



Accumulation of invariant NKT cells into inflamed skin in a novel murine model of nickel allergy



Takanori Eguchi^{a,b,c}, Kenichi Kumagai^{a,b}, Hiroshi Kobayashi^{a,b,d}, Hiroaki Shigematsu^{a,b}, Kazutaka Kitaura^b, Satsuki Suzuki^e, Tatsuya Horikawa^f, Yoshiki Hamada^a, Kouetsu Ogasawara^g, Ryuji Suzuki^{b,*}

^a Department of Oral and Maxillofacial Surgery, School of Dental Medicine, Tsurumi University, 2-1-3, Tsurumi, Tsurumi-ku, Yokohama, Kanagawa 230-8501, Japan

^b Department of Rheumatology and Clinical Immunology, Clinical Research Center for Rheumatology and Allergy, Sagami National Hospital, National Hospital Organization, 18-1, Sakura-dai, Minami-ku, Sagami, Kanagawa 252-0392, Japan

^c Department of Oral and Maxillofacial Surgery, Toshiba Rinkan Hospital, 7-9-1, Kamitsuruma, Sagami, Kanagawa 252-0385, Japan

^d Department of Oral and Maxillofacial Surgery, Nagano Matsushiro General Hospital, 183, Matsushiro, Nagano 381-1231, Japan

^e Section of Biological Science, Research Center for Odontology, Nippon Dental University, 1-9-20, Fujimi, Chiyoda-ku, Tokyo 102-0071, Japan

^f Department of Dermatology, Nishi-Kobe Medical Center, 5-7-1, Kohjindai, Nishi-ku, Kobe, Hyogo 651-2274, Japan

^g Department of Immunobiology, Institute of Development, Aging and Cancer, Tohoku University 4-1, Seiryomachi, Aoba-ku, Sendai, Miyagi 980-8575, Japan

ARTICLE INFO

Article history:

Received 12 November 2012

Accepted 23 July 2013

Available online 2 August 2013

Keywords:

Hypersensitivity

Natural killer T cells

Nickel, T-cell receptor

ABSTRACT

Nickel (Ni) can cause delayed-type hypersensitivity reactions, which are thought to be mediated by the accumulation of T cells into inflamed skin. Accumulated T cells at the developmental stages in metal allergy are poorly characterized because a suitable animal model has not been established. To investigate the accumulated T cells in allergic inflamed skin, we generated a novel murine model of Ni-induced allergy. The murine model of Ni allergy was induced by two sensitizations of Ni plus lipopolysaccharide solution into the groin followed by three challenges with Ni solution into the footpad. Here we show that a specific TCR repertoire bearing V α 14J α 18, called natural killer (NK) T cells, was expanded monoclally in BALB/c or C57BL/6 mice. Accumulation of NKT cells was characterized as CD4⁺ or CD4⁺CD8⁺ T cells. These results suggested that NKT cells are major pathogenic T cells at the elicitation phase of Ni allergy.

© 2013 Elsevier Inc. All rights reserved.

1. Introduction

Nickel (Ni) is a component of several alloy types that are widespread throughout our environment, including implants used in replacement surgery, dentures and orthodontic wires. Hence, we are exposed to Ni from various sources, and Ni is the most frequent contact allergen among metals [1,2].

Metal allergy has been categorized as a delayed-type hypersensitivity (DTH) response due to the recruitment of lymphocytes and inflammatory cells (including T cells, dendritic cells, granulocytes, and macrophages) to the site of allergic inflammation. Various studies on Ni allergy have been carried out *in vitro* using sensitized human lymphocytes [3,4]. Accumulated T cells in inflamed skin are thought to be pathogenic and to be essential for the induction of allergy to metals in humans (similar to that for contact hypersensitivity to classical haptens). Indeed, metal ions can induce the

proliferation of human T cells *in vitro*, and limited TCR repertoires have been observed in human T cells from patients with metal allergies [5–7]. The nature of the T cells that infiltrate tissue during the elicitation phase of DTH is thought to be autoreactive [8], yet the antigen specificity of these T cells has not been determined.

To explore how accumulated T cells in the site of allergic inflammation contribute to the development of metal allergy, establishing suitable animal models is desired. On the basis of previous reports, we generated a novel murine model of Ni allergy by two sensitizations of Ni plus lipopolysaccharide (LPS) solution into the groin and then three challenges of Ni solution into the footpad.

In the present study, we investigated whether the TCR repertoire is skewed and cytokine profiles are specific in accumulated T cells in inflamed skin at the elicitation phase in a novel murine model of Ni allergy.

2. Materials and methods

All experiments were undertaken according to the Guidelines for Care and Use of Laboratory Animals set by the Clinical Research Center for Rheumatology and Allergy, Sagami National Hospital (Sagami, Japan) and Tohoku University (Sendai, Japan). All

Abbreviations: IHC, immunohistochemical; MCA, TCR α -chain C region-specific primer; MCB, TCR β -chain C region-specific primer; TCRAV, TCR α -chain variable; TCRBV, TCR β -chain variable; TCRV, TCR V region.

* Corresponding author. Fax: +81 42 742 7990.

E-mail address: r-suzuki@sagami-hosp.gr.jp (R. Suzuki).

experiments were approved by the Institutional Animal Care and Use Committee at Sagami National Hospital (approval number H22-2010-1) and Tohoku University (approval number 2012kado-45).

2.1. Animals

BALB/c and C57BL/6 mice were obtained from CLEA Japan (Tokyo, Japan). Mice were raised in standard aluminum cages (with a lid made of stainless-steel wire). Food and water were available *ad libitum*.

2.2. Reagents

First-grade NiCl₂ (purity >95%) was purchased from Wako Pure Chemical Industries (Osaka, Japan). LPS from *Escherichia coli* O55:B5 prepared by phenol–water extraction was purchased from Sigma–Aldrich (St Louis, MO, USA). NiCl₂ and LPS were dissolved in sterile saline.

2.3. Sensitization, elicitation and measurement of allergic footpad swelling

2.3.1. Sensitization

Total of 125 µl of 10 mM NiCl₂ with 10 µg/ml LPS in saline was injected twice (at an interval of 7 days) via the intradermal (i.d.) route into the left and right groin of mice (250 µl each time). Seven days after sensitization, mice were challenged for the first time.

2.3.2. Challenge for elicitation

Non-sensitized mice or sensitized mice were challenged by i.d. injection with 25 µl of 10 mM NiCl₂ (without LPS) in saline, into the left and right footpad under anesthesia with tribromoethanol. For full elicitation in the footpad, the challenge was repeated three times at an interval of 14 days. Ni + LPS-sensitized and saline-challenged mice were used as controls.

Footpad swelling was measured at the indicated times using a Peacock Dial Thickness Gauge (Ozaki MFG Co. Ltd, Tokyo, Japan). The induced difference (value after challenge–value before challenge) was recorded.

2.4. Immunohistochemical (IHC) analyses

Footpads were obtained from Ni-allergic mice for histological and IHC analyses. Tissue samples were fixed with 4% of paraformaldehyde-lysine-periodate overnight at 4 °C. After washing with PBS, fixed tissues were penetrated by soaking in 5% sucrose/PBS for 1 h, in 15% sucrose in PBS for 3 h, and 30% sucrose in PBS overnight at 4 °C. Samples were embedded in Tissue Mount (Chiba Medical, Saitama, Japan) and snap-frozen in a mixture of acetone and dry ice. Frozen sections were sliced into 6-µm cryosections, which were air-dried on poly-L-lysine-coated glass slides. For histological analyses, cryosections were stained with hematoxylin & eosin (H&E) by conventional methods. For IHC analyses, antigen retrieval was undertaken. Cryosections were then stained with anti-mouse CD3ε (145-2C11; Pharmingen, San Diego, CA, USA), CD4 (GK1.5, Pharmingen) and CD8α (53-6.7, Pharmingen) mAbs by conventional methods. Briefly, the non-specific signals of cryosections were blocked with PBS containing blocking reagents consisting of a 1:20 dilution of normal goat serum or normal rabbit serum, 0.025% Triton X-100 (Wako Pure Chemicals, Osaka, Japan) and 5% BSA (Sigma–Aldrich) for 30-min incubation at room temperature (RT). Primary mAbs were loaded onto tissue sections and incubated for 1 h at RT. After washing three times with PBS for 5 min, intrinsic peroxidase was quenched by 3% H₂O₂ in methanol. After soaking the sections in distilled water, they were washed twice, as

described above. Sections were treated with a secondary Ab (biotinylated goat anti-hamster IgG antibody or biotinylated rabbit anti-rat IgG antibody) for 1 h at RT. After washing three times, sections were incubated with VECTASTAIN ABC Reagent (Vector Laboratories, Burlingame, CA, USA) for 30 min at RT, followed by 3,3'-Diaminobenzidine (DAB) staining (0.06% DAB, 0.03% H₂O₂ in 0.1 M Tris–HCl buffer pH 7.6; Wako Pure Chemicals). Finally, tissue sections were stained with hematoxylin to visualize nuclei.

