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Original research

Possible involvement of invariant natural killer T cells and mucosal-associated invariant T cells in a murine model of titanium allergy

Kenichi Kumagai^{a,b,c}, Ryota Matsubara^{a,b}, Yasunari Nakasone^{a,b}, Hiroaki Shigematsu^{a,b,c}, Kazutaka Kitaura^b, Satsuki Suzuki^d, Kuniaki Haneji^e, Yoshiki Hamada^{a,c}, Ryuji Suzuki^{b,*}

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

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

^c Center of Oral and Maxillofacial Implantology, Tsurumi University Dental Hospital, 2-1-3 Tsurumi, Tsurumi-ku, Yokohama 230-8501, Japan

^d Section of Biological Sciences, Research Center for Odontology, The Nippon Dental University School of Life Dentistry at Tokyo, 1-9-20 Fujimi, Chiyoda-ku, Tokyo 102-8159, Japan

^e Haneji Oral Surgery Clinic, Medical Corporation, 1062-2 Hayamakai, Hiraazakugai, Miyako 960-0015, Japan

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ABSTRACT

Objective: Titanium alloy has long been regarded as a biocompatible material, and is widely used for implants in medicine and dentistry. Titanium is thought to be a potential cause of metal allergy, however, accumulating T cells during development of titanium allergy have been poorly characterized because basic research based on a suitable animal model has not been performed. This study aimed to investigate the skewing of the T-cell receptor repertoire and cytokine profiles of accumulated T cells in inflamed skin during titanium allergy.

Methods: A novel model of titanium allergy was induced by two sensitizations by injection of TiCl₃ plus lipopolysaccharide solution into the mouse groin followed by two challenges with TiCl₃ into the footpad. Cytokine expression profiles, T cell phenotypes, and T-cell receptor repertoire were determined in the titanium-induced allergic footpads.

Results: Significant swelling and pathological features, such as spongiosis of the dermis, were histologically evident at 7 days after challenge in mice with titanium allergy. Characterization of the TCR repertoire revealed the presence of the TRAV10/TRAJ18 clonotype, indicative of natural killer T cells, and TRAV1/TRAJ33, associated with mucosal-associated invariant T (MAIT) cells in the inflamed skin of both the irritant and allergic mice models.

Conclusions: Our murine model of titanium-induced hypersensitivity shows that NKT cells and MAIT cells participate in titanium allergy.

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Abbreviations: CDR, Complementarity determining region; TRAT, cell receptor alpha; TRAJT, cell receptor alpha joining; TRAVT, cell receptor alpha variable; TRBT, cell receptor beta; TRBJT, cell receptor beta joining; TRBVT, cell receptor beta variable; iNKT, cells invariant NK T cells.

* AsianAOMS: Asian Association of Oral and Maxillofacial Surgeons; ASOMP: Asian Society of Oral and Maxillofacial Pathology; JSOP: Japanese Society of Oral Pathology; JSOMS: Japanese Society of Oral and Maxillofacial Surgeons; JSOM: Japanese Society of Oral Medicine; JAMI: Japanese Academy of Maxillofacial Implants

* Corresponding author.

E-mail addresses: kumagai-kenichi@tsurumi-u.ac.jp

(K. Kumagai), matsubara-ryota@tsurumi-u.ac.jp (R. Matsubara), nakasone-yasunari@tsurumi-u.ac.jp (Y. Nakasone), shigematsu-h@tsurumi-u.ac.jp (H. Shigematsu), k-kitaura@sagamihara-hosp.gr.jp (K. Kitaura),

1. Introduction

Titanium alloy is regarded as a biocompatible material with high corrosion resistance owing to its oxide layer [1]. The use of titanium in medicine and dentistry has increased over the last three decades in applications including dental implants, endoprostheses, orthodontal brackets; pacemakers, and artificial joint replacements. It has been suggested that no metallic material,

satsukis@tky.ndu.ac.jp (S. Suzuki), haneji@cosmos.ne.jp (K. Haneji), hamada-y@tsurumi-u.ac.jp (Y. Hamada), r-suzuki@sagamihara-hosp.gr.jp (R. Suzuki).

