Hycult Biotech, Plymouth Meeting, PA), IL-4 (BVD4-1D11) (BD, Franklin Lakes, NJ), TUG8 (anti-MCP8) and 2G12 (anti-CCR4) (BioLegend, San Diego, CA) were purchased. Intracellular staining was performed with Cytofix/Cytoperm reagents (BD) according to the manufacturer's instructions and analysed with FACS Fortessa (BD). For the pretreatment of IL-4 staining in CD4$^+$ T cells, splenocytes were collected and incubated with PMA (20 ng ml^{-1}) and ionomycin (1 μM) for four hours. For the *in vivo* intracellular staining assay[29], mice were pretreated with intravenous injection with 500 μl of PBS solution containing 100 μg monensin (Sigma-Aldrich) for 6 h.

Epicutaneous sensitization and clinical severity. Epicutaneous sensitization with protein antigens, the clinical severity scoring of skin lesions, and the histological scoring were performed as reported previously[23,30]. In brief, mice were anesthetized with diethylether (Nacalai Tesque, Kyoto, Japan) and shaved with an electric shaver (THRIVE Co. Ltd., Osaka, Japan). A single skin site on each mouse was tape-stripped five times with adhesive cellophane tape (Nichiban, Tokyo, Japan). One-hundred milligram of OVA in 100 μl of normal saline was placed on the patch-test tape (Torii Pharmaceutical Co., Ltd., Tokyo, Japan). Each mouse had a total of three 2-day exposures to the patch, separated by 1-day intervals. The total clinical score for skin lesions was designated as the sum of individual scores (graded none as 0, mild as 1, moderate as 2 and severe as 3) for the symptoms of pruritus, erythema, oedema, erosion and scaling. Immunohistochemical analysis was performed as reported previously[14].

Th2 induction model and ELISA. B6 and basophil-depleted mice were sensitized with intraperitoneal injections of alum (20 mg ml^{-1}) with OVA protein (100 μg ml^{-1}) or OVA peptide (10 μg ml^{-1}) on day 0, day 14, day 21 and day 28. Total serum IgE/IgG1/IgG2a levels were measured using a Bio-Rad (Hercules, CA) Luminex kit according to the manufacturer's instructions. To measure OVA/oxazolone-specific IgE/IgG1/IgG2a levels, the appropriate mouse IgE/IgG1/IgG2a ELISA kit (Bethyl Laboratories, Montgomery, TX) was used with slight modifications. Absorbance was measured at 450 nm. The difference between the sample absorbance and the mean of negative control absorbance was taken as the result.

Repeated oxazolone application. Repeated oxazolone treatment was performed essentially as described[31]. Mice treated with vehicle without oxazolone were used as control. Ear thickness was measured using a micrometre before, 1, 6, 24 and 48 h after challenge to assess inflammation. Ears were then collected for histology.

Quantitative PCR analysis. Quantitative PCR analysis was performed as reported previously[30]. The primer sequences for *TSLP*: 5′-ACGGATGGG GCTAACTTA CAA-3′ (forward) and 5′-AGTCCTCGATTTGCTCGAACT-3′ (reverse).

Generation of BMDC and BMB. Complete RPMI (cRPMI), RPMI 1640 medium (Sigma, St Louis, MO) containing 10% fetal calf serum (FCS) (Invitrogen), was used as the culture medium. For BMDC induction, 5 × 10^6 BM cells from BALB/c mice were cultured supplemented with 10 ng ml^{-1} recombinant murine GM-CSF (PeproTech, Rocky Hill, NJ) for 5 days (>90% expressed CD11c).

For BMB induction by IL-3, 5 × 10^6 BM cells from BALB/c mice were cultured supplemented with 10 ng ml^{-1} recombinant murine IL-3 (PeproTech) containing 20% FCS for 10 days. In some experiments, for BMB induction by TSLP or IL-3, BM cells from 4get mice were cultured in the presence of 1 μg ml^{-1} of TSLP for 5 days as reported previously[15].

Cytoplasmic Ca^{2+} imaging. T cells were incubated with 5 μM Quest Fluo-8 AM (ABD Bioquest, CA), and BMBs and BMDCs were stained with 2 μM CellTracker

Red (Invitrogen). The Fluo-8 image and the transmission image were recorded every 10 s using a back-thinned electron multiplier charge-coupled device (CCD) camera (ImagEM, Hamamatsu Photonics, Japan) and a microscope (Eclipse Ti, Nikon, Japan). The fluorescence intensity was expressed as a ratio to the initial value after subtracting background fluorescence.