2.5. Isolation of total RNA from tissues

Fresh footpads and spleens were obtained from mice and immediately soaked in RNeasy RNA Stabilization Reagent (Qiagen, Hilden, Germany). Total RNAs from footpads and spleens were extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen) according to manufacturer instructions.

2.6. Quantitative PCR (qPCR)

The expression levels of mRNA for the related genes of immune responses, including T cell-related CD antigens, cytokines, and cytotoxic granules, were measured by qPCR using the Bio-Rad CFX96 system (Bio-Rad, Hercules, CA, USA). Specific primers for GAPDH, CD3, CD4, CD8, IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-10, perforin, granzyme A, granzyme B, and Fas ligand (FasL) were as described previously [9,10]. Specific primers for NK T-cell markers CD161c and Vα14Jα18 were also used in the present study. The primer sequences of the genes tested in the present study were:

CD161:F5'-CTCAATATCCCAGGTCTCCAGAA-3',
R5'-TGCAGCAGAAACCAAGAAGAATG-3';
Vα14Jα18:F5'-TGGGAGATACTACCAACTCTGG-3',
R5'-CAGGTATGACAATCAGCTGAGTCC-3',
CD1d:F5'-TGCAGATGCTCTGAATGACAC-3',
R5'-AGACATGACACACCAGCTGCCTA-3'.

Freshly isolated total RNA from the footpads of mice was converted to cDNA using PrimeScript™ RT Reagent Kit (Takara Bio, Otsu, Japan) according to manufacturer instructions. The PCR reaction consisted of 5 µl of SsoFast™ EvaGreen® Supermix (Bio-Rad), 3.5 µl of RNase/DNase-free water, 0.5 µl of 5 µM primer mix, and 1 µl of cDNA in a total volume of 10 µl. Cycling conditions were as follows: 30 s at 95 °C followed by 45 rounds of 1 s at 95 °C, 5 s at 60 °C. At the end of each run, melting curve analyses were undertaken from 65 to 95 °C to confirm the homogeneity of PCR products. All assays were repeated two times and the mean values used as the gene expression levels. Seven points of tenfold serial dilutions of each standard transcript were used for the absolute quantification, specification and amplification efficiency of each primer set. Standard transcripts were generated from each PCR product and cloned into plasmids. The sequences were confirmed by DNA sequencing using the CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA). Their quality and concentration were validated using an Agilent DNA 7500 Kit in an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). GAPDH gene expression was used as the internal control. The expression levels of each target gene were normalized to that of GAPDH expression.

2.7. TCR repertoire analyses

TCR repertoire analyses were carried out with the 2nd sensitization–3rd challenge mice and saline-injected mice samples using the adaptor-ligation-mediated PCR and microplate hybridization assay method [11–13]. Briefly, isolated total RNA was converted to double-stranded cDNA using a Superscript cDNA Synthesis kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer

instructions except that a specific primer (BSL-18E) was used [12]. The P10EA/P20EA adaptors were ligated to the 5' end of the cDNA, and this adaptor-ligated cDNA was cut with *SphI*. PCR was done with TCR α -chain constant region-specific or TCR β -chain constant region-specific primers (MCA1 or MCB1) and P20EA. The second PCR was carried out with MCA2 or MCB2 and P20EA. The third PCR was undertaken using both P20EA and 5'-biotinylated MCA3 or MCB3 primer for the biotinylation of PCR products. Ten picomoles of amino-modified oligonucleotides specific for the TCRAV and TCRBV segments were immobilized onto carboxylate-modified 96-well microplates with water-soluble carbodiimide. Prehybridization and hybridization were carried out in GMCF buffer (0.5 M Na₂HPO₄, [pH 7.0], 1 mM EDTA, 7% SDS, 1% BSA and 7.5% formamide) at 47 °C. One-hundred microliters of the denatured 5'-biotinylated PCR products were mixed with an equivalent volume of 0.4 N NaOH/10 mM EDTA, and the mixture added to 10 ml of GMCF buffer. One-hundred microliters of hybridization solution was used in each well of the microplate containing immobilized oligonucleotide probes specific for V segments. After hybridization, the wells were washed four times with washing buffer (2 $\times$ SSC, 0.1% SDS) at room temperature. The plate was incubated at 37 °C for 10 min for stringency washing. After washing four times with the same washing buffer, 200 μ l of TB-TBS buffer (10 mM Tris-HCl, 0.5 M NaCl, pH 7.4, 0.5% Tween-20 and 0.5% blocking reagent; Roche Diagnostics, Basel, Switzerland) was added to block non-specific binding. Next, 100 μ l of 1:2000-diluted alkaline phosphatase-conjugated streptavidin in TB-TBS was added, and the sample incubated at 37 °C for 30 min. Plates were washed six times in T-TBS (10 mM Tris-HCl, 0.5 M NaCl, pH 7.4, 0.5% Tween-20). For color development, 100 μ l of substrate solution (4 mg/ml *p*-nitrophenylphosphate [Sigma-Aldrich], in 10% diethanolamine, pH 9.8) was added, and absorbance at 405 nm determined. The ratio of the hybridization intensity of each TCRV-specific probe to that of a TCRC-specific probe (V/C value) was determined using the TCR cDNA concentrated samples that contained the corresponding V segment and the universal C segment, respectively. Absorbance obtained with each TCRV-specific probe was divided by the corresponding V/C value. The relative frequency was calculated using the corrected absorbencies by the formula: relative frequency (%) = (corrected absorbance of TCRV-specific probe/the sum of corrected absorbencies of TCRV-specific probes) $\times$ 100.

2.8. Determination of CDR3 nucleotide sequences

PCR was undertaken with 1 μ l of the 1:20-diluted second PCR product using a forward primer specific for the variable region and a reverse primer specific for the constant region (MCA3 or MCB3) under the conditions described above. The primers used were VA14-1: AGACTCCCAGCCAGTGACT, and VB8-2: GCTACC CCCTCTCAGACAT. PCR products were eluted from agarose gel and cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA). The recombinant plasmid DNA was then transfected into DH5 α -competent cells. Sequence reactions were carried out using the GenomeLab DTCS Quick Start kit (Beckman Coulter) and analyzed by the CEQ8000 Genetic Analysis System (Beckman Coulter). In total, 16 clones from each sample of footpad tissues in BALB/c and C57BL/6 mice were examined.

2.9. ELISA assay for the activation of NKT cells

Lymphocytes subsets were purified from splenic or hepatic mononuclear cells isolated from C57BL/6 mice. Isolation was performed by FACS (FACS Aria I, BD Bioscience, San Jose, CA, USA). Briefly, a cell suspension from the spleen was prepared by cutting the tissue into small pieces and gently pressing through a 100 μ m wire mesh. For liver preparation, the portal vein was flushed with

10 ml sodium chloride until the organ became pale. Then the liver was cut into small pieces and passed through a 100 μ m wire mesh. Hepatic lymphocytes were further isolated by density gradient centrifugation using 80% and 40% Percoll (Biochrom, Berlin, Germany). NKT cells were then labeled with FITC-conjugated anti-mouse CD3 (clone: 17A2, rat IgG2b) and PE-conjugated anti-mouse NK1.1 antibodies (clone: PK136, Ms. IgG2a) (all from BD Biosciences) for CD3 + NK1.1 + enrichment by FACS, and are referred to as NKT cells in this study.