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including titanium; is totally resistant to corrosion or ionization within living tissues [1]. Degradation products of metallic biomaterials including titanium cause the elution of ions; which mediate metal hypersensitivity or allergic reactions. In particular, the rate of successful osseointegration of titanium implants is high, however, titanium is thought to be a potential cause of metal allergy in the clinical setting, and hypersensitivity to titanium ion has been suggested as a possible factor responsible for implant failures [2]. Most studies investigating titanium hypersensitivity have been performed in cases of orthopedic surgery, such as total knee joint replacement [3–5]. The results of these studies indicated that titanium debris arising from the friction of titanium implants may cause allergic reactions to titanium, however, because of its biocompatibility, the mechanism of titanium allergy has not been elucidated. Metal allergy is defined as a delayed-type hypersensitivity reaction, and caused by metal ions released from medical or dental materials, and jewelry. Unlike classical haptens, metal ions form geometrically coordinated complexes with partner molecules within the body, which causes them to become antigenic as allergens. In general, nickel (Ni), cobalt (Co), palladium (Pd), and chromium (Cr) have been reported to induce allergic contact dermatitis (ACD) [5]. To date, titanium-positive allergic reactions have only rarely been demonstrated by skin patch tests and no standardized patch test for titanium has been developed [2,6]. Furthermore; the precise immune response that occurs has not been elucidated because a suitable animal model for basic research into titanium allergy has not been established. During an immune response to metal associated antigens; clonal T cells expand due to the antigen-specific immune response. T cells bearing T cell receptor (TCR)s recognize antigens in the form of peptide fragments in the context of major histocompatibility complex (MHC) class I and II molecules on antigen-presenting cells. The high specificity of T cells is determined by the TCRs displayed on their surface; which are heterodimers of an α - and β -chain (TRA and TRB) or a γ - and δ -chain (TRG and TRD). We previously developed metal ion-induced murine allergic models for Ni, Pd, and Cr; and have determined their antigen-specific immune responses in terms of TCR usage [7–9]. These models enabled us to study the precise immune mechanism induced in these metal allergies; and provided new insight into the accumulation of metal-specific T cells in inflamed skin. Accumulated T cells in inflamed skin are thought to be pathogenic and to be essential for the induction of allergy to metals in humans. 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 allergy [10–12]. The restricted usage of TCR genes in metal allergy reflects the prolonged exposure of the host immune system to putative metal-associated antigens, however, TCR usage in titanium allergy has not yet been characterized. In the present study, to explore how accumulated T cells in the site of allergic inflammation contribute to the development of titanium allergy, we generated a titanium-induced murine model, and investigated the pathogenesis of titanium-specific immune response in terms of TCR gene usage.

2. Methods

2.1. Ethics statement

This study was performed in strict accordance with recommendations in the Guidelines for Care and Use of Laboratory Animals of the Clinical Research Center for Sagamihara National Hospital, Japan. All animal experiments were performed according to the relevant ethical requirements and with approval from the committees for animal experiments at the Clinical Research Center for Rheumatology and Allergy, Sagamihara National Hospital, Japan (reference

number: 2010-1). All experiments on mice were performed under tribromoethanol anesthesia and all efforts were made to minimize suffering.

2.2. Animals

BALB/cAJcl mice (5-week-old, female, $n=92$) were obtained from CLEA Japan (Tokyo, Japan). Mice were kept in standard aluminum cages (with a lid made of stainless-steel wire) with food and water available *ad libitum*.

2.3. Reagents

Titanium trichloride (TiCl_3) (purity >99.99%) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Lipopolysaccharide (LPS) from *Escherichia coli* (O55:B5) prepared by phenol-water extraction was purchased from Sigma (St Louis, MO, USA). TiCl_3 and LPS were dissolved in sterile saline.

2.4. Sensitization, elicitation, and measurement of irritant and allergic footpad swelling

2.4.1. Sensitization

A total of 125 μL of 10 mM TiCl_3 containing 10 $\mu\text{g}/\text{mL}$ LPS in saline was injected twice (at an interval of 7 days) intradermally (i.d.) into the left and right groin of the mice (250 μL each). Seven days after sensitization, mice were challenged for the first time.

2.4.2. Challenge for elicitation

Non-sensitized irritant contact dermatitis mice (ICD mice, total $n=40$) or sensitized allergic contact dermatitis mice (ACD mice, total $n=40$) were challenged for elicitation once or twice (at an interval of 7 days) by i.d. injection with 25 μL of 5, 10, 50, or 100 mM TiCl_3 ($n=5$ per treatment) (without LPS) in saline into the left and right footpad under anesthesia with tribromoethanol. BALB/cAJcl mice sensitized with TiCl_3 plus LPS and challenged with saline were used as controls ($n=12$). The footpad swelling was measured using a Peacock Dial Thickness Gauge (Ozaki MFG Co. Ltd, Tokyo, Japan).

2.5. Immunohistochemistry

Footpad samples were taken from titanium ion-induced ICD and ACD mice for histology and immunochemical analyses. These tissue samples were fixed with 4% paraformaldehyde-lysine-periodate overnight at 4°C. After washing with phosphate-buffered saline (PBS), fixed tissues were penetrated by soaking in 5% sucrose/PBS for 1 h, 15% sucrose in PBS for 3 h, and then 30% sucrose in PBS overnight at 4°C. The tissue samples were embedded in Tissue Mount (Chiba Medical, Saitama, Japan) and snap frozen in a mixture of acetone and dry ice. The frozen sections of footpads were cut into 6- μm -thick cryosections and air dried on poly-L-lysine-coated glass slides. For histological analyses, the cryosections were stained with hematoxylin and eosin. For immunochemical analyses, antigen retrieval was performed and the cryosections were stained using anti-mouse F4/80 (diluted 1:1000; Cl-A3-1, Abcam, Cambridge, UK) and anti-CD3 (diluted 1:500; SP7, Abcam, Cambridge, UK) monoclonal antibodies. Non-specific binding of the monoclonal antibodies was blocked by incubation of sections with PBS containing 5% normal goat or rabbit serums, 0.025% Triton X-100 (Wako Pure Chemicals Industries) and 5% bovine serum albumin (Sigma Aldrich) for 30 min at room temperature. The sections were incubated with primary monoclonal antibodies for 1 h at room temperature. After washing three times with PBS for 5 min, intrinsic peroxidase was quenched using 3% H_2O_2 in methanol. After soaking the sections in distilled water, the sections were washed twice and then incubated with a secondary antibody (biotinylated goat

anti-hamster IgG antibody or biotinylated rabbit anti-rat IgG antibody) for 1 h at room temperature. After washing three times, the sections were incubated with Vectastain ABC Reagent (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature, followed by 3,3'-diaminobenzidine (DAB) staining (0.06% DAB and 0.03% H₂O₂ in 0.1 M Tris-HCl buffer pH 7.6; Wako Pure Chemicals). Last, the tissue sections were counterstained with hematoxylin.