Processing of OVA and Th2 differentiation. For measuring internalization and processing of OVA, 1×10^6 cells of BMDCs or BMBs were incubated with DQ-OVA (5 μg ml^{-1}, Invitrogen) for 1 h and analysed with FACS Fortessa. For Th2 differentiation *in vitro*, naive CD4$^+$ T cells were sorted by microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and were plated at a density of 5×10^5 cells per ml in the presence of various ratios of OVA protein (2, 10, 50 μg ml^{-1}), OVA peptide (0.4, 2, 10 μg ml^{-1}) and/or BMDCs (1×10^4, 2×10^4, 1×10^5 cells) in RPMI medium with 10% FCS for 3 days, standard supplements and IL-3 (30 ng ml^{-1}) for basophil survival. BMBs were sorted with anti-PE-ckit and anti-PE-CD11c by anti-PE microbeads and plated at a ratio of 1:5 with CD4$^+$ T cells.

Statistical analysis. Unless otherwise indicated, data are presented as the means ± s.d. and are representative of three independent experiments. *P*-values were calculated with the Wilcoxon signed-rank test. *P*-values < 0.05 are considered to be significantly different between basophil-depleted mice and corresponding B6 mice and are marked by an asterisk in the figures.

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Author contributions

A.O. performed the research and wrote the paper; M.K. Analysed and discussed the data; S.N, B.K., G.E., T.H., A.K., T.N., S.H., C,M. and S.T. performed the experiments and analysed data; T.W and Y.M. provided helpful discussions; K. K. directed experiments, analysed data and wrote the paper.

Additional information

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High frequencies of positive nickel/cobalt patch tests and high sweat nickel concentration in patients with intrinsic atopic dermatitis

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ABSTRACT

Background: Atopic dermatitis (AD) is classified into extrinsic AD with high serum IgE levels and impaired barrier, and intrinsic AD with low serum IgE levels and unimpaired barrier. Intrinsic AD has a lower frequency of *FLG* mutations and a higher frequency of circulating Th1 cells, implying that non-protein antigens, represented by metals, may be an exacerbation factor in intrinsic AD.
Objective: To investigate metal allergy in intrinsic AD.
Methods: Enrolled in this study were 86 Japanese AD patients seen in three university hospitals, consisting of 55 extrinsic and 31 intrinsic AD patients. Patch testing was performed, focusing on nickel, cobalt, and chrome, in parallel with other 14 metals. *FLG* mutations were analyzed in 49 patients (extrinsic, 29; intrinsic, 20). In 17 patients (extrinsic, 12; intrinsic, 5), sweat was collected from the forearms by exercise, and the concentration of nickel was fluorometrically measured.
Results: Nickel, cobalt, and chrome were the major positive metals. Intrinsic AD showed significantly higher percentages of positive reactions than extrinsic AD to nickel (intrinsic 41.9% *vs* extrinsic 16.4%, $P = 0.019$) and cobalt (38.7% *vs* 10.9%, $P = 0.005$). There was no significant difference between *FLG* mutation-bearing and non-bearing patients. The concentration of nickel was higher in the sweat of intrinsic AD than extrinsic AD patients (333.8 *vs* 89.4 ng/g, $P = 0.0005$) and inversely correlated with serum IgE levels.
Conclusions: Nickel and cobalt allergy may be involved in intrinsic AD. Given that the metals are excreted through sweat, intrinsic AD might be exaggerated by highly metal-containing sweat.

1. Introduction

Atopic dermatitis (AD) can be divided into the extrinsic and intrinsic types [1,2]. While extrinsic AD shows high serum IgE levels with specific IgE to some allergens, intrinsic AD has low serum IgE levels without specific IgE. Since total serum IgE value correlates with the allergen-specific IgE status [3], the total IgE can be regarded as a clinically useful parameter to differentiate between the extrinsic and intrinsic types in both adults [4,5] and children [3]. The frequency of intrinsic AD is 10–45% of whole AD patients [6], and it has more recently been reported to be 27% [7], 37% [3], 12% [8], and 22% [5]. The female preponderance is a feature of intrinsic AD [1,4,5,9,10].

Extrinsic AD is characterized by impaired barrier function [11]. Loss-of-function mutations in filaggrin gene (*FLG*) [12] represent a strong predisposing factor for AD, asthma and other allergies [13]. Perturbation of skin barrier function as a result of reduction or complete loss of FLG expression leads to enhanced percutaneous transfer of protein antigens [11,14]. Therefore, *FLG* mutations are closely associated with extrinsic AD [13,15] as well as ichthyosis vulgaris and palmar hyperlinearity. A recent finding also suggests that FLG deficiency results from perturbation of enzymatic processing from profilaggrin to filaggrin monomer [16]. On the other hand, intrinsic AD patients have normal barrier function [4,15] and virtually lack *FLG* mutations and palmar hyperlinearity [9,15].