For lymphocyte stimulation, 96-well cell culture plates (Corning Costar, Sigma Aldrich) were coated with anti-mouse-CD3e antibody (Clone: 145-2C11, BD Biosciences) at 10 μ g/ml and stored overnight at 4 °C. Wells were washed twice with PBS and up to 1×10^6 isolated lymphocytes from spleen and liver were plated in 200 μ l RPMI culture medium (Biochrom). For activation, 50 ng/ml of α -GalCer (Funakoshi Tokyo, Japan) and NiCl₂ at 0, 0.1, 1, 10, 100 or 1000 mM were added. Plates were incubated for the indicated time at 37 °C and 5% CO₂. Activation of NKT cells was confirmed by IFN- γ ELISA from harvested supernatants using a commercially available sandwich ELISA kit (BD Bioscience). Tetramethylbenzidine dihydrochloride was used for detection. ELISA readings were determined by optical density (OD) at 450 nm using a microplate reader (Thermo Labsystems, Helsinki, Finland).

2.10. Statistical analyses

Differences in values were analyzed using the Student's unpaired *t*-test using StatView 5.0 for Windows (SAS Institute Inc., Cary, NC, USA). *P* < 0.05 was considered significant.

3. Results

3.1. Footpad swelling in Ni-induced allergic mice

To address how accumulated T cells in allergic inflamed skin contribute to the development of metal allergy, we generated a new murine model of metal allergy. Footpad swelling was measured every day after Ni injection into the footpad. The footpad swelling seen with 1st sensitization (one-time sensitization) was the same as that of non-sensitization (Fig. 1A) despite multiple challenges (data not shown). In contrast, after 2nd sensitization (two times sensitization), considerable swelling was observed in footpads. In addition, footpad swelling was further increased by multiple challenges in 2nd sensitization mice. The peak of footpad swelling was observed 24 h after challenges in all conditions of sensitization. Footpad swelling was reduced to basal levels 7 days after challenge in no sensitization–3rd challenge (three times challenge) mice and 1st sensitization–3rd challenge mice. Footpad swelling continued to 10 days after challenge in 2nd sensitization–3rd challenge mice (Fig. 1A). A secondary response was observed only in 2nd sensitization–3rd challenge mice compared with no sensitization–multiple challenge mice and 1st sensitization–multiple challenge mice. These results suggested that inflammation (but not allergy) was induced in Ni-challenged–no-sensitization mice and 1st sensitization mice. Therefore, our findings suggested that Ni allergy developed in 2nd sensitization–3rd challenge mice.

3.2. Expression of T-cell markers in the footpads of Ni-induced allergic BALB/c mice by qPCR analyses

To examine the accumulation of T cells into allergic inflamed skin, the mRNA expression levels of CD3 (a component of the TCR) were measured by qPCR. We compared CD3 expression of samples from no sensitization–3rd challenge mice, 1st sensitization–3rd

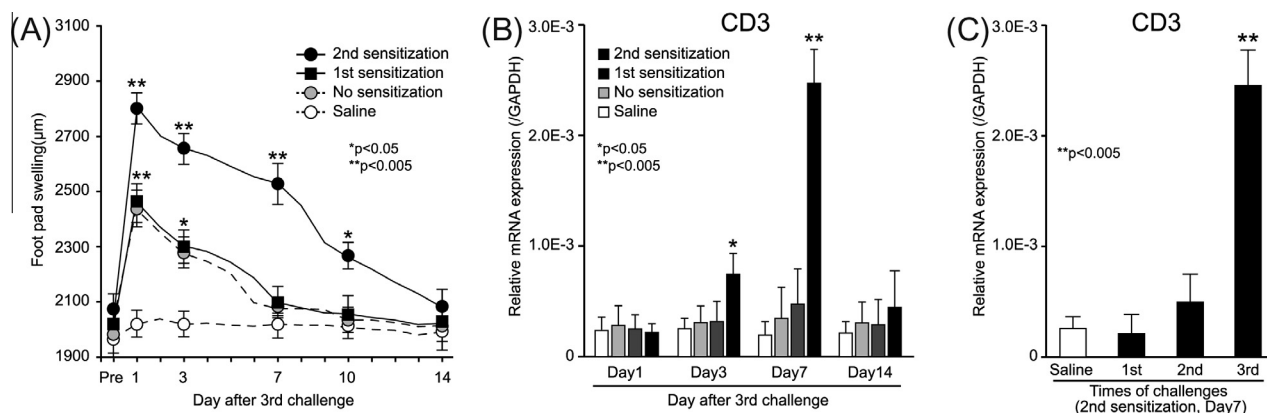


Fig. 1. Footpad swelling in Ni-induced allergic mice (A) Footpad swelling at various time points is shown. These mice underwent were three challenges with Ni. Representative data are shown. (B) Expression levels of CD3 mRNA were dependent upon sensitization conditions at various times after the 3rd challenge. (C) Effect of challenge conditions on CD3 expression levels 7 days after challenge. Each value is the mean $\pm$ SD from seven mice ($n = 7$). Significant differences are shown compared with saline-injected mice using the unpaired Student *t*-test (* $p < 0.05$, ** $p < 0.005$).

challenge mice, 2nd sensitization–3rd challenge mice, and saline-injected mice. In 2nd sensitization–3rd challenge mice, CD3 levels were significantly higher than the other groups 3 days after challenge and reached maximum expression at day 7 (Fig. 1B). By contrast, CD3 expression was not significantly different in the skin of 1st sensitization–3rd challenge mice, no sensitization–3rd challenge mice, and saline-injected mice at each time point (Fig. 1B). Then, we examined whether CD3 expression was dependent upon the time of challenges in 2nd sensitization mice. Expression levels of CD3 in the skin of 2nd challenge mice were not significantly different from those of 1st challenge mice and saline-injected mice (Fig. 1C). In contrast, CD3 levels were expressed considerably in the skins of 3rd challenge mice (Fig. 1C). These results suggested that T cells were accumulated in appreciable numbers in inflamed skin at day 7 in 2nd sensitization–3rd challenge mice.

3.3. IHC analyses of footpads 7 days after the 2nd sensitization–3rd challenge BALB/c mice

To ascertain whether T cells accumulate at the site of inflamed skin, we analyzed the skin of Ni-allergic mice by immunohistochemistry. The histology of Ni-allergic footpads of 7 days after the 2nd sensitization–3rd challenge mice was compared with that of mice receiving saline injections in the footpad. H&E staining of footpads 7 days after the 3rd challenge revealed infiltrates of lymphocytes and liquefaction degeneration in the epithelial basal layer (Fig. 2C and D). IHC staining showed that CD3-positive T cells were present among the infiltrating cells in the epithelial basal layer (Fig. 2F–K). Saline-injected footpads did not show tissue destruction or infiltrating cells (Fig. 2E). CD4 single-positive T cells (but not CD8-positive T cells) were detected in the CD3-positive T cells (Fig. 2H–M). Interestingly, CD4 CD8 double-negative CD3-positive T cells also accumulated in the inflammatory sites of skin (Fig. 2H–M). The population of T cells contained CD4⁺CD8[−], 50/225 cells (22%)/13,600 μm^3 ; CD4[−]CD8⁺, 5/225 cells (2%)/13,600 μm^3 ; and CD4[−]CD8[−], 170/225 cells (76%)/13,600 μm^3 .

These results suggested that T cells accumulated into the sites of inflamed skin in Ni-allergic mice.

3.4. TCR repertoire analyses of Ni-induced allergic diseases in BALB/c mice

To examine whether TCR repertoires are skewed in the inflammatory sites of skin, we analyzed TCRAV and TCRBV repertoires in Ni-induced allergic BALB/c mice. The TCRAV and TCRBV repertoires were not significantly different in the spleens between the 2nd

sensitization–3rd challenge- and the no sensitization–saline-injected mice (data not shown). The percentages of VA14-1 usage were significantly higher in the footpads of 2nd sensitization–3rd challenge mice than in the spleens of no sensitization–saline-injected mice (Fig. 3). Usages of other TCRAVs in the footpads of 2nd sensitization–3rd challenge mice were not significantly different from those in the spleens of no sensitization–saline-injected mice. In TCRBV repertoires, VB8-2 was most frequently utilized in the footpads of 2nd sensitization–3rd challenge mice compared with that in the spleens of no sensitization–saline-injected mice (Fig. 3). These results suggested that specific T cell-bearing VA14-1 and VB8-2 were present for 1 week in the inflamed skin of Ni-allergic mice.