2.6. Isolation of total RNA from tissues

Footpad samples were taken from mice and immediately placed 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 the manufacturer's instructions.

2.7. Quantitative polymerase chain reaction

The mRNA levels of T cell-related CD antigens, cytokines, and cytotoxic granules were measured by quantitative polymerase chain reaction (qPCR) using the Bio-Rad CFX96 system (Bio-Rad, Hercules, CA, USA). The specific primers for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *CD4*, *CD8*, interferon- γ (*IFN* γ), interleukin-4 (*IL4*), tumor necrosis factor- α (*TNF* α), *Fas*, and *Fas* ligand (*FasL*) were as previously described [7–9]. Freshly isolated total RNA from the footpads of mice was converted to cDNA using PrimeScriptTM RT Reagent Kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The PCR reaction consisted of 5 μ L of SsoFastTM EvaGreen[®] Supermix (Bio-Rad), 3.5 μ L of RNase/DNase-free water, 0.5 μ L of 5 μ M primer mix, and 1 μ L of cDNA in a final volume of 10 μ L. Cycling conditions were as follows: 30 s at 95 °C, followed by 45 rounds of 1 s at 95 °C and 5 s at 60 °C. At the end of each program, melting curve analyses were performed from 65 °C to 95 °C to confirm the homogeneity of PCR products. We repeated the assays three times and the mean values were used for gene expression levels. Five rounds of tenfold serial dilutions of each standard transcript were used to determine the absolute quantification, specification, and amplification efficiency of each primer set. Standard transcripts were generated by *in vitro* transcription of the corresponding PCR product into a plasmid. The nucleotide sequences were confirmed by DNA sequencing using the CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA). Sequence quality and concentration were checked using the Agilent DNA 7500 Kit in an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). *GAPDH* gene expression was used as an internal control, with the mRNA levels of the genes of interest normalized to *GAPDH* expression.

2.8. Unbiased amplification of TCR genes, amplicon sequencing and assignment of TRV and TRJ segments in TCR genes

Total RNA was prepared from the footpads of ICD and ACD mice at day 7 after challenge, and converted to complementary DNA (cDNA) with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). Next, TCR genes were amplified using adaptor ligation-mediated PCR [13]. High-throughput sequencing was performed using the Illumina Miseq paired-end platform (2 $\times$ 300 bp) (Illumina, San Diego, CA, USA). Assignment of TRV and TRJ segments in TCR genes was performed based on the international ImmunoGeneTics information system[®] (IMGT) database (<http://www.imgt.org>). Data processing, assignment, and data aggregation were automatically performed using repertoire analysis software originally developed by our group (Repertoire Genesis, Osaka, Japan).

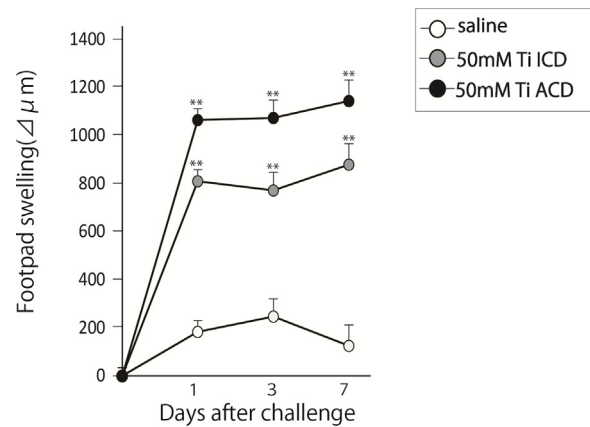


Fig. 1. Footpad swelling in mice with titanium-induced allergy. Footpad swelling from 1 to 7 days after challenge was significantly higher in allergic contact dermatitis (ACD) mice compared with control, and irritant contact dermatitis (ICD) mice. Bars and error bars indicate mean plus standard deviation. ** $p < 0.01$ (Kruskal Wallis test). $N = 20$ for each group.

2.9. TCR data analyses

The amino acid sequences of CDR3 regions between the conserved cysteine at position 104 (Cys104) of IMGT nomenclature and the conserved phenylalanine at position 118 (Phe118) with the following glycine (Gly119) were translated from the nucleotide sequences. A unique sequence read (USR) was defined as a sequence read having no identity in TRV, TRJ and deduced amino acid sequence of CDR3 with other sequence reads. The copy number of identical USRs were automatically counted by repertoire analysis software in each sample and then ranked in order of the copy number. Percentage occurrence frequencies of sequence reads with TRAV, TRAJ, TRBV and TRBJ genes in total sequence reads were calculated.