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Recently, we have shown that the percentage of interferon (IFN)-γ-producing Th1 cells is significantly higher in the peripheral blood of intrinsic AD than extrinsic AD [15]. Protein antigen is known to induce Th2 responses [11,17], and therefore, the impaired barrier of extrinsic AD may allow protein allergens to penetrate the barrier and to evoke Th2 responses. In this scenario, Th1-inducing non-protein antigens, such as metals, might be causative for intrinsic AD [18]. In fact, high frequencies of positive patch test reactions to metals have been reported in AD patients [19–21]. Nickel (Ni), cobalt (Co) and chrome (Cr) are the three major metals, and a high frequency of positive patch test to at least one of them is higher in AD patients than in non-AD patients [21]. However, the intrinsic and extrinsic types were not separately analyzed in those studies. Metals are administered orally as food and may be excreted from sweat at high concentrations as well as urine [22]. This notion raises the possibility that the concentrations of metals are high in the sweat of intrinsic AD patients.

In this study, we performed a multicenter study of patch tests to metals, focusing on Ni, Co and Cr, in extrinsic and intrinsic AD patients. To better assess the patients' background, *FLG* mutations were investigated with other items. We also measured the concentration of Ni in the two groups of AD patients.

2. Materials and methods

2.1. Participants and background assessments

AD was diagnosed according to the criteria of Hanifin and Rajka [23] and divided into the intrinsic and extrinsic types. There are no uniform criteria to distinguish intrinsic AD from extrinsic AD, as IgE values less than 150, 200 or 220 kU/L have been used for an indication of intrinsic AD in the previous studies [2,4,6]. Since Japanese AD patients have high levels of IgE specific to house dust mites [17,24], IgE levels to *Dermatophagoides (D) pteronyssinus* (DP) and *D. farinae* (DF) were used for categorization of extrinsic and intrinsic AD as well as total IgE levels. Serum IgE levels specific to these mites were graded into 7 classes (class 0 to 6; Special Reference Laboratories, Tokyo, Japan). In this study, intrinsic AD was defined as serum IgE levels ≤200 kU/L or 200 < IgE ≤ 400 plus class 0 or 1 of IgE specific to DP or DF, and extrinsic AD was defined as 400 < IgE levels or 200 < IgE ≤ 400 plus class 2 or more of the specific IgE.

Enrolled in this study were 31 patients with intrinsic AD (aged 9–59 years; 10 men and 21 women) and 55 patients with extrinsic AD (aged 6–78 years; 22 men and 33 women) (Table 1). We first selected 31 intrinsic AD patients who visited the hospitals in 2009–2012, and as the control counterpart, 55 extrinsic AD patients were then randomly selected. The ages of the two groups were statistically comparable, but the female dominancy was observed in intrinsic AD as well known [1,2,15]. The patients were seen as outpatients or inpatients in Departments of Dermatology, Hamamatsu University School of Medicine, University of Occupational and Environmental and Health, and Kobe University.

The severity of AD was assessed by Severity Scoring of AD index (SCORAD) [25] and a 100 mm visual analog scale (VAS) was used for their itch. Serum CCL17/thymus and activation-regulated chemokine (TARC), strongly associated with severity of AD [26,27], was measured by ELISA (Special Reference Laboratories) in 26 extrinsic AD patients and 11 intrinsic AD patients.

2.2. Intracellular cytokine staining and flow cytometric analysis of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll-Paque method. Intracellular cytokines were stained according to the protocol of Cytostain (Immunotech, Marseille, France) [15]. Briefly, cells (2×10^6 cells/ml) were stained with PerCP-conjugated anti-CD8 monoclonal antibody (mAb), APC-conjugated anti-CD3 mAb (BD Biosciences), phycoerythrin-labeled anti-IL-4 or IL-5, and FITC-labeled anti-IFN-γ mAb (all from BD Biosciences). Fluorescence profiles were analyzed by flow cytometry in FACSCanto (BD Biosciences). The percentage of $CD3^+CD8^-$ cells bearing each cytokine was counted.