3.5. Determination of CDR3 nucleotide sequences in Ni-allergic BALB/c mice

To investigate whether specific CDR3 sequences are used in accumulated T cells bearing VA14-1 and VB8-2, we determined CDR3 nucleotide sequences from cDNA clones obtained from the footpads of 2nd sensitization–3rd challenge mice. Predicted amino-acid sequences based on the CDR3 sequences of VA14-1 cDNA clones and the clonal frequencies are shown in Table 1. Surprisingly, the clonotype (CVV-G-DRGSALGRLHFG) was most frequently utilized in accumulated T cells and the clonotype (CVV-G-DRGSALGRLHFG) of CDR3 was obtained from the footpads of all Ni-allergic mice (Table 1). This clonotype has identical CDR3 sequences and common AJ18 segments (Table 1). In contrast, VB8-2 showed highly diverse CDR3 sequences in the footpads of all mice (data not shown). Interestingly, the BJ2.7 segment was detected in footpads of all mice at high frequencies: 9 of 12 clones in mouse #1; 7 of 15 clones in mouse #2; 6 of 14 clones in mouse #3; 3 of 12 clones in mouse #4; 5 of 14 clones in mouse #5; 7 of 11 clones in mouse #6; and 7 of 16 clones in mouse #7 (Table 2). Frequency of the TCRBJ 2.7 usage of footpad was significantly increased than those of spleen (Table 2). V α 14J α 18 (VA14-1 AJ18) and V β 8.2J β 2.7 (VB8-2 BJ2.7) have been reported as the TCR of invariant NKT (iNKT) cells. These results suggested that NKT cells are major pathogenic T cells involved in the allergy to Ni observed in mice.

3.6. TCR repertoire analyses of Ni-induced allergic diseases in C57BL/6 mice

To confirm that specific NKT cells are commonly accumulated in other types of murine Ni allergy, we examined the TCR repertoire of accumulated T cells in inflamed skin from Ni-allergic C57BL/6

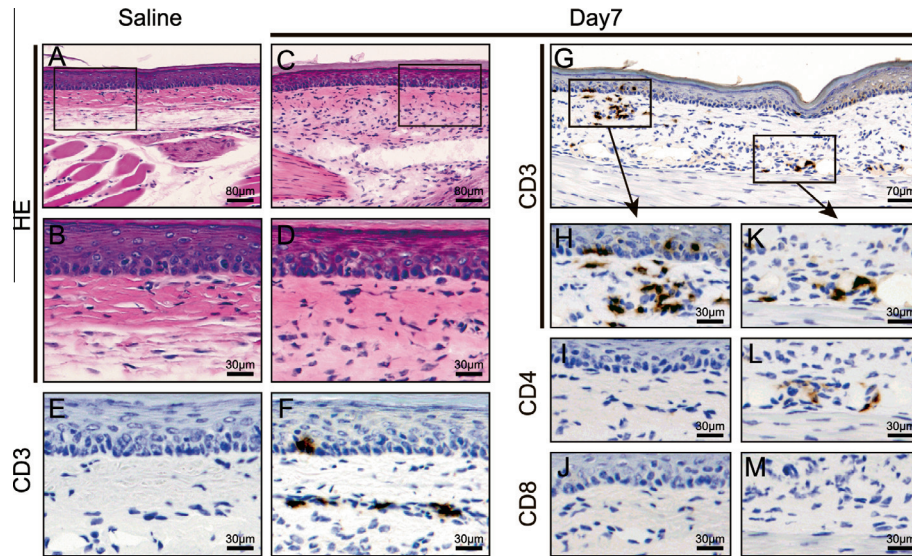


Fig. 2. Immunohistochemical analyses of footpads from Ni-induced allergic mice. Histopathology and IHC analysis were undertaken to identify CD3⁺, CD4⁺ and CD8⁺ cells in footpads. Representative photomicrographs of footpad sections from saline-injected mice or mice 7 days after the 2nd sensitization–3rd challenge stained with H&E (A–D) and CD3, CD4 and CD8 (E–M). Liquefaction degeneration and infiltration of inflammatory cells into the epithelial basal layer and dermis are shown in the footpads of mice 7 days after the 2nd sensitization–3rd challenge, but not in those of saline-injected mice (A–D). For IHC analyses, localization of CD3⁺ cells is shown (brown stain) in the epithelial basal layer of footpads from mice 7 days after the 2nd sensitization–3rd challenge, but not in that of saline-injected mice (E and F). In the footpads of mice 7 days after the 2nd sensitization–3rd challenge, CD3⁺ cells were mainly CD4⁺ CD8[−] (H–K). CD4⁺ T cells or few CD8⁺ cells were observed (K–M). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

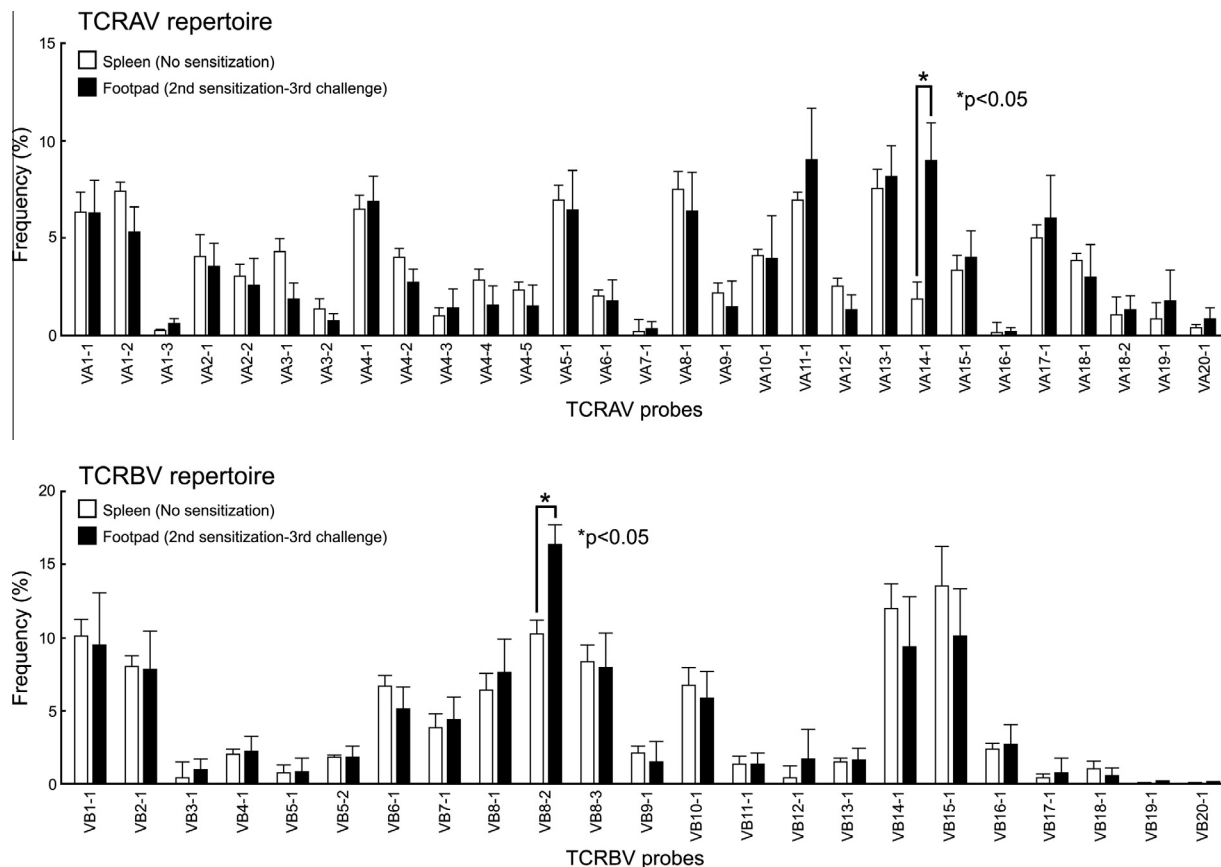


Fig. 3. Tcr repertoire analyses in Ni-induced allergic BALB/c mice. Tcr repertoire analyses were performed in the spleen of no sensitization–saline-injected mice and in the footpads of 2nd sensitization–3rd challenge. TCRAV and TCRBV repertoires were analyzed by microplate hybridization assay. The vertical bars and error bars indicate the mean $\pm$ SD of TCRAV and TCRBV frequencies in seven mice ($n = 7$). In the footpads of mice 7 days after the 2nd sensitization–3rd challenge, the percentage frequencies of T cells bearing VA14-1 and VB8-2 were significantly increased compared with those from normal mouse spleens ($*p < 0.05$, unpaired Student t -test). All experiments were performed in triplicates.