2.10. Statistical analysis

The differences between groups were analyzed statistically using the Student's unpaired *t*-test, and Kruskal Wallis test using StatView 5.0 for Windows (SAS Institute Inc., Cary, NC, USA). A *P* value less than 0.05 was determined to be statistically significant.

3. Results

3.1. Footpad swelling in titanium ion-induced allergic mice

To examine the accumulated inflammatory cells in inflamed skin, we generated a novel murine model of titanium-induced allergic mice (Fig. 1). In ACD mice, TiCl₃ containing 10 μ g/mL LPS in saline was injected twice by i.d. into the left and right groins. To differentiate between allergic contact and irritant contact reactions, a second cohort were not presensitized (ICD mice). Both groups were challenged for elicitation of an immune response by i.d. injection with TiCl₃ (without LPS) in saline into the left and right footpads. Mice sensitized with titanium plus LPS and challenged with saline were used as controls. Footpad swelling was measured every day after titanium injection into the footpad. The peak of footpad swelling was observed 3 days after the challenge in control mice. Footpad swelling from 1 to 7 days after challenge was significantly higher in ACD mice compared with control and ICD mice (Figs. 1 and 2). Footpad swelling was significantly higher in ACD mice challenged with 50 mM TiCl₃ compared with 5 or 10 mM TiCl₃ (Supplement Fig. 1). In the ACD mice challenged with 100 mM TiCl₃,



Fig. 2. Macroscopic findings of footpad swelling in mice with titanium-induced allergy. Significant redness and swelling are evident in the footpad of mice with ACD compared with those of control mice at 7 days challenged by saline, and ICD at 7 days after challenge. N = 10 for each group.

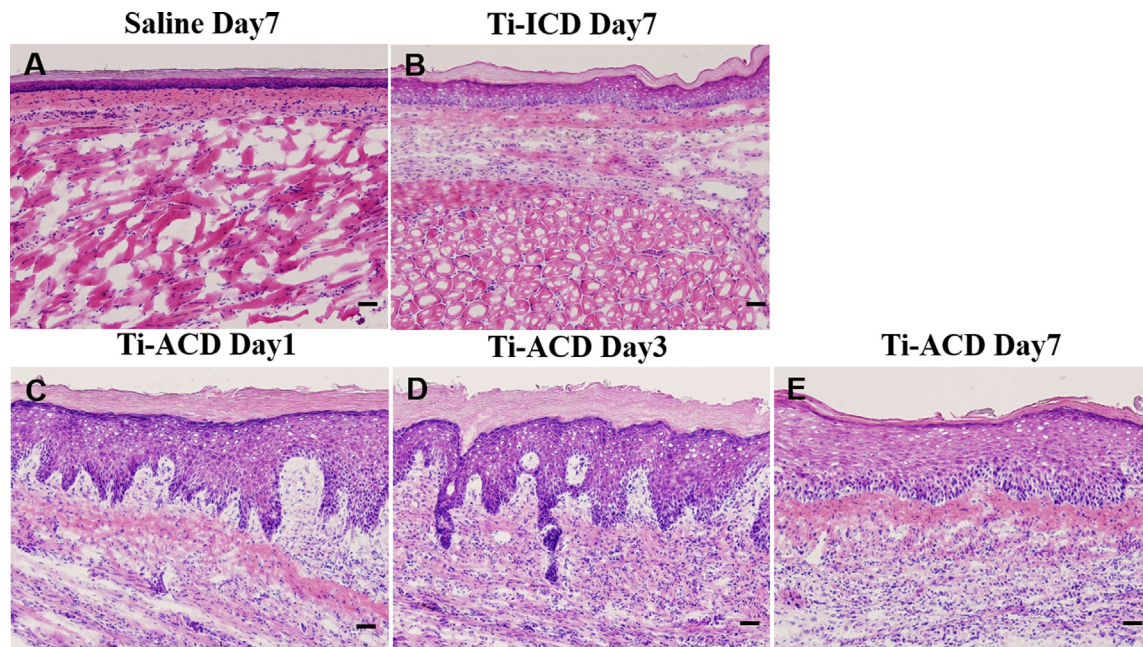


Fig. 3. Histopathological analyses of hematoxylin and eosin staining in the footpads of mice with titanium-induced allergy. Footpad tissue sections were stained with hematoxylin and eosin at 7 days challenged by saline (A), ICD (B), and ACD (C–E) mice. At 3 days after challenge, the epithelial ridges become elongated and broadened, and at 7 days after challenge, hyperkeratosis and acanthosis with abundant infiltration of dermal lymphocytes can be seen in ACD mice; however, the degree of inflammatory reaction in the footpads was less in the ICD mice (G–I). Scale bar = 40 μ m. Images are representative mice of each group.

the footpad tissue became necrotic, and some mice died. Therefore, we selected 50 mM as the most suitable dose of TiCl_3 for challenges.

In the twice challenged ACD mice, the footpad became obviously swollen compared with those of once challenged ACD mice during the 14 days after the challenge (Supplement Fig. 2). After two challenges, footpad swelling in ACD mice reached a maximum at 7 days after the challenge, and reduced to basal levels at 14 days. (Supplement Fig. 2). Mice were further challenged with a dose of 10 mM TiCl_3 at 7 days after the final challenge, but footpad swelling was not enhanced.