2.3. FLG mutation

Mutation in *FLG* has been identified as one of the most important factors of AD [12,26,28]. Blood was collected from 20 intrinsic and 29 extrinsic AD patients for detection of *FLG* mutation by polymerase chain reaction (PCR). Former reported mutations specific to Japanese were eight types (R501X, 3321delA, S1695X, Q1701X, S2554X, S2889X, S3296X and K4022X) [29,30]. Genotyping for the 8 types of *FLG* was performed using PCR and restriction enzyme digest analysis [31]. The analysis was approved by the ethical committee of Hamamatsu University School of Medicine. Patients gave written informed consent for the participation in this study, which followed the principles of the Declaration of Helsinki.

2.4. Patch testing with metals

A multicenter study was performed in the Departments of Dermatology of the three university hospitals. Patch testing with

Table 1
Patients' background in this study.

	Intrinsic AD	Extrinsic AD	
Patients	31 (10 men and 21 women)	55 (22 men and 33 women)	
Age (years, mean ± SD)	30.9 ± 11.5	28.9 ± 13.5	$P=0.501$
Sex (male:female, %)	32.3%:67.7%	40.0%:60.0%	$P=0.498$
IgE ($kU\,L^{-1}$, mean ± SD)	168.7 ± 124.3 ($n=31$)	8829.5 ± 12508.0 ($n=55$)	$P<0.001$
LDH ($IU\,L^{-1}$, mean ± SD)	243 0 ± 143.5 ($n=22$)	266.3 ± 118.7 ($n=43$)	$P=0.489$
Eosinophil (%, mean ± SD)	7.0 ± 6.7 ($n=25$)	9.57 ± 6.3 ($n=42$)	$P=0.115$
TARC ($pg\,mL^{-1}$, mean ± SD)	783.1 ± 826.1 ($n=11$)	2223.1 ± 3596.1 ($n=26$)	$P=0.200$
Circulating Thl/Th2 cells (%) (%, mean ± SD)			
IFN-γ	12.94 ± 4.62 ($n=19$)	8.66 ± 6.51 ($n=31$)	$P=0.016$
IL-4	0.35 ± 0.15 ($n=19$)	0.35 ± 0.23 ($n=33$)	$P=0.905$
IL-5	0.47 ± 0.23 ($n=18$)	0.58 ± 0.26 ($n=33$)	$P=0.145$
VAS of pruritus (mean ± SD)	56.1 ± 31.2 ($n=20$)	55.4 ± 22.7 ($n=33$)	$P=0.931$
SCORAD (mean ± SD)	36.0 ± 15.2 ($n=20$)	40.2 ± 17.3 ($n=34$)	$P=0.369$
FLG mutation	5.0% (1/20:M0/6,F 1/14)	31.0% (9/29: M 6/11, F 3/18)	$P=0.065^*$
Sweat sample collection	5 (3 men and 2 women)	12 (6 men and 6 women)	
Piercing history in female patients	40.0% (6/15)[a]	13.0% (3/23)[a]	$P=0.115^*$

* Fischer's exact test.
[a] In the piercing history-positive patients, 3 of 6 in intrinsic AD and none of 3 in extrinsic AD experienced contact dermatitis due to piercing metals.

metals was done in all 86 patients (31 intrinsic and 55 extrinsic AD patients) with three different patch test materials (PTMs), focusing on Ni, Co, and Cr as the major metals tested. Three different PTMs were used in each of the three hospitals. PTM-1, used in University of Occupational and Environmental Health, was obtained from Torii Pharmaceutical Co. Ltd., Tokyo, Japan, as Japanese Standard Metal Allergen Series, and other fourteen metals were also tested with PTM-1, including aluminum chloride, stannous chloride, ferric chloride, platinous chloride, palladium chloride, manganese chloride, indium trichloride, iridium tetrachloride, silver bromide, potassium dichromate, zinc chloride, gold trichloride, cupric sulfate, and mercuric chloride. PTM-2, used in Hamamatsu University School of Medicine, was purchased from Sato Pharmaceutical Co. Ltd., Tokyo, Japan. PTM-3, used in Kobe University, was provided by The Japanese Society for Dermatoallergology and Contact Dermatitis. The concentrations of Ni, Co and Cr were different among the three PTMs. PTM-1 is of liquid type, containing 5% Ni, 2% Co, and 0.5% Cr. PTM-2 is of plaster type, containing 34.8% Ni, 52.3% Co, and 31.2% Cr. PTM-3 is of petrolatum type, containing 2.5% Ni, 1.0% Co, and 0.5% Cr. The patch tests were applied to the upper back and occluded for 48 h. Reactions were assessed on day 2 and day 7 in most cases, with ±1 day variations in some cases, according to the International Contact Dermatitis Research Group and scored as 1+, 2+, and 3+. A 1+, 2+ or 3+ reading was interpreted as a positive response.