Table 1

Amino-acid sequences of the CDR3 regions of VA14-1 in the footpads of mice 7 days after the 2nd sensitization–3rd challenge.

Footpad	Clonal frequency	V	N	J	J gene
#1	14/14	CVV	G	DRGSALGRLHFG	AJ18 ^a
#2	12/13	CVV	G	DRGSALGRLHFG	AJ18 ^a
	1/13	CVG	LL	NSGGSNYKLTFG	AJ53
#3	13/14	CVV	G	DRGSALGRLHFG	AJ18 ^a
	1/14	SVW	W	ASSFSKLTVFG	AJ50
#4	16/16	CVV	G	DRGSALGRLHFG	AJ18 ^a
#5	9/11	CVV	G	DRGSALGRLHFG	AJ18 ^a
	1/11	CVV	G	ATGGNNKLTFG	AJ56
	1/11	CVV	GA	DSGYNNKLTFG	AJ11
#6	15/15	CVV	G	DRGSALGRLHFG	AJ18 ^a
#7	12/15	CVV	G	DRGSALGRLHFG	AJ18 ^a
	1/15	CVV	V	DRGSALGRLHFG	AJ18
	1/15	CAV	G	DRGSALGRLHFG	AJ18
	1/15	CV	V	VNTGNYKYVFG	AJ40

^a Identical sequences were detected in different individual mice.

mice. C57BL/6 mice as well as BALB/c mice developed Ni allergy by treatment with two sensitizations and three challenges (data not shown). The percentages of usage of VA14-1 and VB8-2 were significantly higher in the footpads of 2nd sensitization–3rd challenge mice than in the spleens of no sensitization–saline-injected mice (Fig. 4A). Unexpectedly, the CDR3 sequence (CVV-G-DRGSALGRLHFG) of VA14-1 AJ18 from BALB/c NKT cells was identical to the CDR3 sequences of VA14-1 AJ18 from C57BL/6 NKT cells in Ni-allergic diseases (Fig. 4B).

3.7. Quantification of NKT cell related markers and cytokines in Ni-allergic BALB/c mice

We measured several factors related to NKT cells by qPCR to determine the role of NKT cells in Ni-allergic mice. Expression levels of NKT cell related markers such as V α 14J α 18, CD161c, NKG2D and CD1d were significantly higher at day 7 in the footpads of 2nd sensitization–3rd challenge mice compared with those of saline-injected mice (Fig. 5A). Then, we examined the expression levels of TNF- α , IFN- γ , IL-2, IL-4, IL-5, and IL-10 mRNA to determine the cytokine environment in the footpad tissues of Ni-allergic mice (Fig. 5B and C). A significant increase in the expression of IFN- γ , TNF- α , and IL-2 (Th1 cytokines) was observed in the footpads of

2nd sensitization–3rd challenge mice compared with saline-injected mice at 7 days. However, expression of IL-4, IL-5, and IL-10 (Th2 cytokines) was not significantly increased. Furthermore, we examined the expression levels of cytotoxic molecules to determine the apoptotic environment in the footpad tissues of Ni-allergic mice. Expression levels of FasL, perforin, granzyme A and granzyme B were significantly higher at day 7 in the footpads of 2nd sensitization–3rd challenge mice compared with those of saline-injected mice (Fig. 5D). These results suggested that NKT cell were accumulated into footpad at late phase (3–7 day), moreover Th1-type cytokines and cytotoxic molecules such as FasL, granzyme A, granzyme B and perforin are positively correlated with the pathogenesis of Ni allergy in the footpads of mice.

3.8. ELISA assay for the activation of NKT cells

To examine the response of NKT cells to Ni, we measured the secretion of IFN- γ at various concentrations of Ni and 50 ng/ml α -GalCer solutions by ELISA. There was no significant change compared with 0.1 or 1 mM Ni solution and α -GalCer solution alone (Fig. 6). In contrast, secretion levels of IFN- γ were significantly higher at 10 mM Ni solution than α -GalCer alone (Fig. 6). In addition, the secretion of IFN- γ was not detected at 100 mM Ni solution (Fig. 6). These data indicate that NKT cells were strongly activated by 10 mM Ni solution and inactivated by 100 mM Ni solution.

4. Discussion

We found that the TCR repertoire was skewed in accumulated T cells in inflamed skin at the elicitation phase in a murine model of Ni allergy. Unexpectedly, the TCR repertoire was found to be specific: TCR V α 14J α 18 and V β 8.2 was commonly utilized in BALB/c and C57BL/6 mice by Ni-induced allergic diseases. TCR V α 14J α 18 and V β 8.2 are a major subset of mouse iNKT cells [14], and we found that iNKT cells accumulated in the inflamed skin of Ni-allergic mice. NKT cells are also known to express an invariant V α 14J α 18 TCR α -chain that pairs predominantly with V β 7, V β 8.2 or V β 2 TCR β -chains [15]. Our findings suggest that iNKT cells bearing TCR V α 14J α 18 are involved in the pathogenesis of Ni metal allergy. The accumulated NKT cells were CD4⁺ or CD4⁺CD8⁺, and expressed high levels of NKG2D and IFN- γ , which are candidate effector molecules for the development of metal allergy. Several

Table 2

Comparison of TCRBJ usage between footpad and spleen in TCRBV8-2.

Sample			Number of clone	TCRBJ gene usage in TCRBV8-2 (%)											
				1.1	1.2	1.3	1.4	1.5	1.6	1.7	2.1	2.2	2.3	2.4	2.5
Footpad	#1	12	0.0	0.0	0.0	8.3	0.0	8.3	0.0	8.3	0.0	0.0	0.0	0.0	75.0
	#2	15	0.0	0.0	13.3	13.3	0.0	13.3	0.0	0.0	0.0	0.0	13.3	0.0	46.7
	#3	14	21.4	0.0	14.3	0.0	0.0	7.1	0.0	0.0	0.0	0.0	14.3	0.0	42.9
	#4	12	8.3	0.0	41.7	0.0	0.0	8.3	0.0	8.3	0.0	0.0	8.3	0.0	25.0
	#5	14	14.3	0.0	35.7	7.1	0.0	0.0	0.0	0.0	0.0	0.0	7.1	0.0	35.7
	#6	11	9.1	9.1	9.1	0.0	0.0	9.1	0.0	0.0	0.0	0.0	0.0	0.0	63.6
	#7	16	18.8	0.0	12.5	0.0	6.3	6.3	0.0	6.3	0.0	6.3	0.0	0.0	43.8
	Mean		10.3	1.3	18.1	4.1	0.9	7.5	0.0	3.3	0.0	0.9	6.2	0.0	47.5 ^a
	SD		8.5	3.4	15.0	5.5	2.4	4.0	0.0	4.1	0.0	2.4	6.3	0.0	16.8
Spleen	#1	12	8.3	0.0	50.0	8.3	0.0	8.3	0.0	0.0	0.0	0.0	16.7	0.0	8.3
	#2	14	14.3	7.1	14.3	7.1	0.0	21.4	14.3	0.0	7.1	7.1	0.0	7.1	0.0
	#3	16	0.0	12.5	6.3	0.0	12.5	6.3	6.3	12.5	6.3	12.5	6.3	6.3	12.5
	#4	11	9.1	0.0	9.1	9.1	0.0	18.2	0.0	9.1	18.2	9.1	9.1	9.1	0.0
	#5	12	0.0	16.7	8.3	16.7	8.3	0.0	16.7	8.3	8.3	0.0	8.3	0.0	8.3
	#6	14	14.3	0.0	14.3	7.1	7.1	7.1	0.0	7.1	0.0	14.3	14.3	0.0	0.0
	#7	15	13.3	6.7	0.0	0.0	13.3	0.0	6.7	13.3	13.3	6.7	6.7	13.3	6.7
	Mean		8.5	6.1	14.6	6.9	5.9	8.8	6.3	7.2	7.6	7.1	8.8	5.1	5.1
	SD		6.3	6.7	16.4	5.7	5.9	8.3	7.0	5.4	6.6	5.6	5.5	5.3	5.1

^a The TCRBJ 2.7 gene usage of footpad was significantly increased than those of spleen (unpaired *t*-test *p* < 0.05).