3.2. Histological and immunohistochemical analyses of CD3 and F4/80 in the footpads of titanium-induced ICD and ACD mice

To verify whether antigen-presenting cells (APCs) and T cells infiltrated into the inflamed skin, we analyzed the footpad skin of titanium-induced ICD and ACD mice and control mice at 1, 3, and 7 days after challenge (Fig. 3). Hematoxylin and eosin staining showed dense mononuclear infiltrates in the epithelial basal layer and upper dermis, and epidermal spongiosis and liquefaction degeneration of the epithelial basal layer in the ACD mice at 1 day after challenge. At 3 days after challenge, the epithelial ridges become elongated and broadened, and at 7 days after challenge, hyperkeratosis and acanthosis with abundant infiltration of dermal lymphocytes can be seen in ACD mice; however, the degree

of inflammatory reaction in the footpads was less in the ICD mice (Fig. 3). Immunohistochemical staining of sections from ACD mice showed that CD3-positive T cells were present in the epithelial basal layer and the upper dermis, but not in the control or ICD mice (Fig. 4A–F). T cells in ACD mice 7 days after challenge were predominantly CD4-positive (Fig. 5). In contrast, there were few CD8-positive T cells detected 7 days after challenge (Fig. 5). Notably, small numbers of F4/80-positive APCs were found in the epithelial layer in ICD mice, however, they were abundant in ACD mice from 1 to 7 days after challenge (Fig. 4E and F).

3.3. Expression levels of T cell markers, related cytokines, and apoptotic-related genes in footpads of titanium induced allergic mice

Footpad mRNA levels of CD3 were significantly higher in twice challenged ACD mice than in once challenged ACD mice (Supplement Fig. 3). CD4 and CD8 expression at 7 days after the final challenge were measured by qPCR as CD3 positive T cells had infiltrated into inflamed skin in ACD mice (Fig. 6). Consistent with the results of the histological examination, CD4 levels were significantly higher in ACD mice than in control and ICD mice; however, CD8 levels in skin were not significantly different between ICD, ACD, and control mice. We also compared the expression levels of a Th1-related gene (*IFN γ*), and Th2-related gene (*IL4*) in foot-

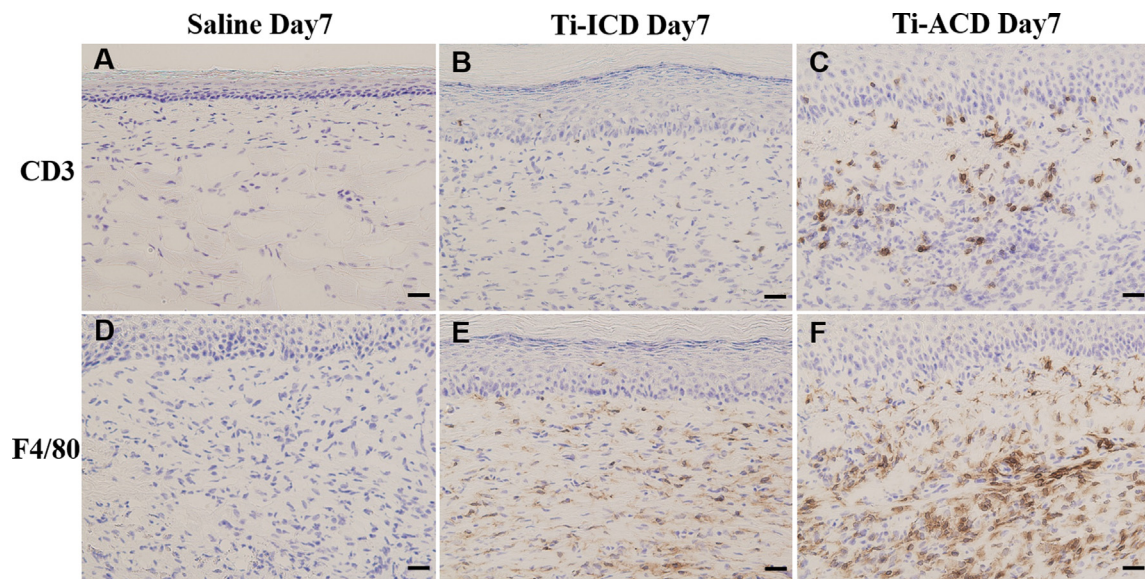


Fig. 4. Immunohistochemical analyses of accumulating T cells and antigen-presenting cells in the footpads of mice with titanium-induced allergy. Footpad tissue sections were stained for CD3 (A–C) and F4/80 (D–F). Infiltrating CD3-positive T cells are present in the epithelial basal layer and the upper dermis in mice with ACD, but not in control mice or those with ICD (A–C). Few F4/80-positive antigen-presenting cells are present in the epithelial layer in ICD; however, they were abundant in ACD (D–F). Scale bar = 100 μ m. Images are representative of each group.