2.5. Quantitative analysis of Ni in sweat

Sweat samples of 12 extrinsic AD patients and 5 intrinsic AD patients were collected. The forearms of the patients were washed clean, and the patients ran on the treadmill with his forearms wrapped with plastic bag for 10–15 min. The collected sweat was filtered with 0.45 μm diameter membrane (Millex, Merck Millipore, Billerica, MA). The concentrations of Ni were measured fluorometrically with Inductively Coupled Plasma Atomic Emission Spectroscopy (Mitsubishi Chemical Analytec Co. Ltd., Tokyo, Japan).

2.6. Statistical analyses

Student's t test and Fisher's exact test were applied to determine calculating differences. P-value <0.05 was defined statistically significance.

3. Results

3.1. Patients' demographics

Patients' background is summarized in Table 1. The ages of the intrinsic and extrinsic groups were statistically comparable. The female preponderance was observed in intrinsic AD as reported previously [1,2,4,9,10,15], but the extrinsic AD patients in this study also consisted of a higher number of women. The extrinsic AD group tended to have high values of serum LDH, eosinophils counts, VAS of pruritus, SCORAD, and serum TARC, compared with the intrinsic AD group, without statistical significance. Both groups showed high frequencies of IL-4$^+$ and IL-5$^+$ T cells in the peripheral blood, but IFN-γ$^+$ T cells were higher in intrinsic AD than in extrinsic AD [15]. The higher levels of SCORAD and VAS in extrinsic than intrinsic AD have also been observed [4,8,32]. As to *FLG* mutation, 3.7% of normal Japanese individuals have *FLG* mutation [30], and extrinsic AD patients have higher frequency of FLG mutations than intrinsic AD patients [15]. In our patients, 9 of 29 extrinsic AD patients had one of the eight mutations common to Japanese AD patients, whereas only one of 20 intrinsic AD patients had a mutation of *FLG*. Piercing might bypass the epidermal barrier and induce metal allergy, in particular Ni allergy [33]. Totally 70 patients were surveyed in their histories of piercing. Since only one male patient had a history of piercing, data from the female patients are shown in Table 1. Six of 15 female intrinsic AD patients had piercing histories, while 3 of 23 female extrinsic AD patients had piercing. Three of 6 female intrinsic patients experienced contact dermatitis to piercing metals, while none of 3 female extrinsic patients did so.

3.2. High frequencies of positive patch test reactions to Ni and Co in intrinsic AD than extrinsic AD patients

Patch testing to metals was performed with the three different materials, PTM-1 (liquid type), PTM-2 (plaster type), and PTM-3 (petrolatum type). First, we examined the positive reactions to 17 metals by using PTM-1 in 14 intrinsic and 21 extrinsic AD patients. Among 17 metals, high percentages of positive reactions were found with Ni (intrinsic 4/14, 28.6%; extrinsic, 6/21, 28.6%), Co (intrinsic 8/14, 57.1%; extrinsic, 2/21, 9.5%), and Cr (intrinsic 4/14, 28.6%; extrinsic, 4/21, 19.1%). However, there were low positive frequencies in manganese (intrinsic 2/14, 14.3%; extrinsic, 1/21, 4.8%), palladium (intrinsic 1/14, 7.1%; extrinsic, 1/21, 4.8%), zinc (intrinsic 1/14, 7.1%: extrinsic, 2/21, 9.5%), aluminum (0%), stannous (0%), ferric (0%), platinous (0%), indium (0%), iridium (0%), silver (0%), potassium (0%), gold (0%), cupric (0%), and mercuric (0%). Thus, Ni, Co, and Cr were the major metals that evoke positive patch test reactions.

We therefore focused on Ni, Co, and Cr as the metals to be tested by using the three different PTMs, which provided virtually similar results. However, there were considerable variations among the PTMs, presumably because of differences in metal concentrations and vehicle materials (Table 2). When analyzed collectively with the three PTMs, Ni, Co, and Cr exhibited positive reactions at 25.6%, 20.9%, and 16.3%, respectively, in the 86 AD patients.

We then analyzed the positive frequencies in the intrinsic and extrinsic types of AD. Intrinsic AD showed significantly higher percentages of positive reactions than extrinsic AD to Ni and Co (Table 3). The positivity to Co was also higher in intrinsic than extrinsic AD, but statistically not significant. The prevalence of metal allergy to one or more of the three metals was more than twice higher in intrinsic AD (61.3%) than extrinsic AD (25.5%).