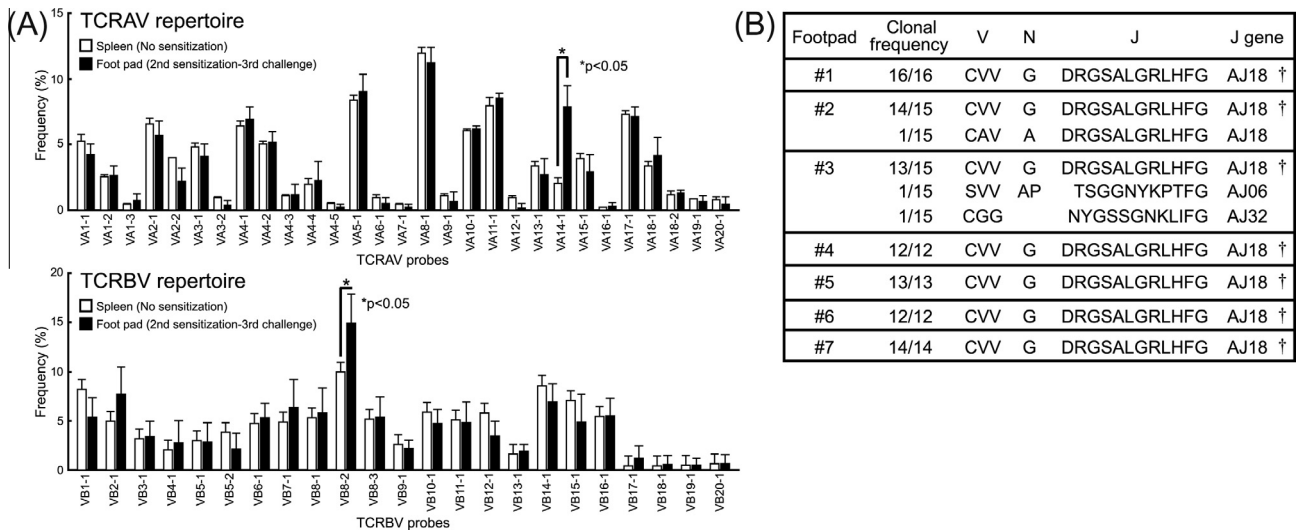


Fig. 4. Tcr repertoire analyses in Ni-induced allergic C57BL/6 mice (A) We analyzed TCRV and TCRBV repertoires in the footpads of 7-day 2nd sensitization–3rd challenge C57BL/6 mice. The vertical bars and error bars indicate the mean $\pm$ SD of TCRV and TCRBV frequencies in seven mice ($n = 7$). The percentage frequencies of T cells bearing VA14-1 or VB8-2 were significantly higher than those in the footpads of saline-injected mice ($*p < 0.05$, unpaired Student t -test). All experiments were performed in triplicates. (B) Monoclonal peak patterns detected limited CDR3 sequences in VA14-1. Identical sequences were detected in different individual mice are indicated by†.

authors have suggested that IL-4 is involved in metal allergy [16], but we did not detect IL-4 production from pathogenic lymphocytes in our animal model.

NKT cells (also called “iNKT cells” or “V α 14i NKT cells”) were originally defined as T lymphocytes that expressed CD161c and other receptors typical of NK cells. This definition has subsequently been narrowed down to specify a subpopulation of T cells that express a TCR with an invariant V α 14J α 18 TCR α chain paired with a restricted subset of TCR V chains [17]. NKT cells recognize glycolipids (microbial derived or self-glycolipids) presented in the context of CD1d (a class-I MHC-like molecule) [18]. After activation, NKT cells produce Th1- and Th2-type molecules such as IFN- γ and IL-4, so they could regulate adaptive immune responses [19]. Mature NKT cells in mice are mainly CD4 single-positive or CD4 CD8 double-negative. NKT cells predominantly express V β 8.2 joined with a bias of a particular J β usage, including J β 2.1, J β 2.5 or J β 2.7 [20,21]. Consistent with previous studies, we found accumulated NKT cells bearing V α 14J α 18 to be CD4 single-positive or CD4 CD8 double-negative at the site of inflamed skin in Ni-allergic mice.

The response to LPS (long considered to be a paradigm for innate immunity) provides a model system for understanding how V α 14i NKT cells may respond to microbes in the absence of TCR engagement by a foreign Ag (even though V α 14i NKT cells do not respond directly to LPS). LPS, a component of the cell walls of many Gram-negative bacteria, signals through a complex consisting of CD14, TLR4, and MD-2 [22], leading to secretion of pro-inflammatory cytokines and potent activation of the innate immune system [23]. Recently, Schmidt et al. reported that Ni directly stimulates human TLR4, but not mouse TLR4, to produce inflammatory cytokines, which may be crucial for the development of contact allergy [24]. Ni itself therefore has insufficient sensitization ability in mice. Hence, the co-stimulation of TLR4 with LPS during afferent phases seems to be essential for the development of Ni sensitization. We then hypothesized that IFN- γ production by V α 14 iNKT cells was (at least in part) responsible for augmentation of the innate immune response to LPS. IFN- γ is important for the induction of TNF- α in inflammatory responses [22]. A vital characteristic of NKT cells is their ability to rapidly produce large amounts of Th1-type cytokines (IFN- γ and TNF- α) and Th2-type cytokines (IL-4 and IL-5) upon activation [14]. NKT cells also have cytotoxic properties and can kill other cells through FasL- and perforin-dependent

pathways [25]. We found that the expression levels of the cytotoxic molecules FasL, granzyme A, granzyme B and perforin were abundantly expressed at day 7. It is likely that Th1-type cytokines and cytotoxic molecules from NKT cells are positively correlated with the pathogenesis of metal allergy in mice footpads.

In addition to T cells, mast cells, NK cells and granulocytes have been shown to be involved in metal-induced inflammation in other animal models [26–28]. Ear swelling was observed even in nude mice deficient in T cells by treatment with Ni and LPS solutions [29]. In the present study, we demonstrated that iNKT cells appeared in lymphocytic infiltrates at a high frequency during the elicitation phase of Ni allergy. NKT cells have been shown to infiltrate in the lesional skin of contact dermatitis (including Ni allergy) in humans [30]. NKT cells may thus be involved in the pathogenesis of contact hypersensitivity reactions, possibly by recognizing endogenous antigens such as self-lipids. Water-soluble Ni salts can pass through the ion channels of cell membranes or can be taken up by cells along with other molecules such as proteins and amino acids, so Ni can be incorporated readily into the body [31]. We hypothesize that Ni allergy can be triggered by intracellular Ni, which can combine with self-lipids on cellular membranes. We therefore speculate that Ni-modified self-lipids may activate NKT cells in the lesions of Ni-induced dermatitis in our model.