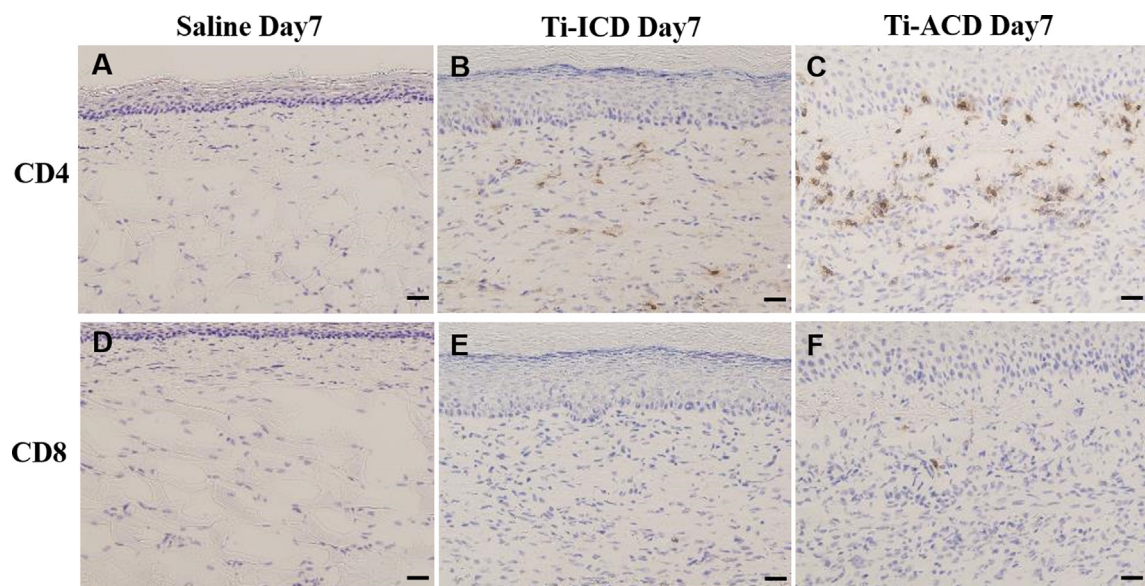


Fig. 5. Immunohistochemical analyses of CD4/CD8 T cell phenotype in the footpads of mice with titanium-induced allergy. Sections were stained for CD4 (A–C) and CD8 (D–F). Infiltrating CD4-positive cells were present in the epithelial basal layer and the upper dermis in mice with ACD, but not in control mice or those with ICD (A–C). Few CD8-positive cells were detected in any mice. The arrows show the CD8-positive cells (D–F). Scale bar = 100 μ m. Images are representative of each group.

pads of titanium ion-induced ACD mice (Fig. 6). In ACD mice, the expression level of *IFN γ* was significantly higher in ACD mice than in control and ICD mice. In contrast, the expression level of *IL4* was significantly lower in ACD mice than in control and ICD mice. The mRNA levels of the apoptosis-related genes *Fas* and *FasL* were not significantly higher in ACD mice than in control and ICD mice (Fig. 6).

3.4. TCR repertoire usage in ICD and ACD mice at Day 7 after challenge

To determine the TCR repertoire of T cells infiltrating the footpads of ICD and ACD mice at day 7 after challenge, we produced

3D pictures of the TCR repertoire bearing a combination of TRV with TRJ genes (Figs. 7 and 8). The advantage of 3D representations is that the dominance of certain combinations of TRV with TRJ genes as well as the extent of diversity of TCR usage can easily be observed. 3D imaging of the TRA repertoire indicated a low level of expression with a wide distribution of TRAV and TRAJ. The occupancy was lower than 1% for all combinations. Of note, TCR gene usage of AV10/AJ8 was high both in ICD and ACD mice (Fig. 7).

In contrast, there was little preferential usage of recombination between TRBJ and TRBV genes. The frequency of each recombination depended on the usage of TRBV or TRBJ. Some TRBV/TRBJ subfamilies were frequently used in all combinations while others were expressed at a low frequency (Fig. 8).