We found the relationship between the stratification of IgE levels and the prevalence of positive metal patch tests (Fig. 1). In the IgE ≤ 100 group, the incidence of positive reactions to one or more of Ni, Co, and Cr was 63.6%, while the 400 < IgE group exhibited 25.0% positivity ($P = 0.029$). The group of 100 < serum IgE ≤ 400 (IU/L) consisted of the two types of AD (20 intrinsic and 3 extrinsic AD patients), and their positive frequencies of Ni, Co and Cr were 40, 35 and 20% in the intrinsic AD patients, and 33.3, 0 and 0% in the extrinsic AD patients. The positive frequencies of one or more metals were 65 and 33.3%, respectively.

Table 2
Frequencies of positive patch tests to metals using three different PTMs.

	Nickel	Cobalt	Chrome
PTM-1	10/35 (28.6%)	10/35 (28.6%)	8/35 (22.8%)
PTM-2	6/35 (17.7%)	4/35 (11.4%)	3/35 (8.6%)
PTM-3	6/16 (37.5%)	4/16 (25.0%)	3/16 (18.8%)
Total	22/86 (25.6%)	18/86 (20.9%)	14/86 (16.3%)

PTM: Patch test material.

Table 3
Frequencies of positive patch tests to metals in intrinsic and extrinsic AD patients.

	Nickel	Cobalt	Chrome	At least one metal
Intrinsic	13/31 (41.9%)	12/31 (38.7%)	7/31 (22.6%)	19/31 (61.3%)
Extrinsic	9/55 (16.4%)	6/55 (10.9%)	7/55 (12.7%)	14/55 (25.5%)
Fisher's exact test	$P=0.019$	$P=0.005$	$P=0.243$	$P=0.001$

When we divided 54 patients into two groups, the Ni, Co, Cr, or one or more positive group, and the negative group, there were no significant differences in SCORAD or VAS of pruritus. Thus, the severity of eruptions did not affect metal patch test positivity.

It was reported that FLG deficiency may represent a risk factor for contact sensitization to allergens, such as Ni [14]. Therefore, the frequencies of positive metal patch test were analyzed in relation to *FLG* mutation. In the total patients, there was no significant difference in the patch testing results of three metals between the *FLG* mutation-bearing and non-bearing groups (Table 4). Even when analysis was limited to extrinsic AD, no difference was found between *FLG* mutation-positive and negative groups.

3.3. High Ni concentration in sweat from intrinsic AD patients

In 17 AD patients (extrinsic, 12; intrinsic, 5), sweat was collected from the forearms by exercise, and the amount of Ni and Co in the sweat was measured. The concentration of Ni was significantly higher in the sweat of intrinsic AD than extrinsic AD patients (Fig. 2a). When the sweat Ni concentration was analyzed as the function of serum IgE values, there was an inverse correlation between them (Fig. 2b). In Fig. 2, patients with positive and negative Ni patch test results are shown as closed and open rectangles. Notably, one patient with negative Ni patch test in the intrinsic AD group had a low Ni concentration in the sweat.

4. Discussion

The intrinsic type of AD, characterized by unimpaired skin barrier, is enigmatic in its causes and pathophysiology. Immunologically, both extrinsic and intrinsic AD patients may exhibit increased production of IL-5 and IL-13 in the blood or skin, but when peripheral lymphocytes are stimulated with anti-CD3 antibody, extrinsic AD patients have a decreased capacity to produce IFN-γ [34]. Accordingly, we have shown that the circulating IFN-γ^+ Th1 cell frequency is higher in intrinsic than extrinsic AD. There are tendencies that Th2 and Th17 cell frequencies are higher in extrinsic and intrinsic AD patients, respectively [34]. Thus, all the data collectively suggest that intrinsic AD has a relatively Th1-preponderant state as compared with extrinsic AD.

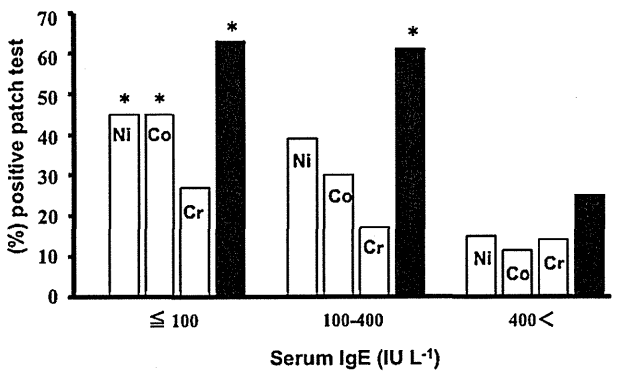

Fig. 1. Relationship between IgE level subgroups and frequencies of positive metal patch tests. The closed bars represent the positive patch test to one or more of the three metals. *$P < 0.05$, compared with the corresponding the 400< groups.