NKG2D is a co-stimulatory receptor expressed on NK cells, CD8 $^{+}$ T cells, NKT cells and $\gamma\delta$ T cells. Although NKG2D is expressed on activated/memory mouse CD8 $^{+}$ T cells, it is not expressed by naïve mouse CD8 $^{+}$ T cells and CD4 $^{+}$ T cells. NKG2D carries out two functions in iNKT cells: direct TCR-independent activation of NK-like cytotoxicity and co-stimulation of activation by CD1d. The ligands of NKG2D are “stress-inducible” molecules expressed in tissues during inflammation and in transformed cells. In mice, retinoic acid early inducible-1 family proteins have been identified as high-affinity NKG2D ligands [32]. We demonstrated previously that pathogenic CD8 $^{+}$ T cells express NKG2D, and that this co-stimulatory molecule is crucial for the development of inflammatory disease [33]. However, in metal allergy, the co-stimulatory molecules that are involved in the development of disease are not known. Further studies based on a specific TCR, co-stimulatory molecules, and effector molecules could help to clarify the mechanism of Ni allergy. We propose that, in addition to acting as a “bridge” to adaptive immunity, NKT cells act as an early

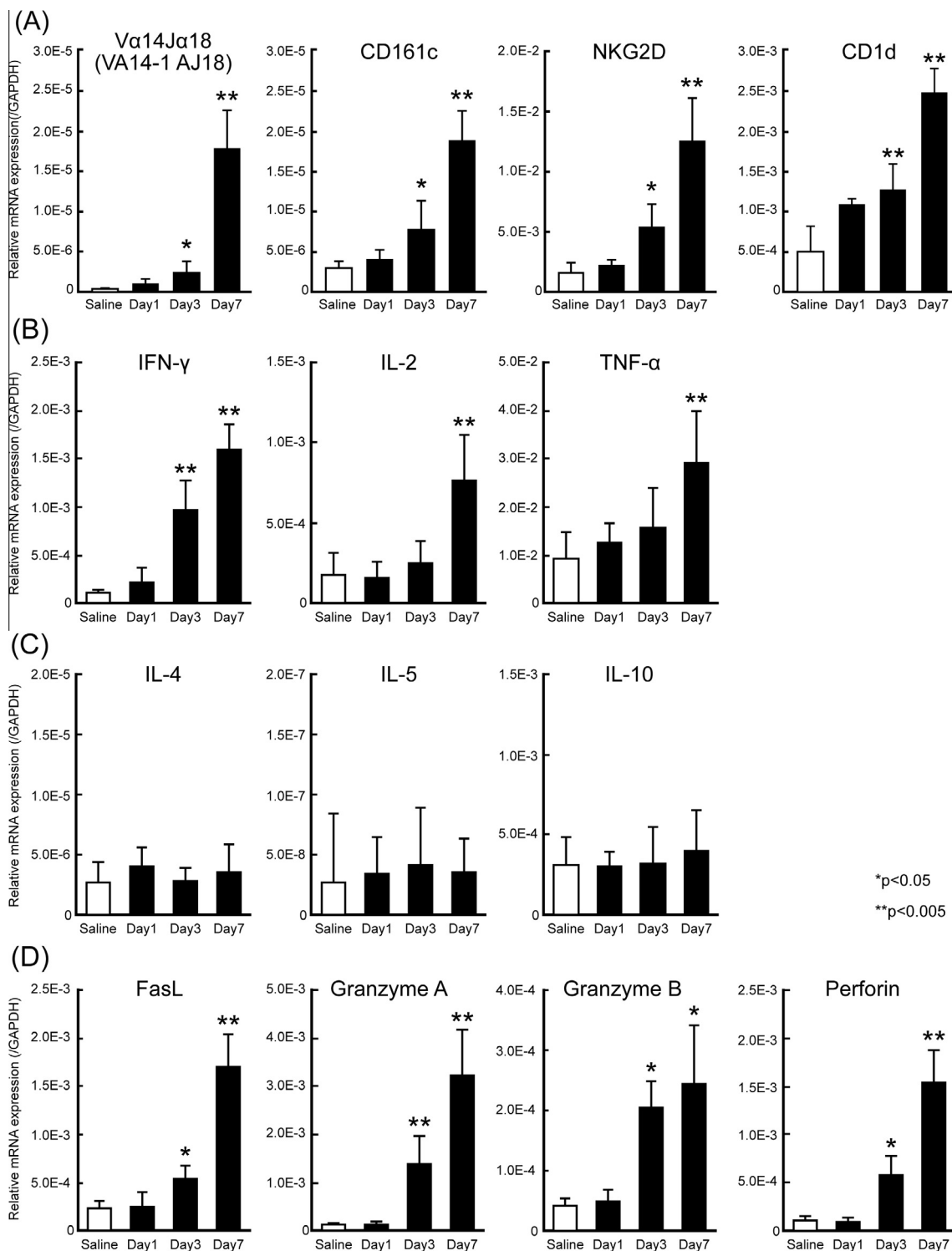


Fig. 5. Quantification of NKT cell related markers and cytokines by qPCR. The mRNA levels of (A) Vα14Jα18 (VA14-1AJ18), CD161c, NKG2D and CD1d (B) IFN-γ, TNF-α and IL-2, (C) IL-4, IL-5 and IL-10 and (D) the cytotoxic molecules FasL, perforin, granzyme A and granzyme B are shown. RNA was extracted from saline-injected footpads (open bars) or Ni-injected footpads 1, 3 and 7 days after the 2nd sensitization–3rd challenge (filled bars). GAPDH gene expression was used as an internal control. Open or filled bars and vertical error bars indicate the mean $\pm$ SD of seven mice ($n = 7$). Significant differences are shown when compared with saline-injected footpad using the unpaired Student t -test (* $p < 0.05$, ** $p < 0.005$). All experiments were performed in duplicates.

amplification step in the innate immune response, and that the rapid and complete initiation of this innate response is dependent upon the early production of IFN-γ by NKT cells.

A previous study showed that the Th1 subset of NKT cells has a dominant and critical role in allergy. In addition, the immune

response in nickel allergy was skewed towards a Th1 response with a minimal Th2 response [34]. Thus, we concluded the Th1 subset of NKT cells expanded locally in response to Ni. In addition, the regulation of excessive NKT cell immune responses might be a new therapeutic target for Ni allergy. In particular, OCH, an altered

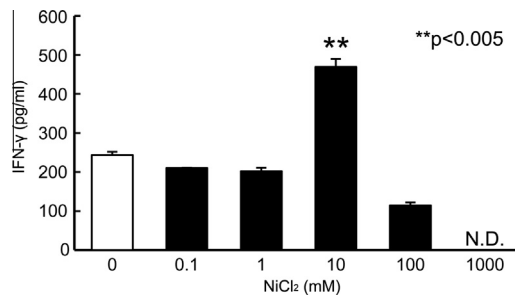


Fig. 6. Elisa for the activation of NKT cells. The secretion levels of IFN- γ at various concentrations of Ni and 50 ng/ml α -GalCer is shown. Open bar represents the α -GalCer only group. Filled bar represents various Ni solutions and α -GalCer. Open or filled bars and vertical error bars indicate the mean $\pm$ SD. Significant differences are shown when compared with α -GalCer alone using the unpaired Student *t*-test (**p* < 0.05). Experiments were repeated at least three times.

α -GalCer compound that promotes Th2 cytokine secretion from NKT cells, could be a new target molecule for Ni-allergic patients [35].

In conclusion, we clarified that V α 14i NKT cells are present during Ni-induced metal allergy in mice. Bridging the innate and adaptive immune response, V α 14i NKT cells can act rapidly to amplify innate immune responses initiated by APCs, even in the absence of a TCR-mediated signal.

Disclosures

This study was supported by a Grant-in-Aid for Scientific Research C 70373470, and 23593004 from the Ministry of Education, Culture, Sports, Science and Technology, Japan (Y.H. and R.S.); and by Grants-in-Aid for Scientific Research from the Ministry of Health, labor and Welfare of Japan H22-meneki-ippan-004 (K.O. and R.S.).

Acknowledgments

We thank Dr. Kazuyoshi Takeda and Dr. Masafumi Nakayama for helpful discussions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.cellimm.2013.07.010>.