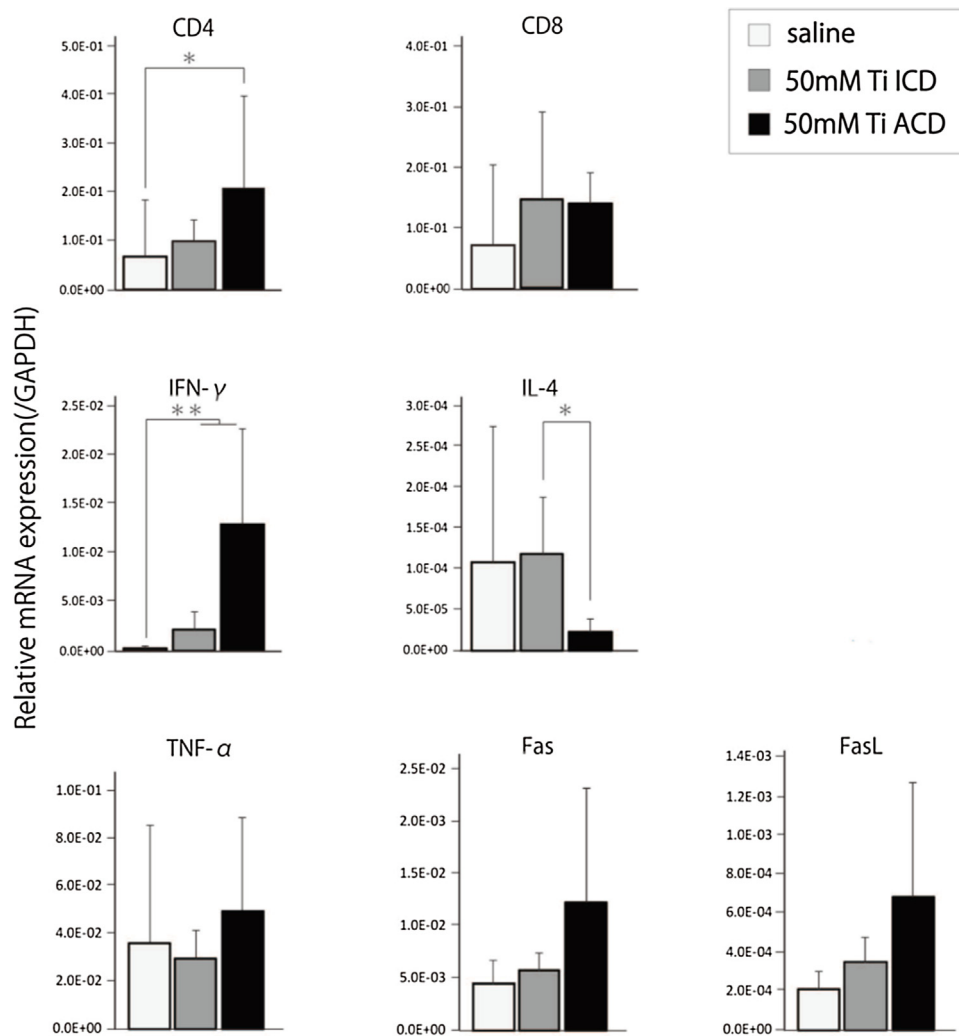


Fig. 6. mRNA expression of T cell phenotypes, T cell-related cytokines, and apoptosis-related genes in mice with titanium-induced allergy.

Footpad mRNA levels of *IFN γ* were significantly higher in mice with ACD compared with control mice and those with ICD. However, the mRNA levels of the apoptosis-related genes *Fas* and *FasL* were not significantly higher in ACD mice than in control and ICD mice. Bars and error bars indicate mean plus standard deviation. * $p < 0.05$, ** $p < 0.01$ (Kruskal Wallis test). $N = 10$ for each group.

Next, we investigated the common CDR3 sequences of TRA and TRB between ICD and ACD mice. Interestingly, shared TRA sequences contained a high proportion of the invariant TCR α s indicative of iNKT cells or MAIT cells (Fig. 7, Supplement Tables 1 and 2). It was reported that MAIT cells express TRAV1 and TRAJ33 while iNKT express TRAV10 and TRAJ18. The total percentage frequencies of iNKT TRAs bearing TRAV10 and TRAJ18, and MAIT TRAs bearing TRAV1 and TRAJ33 were 5.86% and 0.93% in ICD, and 4.11% and 0.41% in ACD respectively. Of shared TRA sequences, TRAV7D-2/AJ30 was observed at 0.72% in ICD, and 0.40% of ACD (Fig. 7, Tables 1, 2). However, there was no shared TRB clone in ICD and ACD mice (supplement Tables 3 and 4).

4. Discussion

This study demonstrated the successful establishment of a murine model of titanium ion-induced allergy and revealed the histopathological features of delayed type hypersensitivity, including spongiosis and liquefaction degeneration, in the epidermis, caused by intercellular edema and lymphocytic infiltration. In addition, we identified a titanium-allergen specific immune response with NK T cells, and MAIT cells in titanium-induced allergic mice. This is the first report regarding the establishment of a titanium

allergic murine model with involvement of innate lymphocytes in metal hypersensitivity.

MAIT cells are a novel class of innate like T cells, expressing a semi-invariant TCR and able to recognize small molecules presented on the non-polymorphic MHC related protein MR-1 [14]. NK T cells (also called iNKT cells and Va14 iNKT cells) were originally defined as T cells that expressed CD161c and other receptors typical of NK cells. This definition has subsequently been refined to specify a subpopulation of T cells that express a TCR with an invariant TCR Va chain paired with a restricted subset of TCR Vb chains [15]. Recently, we successfully identified distinctive metal-specific TCR repertoires in the inflamed skin at the elicitation phase in Pd-, Ni-, and Cr-allergic mice [7–9]. In Ni and Cr allergies, we observed iNKT cells in lymphocytic infiltrates at a high frequency during the elicitation phase, and have suggested that metal-specific T cells driven by invariant NK T cells might contribute to the pathogenesis of Ni and Cr allergy.