The normal barrier function and Th1-activating potency of intrinsic AD [15,35] raise the possibility that the patients are not sensitized with protein allergens capable of inducing Th2 responses, but with other antigens. It is assumed that protein antigens are not allowed to penetrate the skin of intrinsic AD with normal barrier. Instead, non-protein antigens, such as metals [19], can enter through the barrier and may induce Th1 responses. In 137 atopic children, 19.3% patients were positive to metals [20], although the two types of AD were not separately studied. In 1965, Shanon reported a patient with metal allergy exhibiting a skin manifestation indistinguishable from AD under the name of "pseudo-atopic dermatitis" [19,36].

In this study, we used three different PTMs, which gave similar but considerably variable positive patch test results. Nevertheless, we found that intrinsic AD showed positive patch tests to Ni and Co at higher percentages than extrinsic AD. Metals such as Ni, Co and Cr are known to cross-react with each other [37,38]. Cross reactivity may occur in our patch tests depending on the concentration of metals and the moieties of vehicles. The frequency of positive patch tests to at least one of the three metals was more than twice higher in intrinsic than extrinsic AD, suggesting that metal allergy is one of the potential causes of intrinsic AD. Interestingly, Co allergy is more prevalent in females than males [15], which is in accordance with the female preponderance of intrinsic AD.

Haptens and metals are representatives of non-protein, small antigenic molecules. The mechanisms underlying Th1-polarizing action of metals remain unclear. Recently, Ni has been shown to activate Toll-like receptor 4 (TLR4) signaling in antigen presenting cells (APCs) such as dendric cells [39]. The same TLR4 simulation occurs with Co, and the necessity of histidines H456 and H458 of human TLR4 is evident for activation of APCs by Co [40]. Thus, metals can interact with not only major histocompatibility/self peptide complex [41] but also with TLR4. TLR4 stimulation induces NF-κB activation and conversion of proIL-1β to IL-1β [39,40], which has no skewing ability to Th1 or Th2 cells. Accordingly, Ni, Co, and Cr show a mixed Th1- and Th2-type cytokine response in peripheral T cells from sensitized patients [42], which is different from Th2-stimulatory protein antigens [11].

The high incidence of contact sensitization to metals in AD patients can be explained by three mechanisms [20]. First, barrier dysfunction of *stratum corneum*, as represented by *FLG* mutation, allows allergens to penetrate below the stratum corneum [43]. FLG is histidine-rich polypeptide, which chelates Ni in the epidermis [44]. However, we did not find a difference in the positive metal patch test between *FLG* mutation positive and negative patients, as reported in another study [45]. Rather, the positive incidence tended to be higher in *FLG* mutation negative patients. Therefore, metal allergy is unlikely associated with the barrier condition. Second, individuals highly ingesting or exposed to metals in daily life possibly develop AD *via* metal allergy. It has been shown that environmental Ni exposure is more important than genetic disposition, such as *FLG* mutation, in the development of Ni allergy [46]. Finally, APCs expressing TLR4 in AD skin might be different from normal APCs and prone to TLR4-mediated stimulation.

Metals are administered with foods and applied to the skin with jewelry [22,47]. Personal habits may increase the risk of development of contact dermatitis to metals. For example, women show a higher sensitization rate to Ni than men perhaps by wearing Ni-containing jewelry [48], which might result in the female preponderance of Ni allergy in intrinsic AD. It was reported

Table 4
Frequencies of positive patch tests to metals in patients with or without *FLG* mutation.

	Total AD patients				Extrinsic AD patients			
	Nickel	Cobalt	Chrome	At least one metal	Nickel	Cobalt	Chrome	At least one metal
FLG mutation positive patients	1/10 (10.0%)	1 10 (10.0%)	1/10 (10.0%)	2/10 (20.0%)	1/9 (11.1%)	0/9 (0.0%)	0/9 (0.0%)	1/9 (11.1%)
FLG mutation negative patients	9/39 (23.1%)	9/39 (23.1%)	6/39 (23.1%)	15/39 (38.5%)	2/20 (10.0%)	2/20 (10.0%)	2/20 (10.0%)	3/20 (15.0%)
Fisher's exact test	$P=0.663$	$P=0.663$	$P=1$	$P=0.459$	$P=1$	$P=1$	$P=1$	$P=1$

that only piercing women in AD group had a higher incidence of sensitization to Ni but AD patient without piercing had no increased risk of Ni allergy [33]. In our study, 40% of female intrinsic and 13% of female extrinsic AD patients had piercing histories. Piercing is not so popular in Japan as European and US countries, as piercing is usually prohibited in Japanese high schools. Given this circumstance, the piercing frequency may be high in intrinsic AD.