References

- [1] L. Budinger, M. Hertl, Immunologic mechanisms in hypersensitivity reactions to metal ions: an overview, *Allergy* 55 (2000) 108–115.
- [2] L.A. Garner, Contact dermatitis to metals, *Dermatol. Ther.* 17 (2004) 321–327.
- [3] D. Pennino, K. Eyerich, C. Scarponi, T. Carbone, S. Eyerich, F. Nasorri, S. Garcovich, C. Traidl-Hoffmann, C. Albanesi, A. Cavani, IL-17 amplifies human contact hypersensitivity by licensing hapten nonspecific Th1 cells to kill autologous keratinocytes, *J. Immunol.* 184 (2010) 4880–4888.
- [4] J.T. Minang, I. Arestrom, M. Troye-Blomberg, L. Lundeberg, N. Ahlborg, Nickel, cobalt, chromium, palladium and gold induce a mixed Th1- and Th2-type cytokine response in vitro in subjects with contact allergy to the respective metals, *Clin. Exp. Immunol.* 146 (2006) 417–426.
- [5] H. Hashizume, N. Seo, T. Ito, M. Takigawa, H. Yagi, Promiscuous interaction between gold-specific T cells and APCs in gold allergy, *J. Immunol.* 181 (2008) 8096–8102.
- [6] L. Budinger, N. Neuser, U. Totzke, H.F. Merk, M. Hertl, Preferential usage of TCR-V β 17 by peripheral and cutaneous T cells in nickel-induced contact dermatitis, *J. Immunol.* 167 (2001) 6038–6044.
- [7] S. Silvennoinen-Kassinen, J. Karvonen, I. Ikaheimo, Restricted and individual usage of T-cell receptor beta-gene variables in nickel-induced CD4⁺ and CD8⁺ cells, *Scand. J. Immunol.* 48 (1998) 99–102.
- [8] R.S. Kalish, Recent developments in the pathogenesis of allergic contact dermatitis, *Arch. Dermatol.* 127 (1991) 1558–1563.
- [9] Y. Fujii, K. Kitaura, K. Nakamichi, T. Takasaki, R. Suzuki, I. Kurane, Accumulation of T-cells with selected T-cell receptors in the brains of Japanese encephalitis virus-infected mice, *Jpn. J. Infect. Dis.* 61 (2008) 40–48.
- [10] K. Kitaura, Y. Fujii, D. Hayasaka, T. Matsutani, K. Shirai, N. Nagata, C.K. Lim, S. Suzuki, T. Takasaki, R. Suzuki, I. Kurane, High clonality of virus-specific T lymphocytes defined by TCR usage in the brains of mice infected with West Nile virus, *J. Immunol.* 187 (2011) 3919–3930.
- [11] T. Matsutani, T. Yoshioka, Y. Tsuruta, S. Iwagami, R. Suzuki, Analysis of TCRAV and TCRBV repertoires in healthy individuals by microplate hybridization assay, *Human Immunol.* 56 (1997) 57–69.
- [12] Y. Tsuruta, S. Iwagami, S. Furue, H. Teraoka, T. Yoshida, T. Sakata, R. Suzuki, Detection of human T cell receptor cDNAs (alpha, beta, gamma and delta) by ligation of a universal adaptor to variable region, *J. Immunol. Methods* 161 (1993) 7–21.
- [13] R. Yoshida, T. Yoshioka, S. Yamane, T. Matsutani, T. Toyosaki-Maeda, Y. Tsuruta, R. Suzuki, A new method for quantitative analysis of the mouse T-cell receptor V region repertoires: comparison of repertoires among strains, *Immunogenetics* 52 (2000) 35–45.
- [14] D.I. Godfrey, M. Kronenberg, Going both ways: immune regulation via CD1d-dependent NKT cells, *J. Clin. Invest.* 114 (2004) 1379–1388.
- [15] R.C. Budd, G.C. Miescher, R.C. Howe, R.K. Lees, C. Bron, H.R. MacDonald, Developmentally regulated expression of T cell receptor beta chain variable domains in immature thymocytes, *J. Exp. Med.* 166 (1987) 577–582.
- [16] S. Niiyama, H. Tamauchi, Y. Amoh, M. Terashima, Y. Matsumura, M. Kanoh, S. Habu, J. Komotori, K. Katsuka, Th2 immune response plays a critical role in the development of nickel-induced allergic contact dermatitis, *Int. Arch. Allergy Immunol.* 153 (2010) 303–314.
- [17] M. Taniguchi, M. Harada, S. Kojima, T. Nakayama, H. Wakao, The regulatory role of Valpha14 NKT cells in innate and acquired immune response, *Annu. Rev. Immunol.* 21 (2003) 483–513.
- [18] P.A. Sieling, CD1-Restricted T cells: T cells with a unique immunological niche, *Clin. Immunol.* 96 (2000) 3–10.
- [19] A. Bendelac, M.N. Rivera, S.H. Park, J.H. Roark, Mouse CD1-specific NK1 T cells: development, specificity, and function, *Annu. Rev. Immunol.* 15 (1997) 535–562.
- [20] B.J. Fowlkes, A.M. Kruisbeek, H. Ton-That, M.A. Weston, J.E. Coligan, R.H. Schwartz, D.M. Pardoll, A novel population of T-cell receptor alpha beta-bearing thymocytes which predominantly expresses a single V beta gene family, *Nature* 329 (1987) 251–254.
- [21] R. Ceredig, F. Lynch, P. Newman, Phenotypic properties, interleukin 2 production, and developmental origin of a “mature” subpopulation of Lyt-2-L3T4- mouse thymocytes, *Proc. Natl. Acad. Sci. USA* 84 (1987) 8578–8582.
- [22] N.A. Nagarajan, M. Kronenberg, Invariant NKT cells amplify the innate immune response to lipopolysaccharide, *J. Immunol.* 178 (2007) 2706–2713.
- [23] L.D. Hazlett, Q. Li, J. Liu, S. McClellan, W. Du, R.P. Barrett, NKT cells are critical to initiate an inflammatory response after *Pseudomonas aeruginosa* ocular infection in susceptible mice, *J. Immunol.* 179 (2007) 1138–1146.
- [24] M. Schmidt, B. Raghavan, V. Muller, T. Vogl, G. Fejer, S. Tchapchet, S. Keck, C. Kalis, P.J. Nielsen, C. Galanos, J. Roth, A. Skerra, S.F. Martin, M.A. Freudenberg, M. Goebeler, Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel, *Nat. Immunol.* 11 (2010) 814–819.
- [25] M.G. Swain, Hepatic NKT cells: friend or foe?, *Clin. Sci. (Lond.)* 114 (2008) 457–466.
- [26] N. Sato, M. Kinbara, T. Kuroishi, K. Kimura, Y. Iwakura, H. Ohtsu, S. Sugawara, Y. Endo, Lipopolysaccharide promotes and augments metal allergies in mice, dependent on innate immunity and histidine decarboxylase, *Clin. Exp. Allergy* 37 (2007) 743–751.
- [27] K. Ishihara, Y. Goi, J.J. Hong, T. Seyama, H. Ohtsu, H. Wada, K. Ohuchi, N. Hirasawa, Effects of nickel on eosinophil survival, *Int. Arch. Allergy Immunol.* 149 (Suppl 1) (2009) 57–60.
- [28] J.Y. Kim, K. Huh, K.Y. Lee, J.M. Yang, T.J. Kim, Nickel induces secretion of IFN-gamma by splenic natural killer cells, *Exp. Mol. Med.* 41 (2009) 288–295.
- [29] M. Kinbara, N. Sato, T. Kuroishi, T. Takano-Yamamoto, S. Sugawara, Y. Endo, Allergy-inducing nickel concentration is lowered by lipopolysaccharide at both the sensitization and elicitation steps in a murine model, *Br. J. Dermatol.* 164 (2011) 356–362.
- [30] M.D. Gober, R. Fischelevich, Y. Zhao, D. Unutmaz, A.A. Gaspari, Human natural killer T cells infiltrate into the skin at elicitation sites of allergic contact dermatitis, *J. Invest. Dermatol.* 128 (2008) 1460–1469.
- [31] X. Lin, M. Costa, Transformation of human osteoblasts to anchorage-independent growth by insoluble nickel particles, *Environ. Health Perspect.* 102 (Suppl 3) (1994) 289–292.
- [32] A. Cerwenka, L.L. Lanier, Ligands for natural killer cell receptors: redundancy or specificity, *Immunol. Rev.* 181 (2001) 158–169.
- [33] K. Ogasawara, J.A. Hamerman, L.R. Ehrlich, H. Bour-Jordan, P. Santamaria, J.A. Bluestone, L.L. Lanier, NKG2D blockade prevents autoimmune diabetes in NOD mice, *Immunity* 20 (2004) 757–767.
- [34] A. Almogren, M.H. Adam, Z. Shakoor, M.O. Gadelrab, H.A. Musa, Th1 and Th2 cytokine profile of CD4 and CD8 positive peripheral blood lymphocytes in nickel contact dermatitis, *Cent. Eur. J. Immunol.* 38 (1) (2013) 100–106.
- [35] Y. Ueno, S. Tanaka, M. Sumii, S. Miyake, S. Tazuma, M. Taniguchi, T. Yamamura, K. Chayama, Single dose of OCH improves mucosal T helper type 1/T helper type 2 cytokine balance and prevents experimental colitis in the presence of valpha14 natural killer T cells in mice, *Inflammatory Bowel Dis.* 11 (1) (2005 Jan) 35–41.