Ni or Co-rich food items include peanuts, hazelnuts, almond, chocolate, cocoa, sunflower seeds, beans, dried beans, porridge oats, licorice, lucerne seeds, oat meal, wheat bran [49–51]. Excess intake of these foods allows metal ions to be extraordinarily administered. In addition, aging, taking dietary supplement, and drinking tap water contribute to the total load of Ni in body [22,47]. Ni is present in most of daily foods and an average diet supplies 0.3–0.6 mg of Ni to the body [52]. Nickel concentration range between 2.348 ± 1.716 mg kg^{-1} in nuts and 0.002 mg L^{-1} in water [53]. Intestinal absorption of food is thought to be about 1% [54]. Accordingly, serum Ni levels correlate with Ni-rich food items. When Ni allergic patients avoided Ni intake, their serum Ni levels were lowered compared to controls [48]. It is thought that metals are excreted through sweat, and therefore, sweating possibly may elicit dermatitis by serving as contactants. We found that the concentration of Ni was higher in the sweat of intrinsic than extrinsic AD patients, suggesting that metal allergy may be more significant in intrinsic AD.

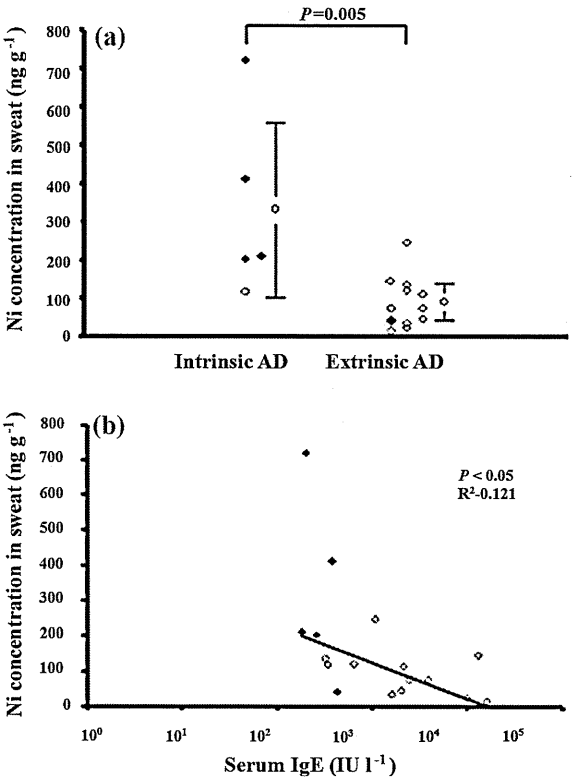

Fig. 2. Concentration of Ni in sweat of intrinsic and extrinsic AD patients. (a) Concentration of Ni in sweat from intrinsic and extrinsic AD patients. (b) Inverse correlation between Ni concentration and serum IgE levels. The closed and open rectangles represent patients with positive and negative Ni patch test.

In our study, the patients' sweat samples were collected from the forearms. Eczematous lesions preferentially occur on the flexor aspects of the limbs and around the neck of AD patients [55]. The degree of sweat secretion has been variously reported in AD [56]. It is also unclear that sweating is different between lesional and non-lesional skin of AD patients. There is an observation that the lesional skin has poor amount of sweat caused by dysfunction of autonomic nervous system [55]. It is an issue, therefore, that the metal concentration is uniform or different in the various skin sites [57]. The possibility remains that Ni concentration is different depending on the skin sites, which might explain the predilection areas of intrinsic AD.

Metal-free diet and elimination of metals improve skin eruptions in some AD patients [19,36]. Direct association between the metal concentrations of sweat and serum is an important issue to be clarified. It has been reported that serum Ni levels correlate with Ni-rich food items. When Ni allergic patients avoided Ni intake, their serum Ni levels were lowered compared to controls [48]. In addition, in 6 patients with palmar pompholyx showing positive patch tests to Ni, Co and Cr, oral metal challenge deteriorated their eruptions [58]. Further investigation on metal concentrations of food, serum and sweat might clarify the important aspect of pathophysiology of intrinsic AD, which is possibly evoked by Th1-inducing substances represented by metals.

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