Little is known of the exact population of MAIT cells in disease, because specific immunological tools for their analysis are limited. Although MAIT cells suggested to be relatively rare in mice, however, they dominate a very significant population of T cells in humans, accounting for between 1% and 10% of T cells in peripheral blood, human liver and mucosal tissues [16–19]. MAIT

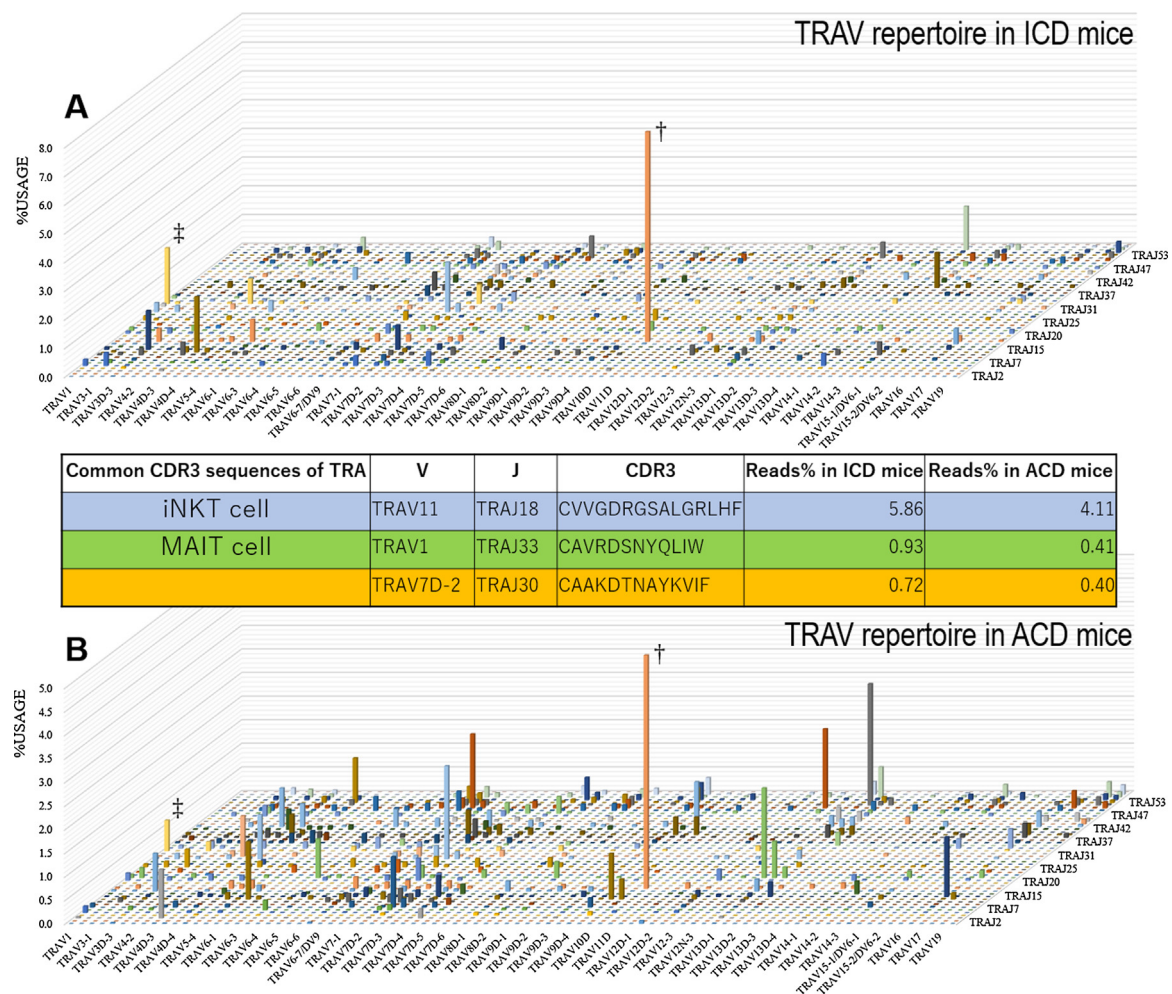


Fig. 7. 3D Graphic analysis of TRAV repertoire in ICD and ACD mice at Day 7 after final challenge.

3D pictures of the TCR repertoire bearing a combination of TRV with TRJ genes show the skewing of T cells infiltrating the footpads of ICD and ACD mice at day 7 after challenge. 3D imaging of the TRA repertoire indicated a low level of expression with a wide distribution of TRAV and TRAJ. Of note, TCR genes for AV10/AJ8 were highly expressed both in ICD and ACD mice. †:TRAs bearing TRAV10 and TRAJ18, and ‡:TRAs bearing TRAV1 and TRAJ33.

cells comprise a very small population of cells in mice, making functional analysis very difficult in this experimental animal. In the present study, we conducted TCR repertoire analysis using next-generation sequencing (NGS) of TCR VA and VB genes. This NGS method enables a comprehensive quantitative analysis of TCR repertoires at a clonal level, and is also useful for the population and new roles of invariant TCR α chains including MAIT cells.

It has also been suggested that MAIT cells are involved in the recruitment of NK T cells, and consequently control the delayed type hypersensitivity [20]. Thus, the expression of innate lymphocytes including MAIT cells and NK T cells would act as an early amplification step in the innate immune response in the sensitization phase, and provide a bridge to adaptive immunity in the elicitation phase in the titanium-specific immune response.

Titanium has long been regarded as having high corrosive resistance owing to its oxide layer, which spontaneously forms and covers the surface [21,22]. In general, metal ions can be released under conditions such as corrosion and wear, which can lead to either failure of implants to function as they are intended or the induction of an adverse allergic reaction in the patient. In the clinical setting of implant-related hypersensitivity, aseptic loosening of articular implants was recently investigated by Huber et al. [23]. The periprosthetic tissues from 11 cases containing unusual solid deposits were analyzed. The corrosion products and hypersensitivity-associated tissue reactions were histologically

observed, indicating the possible relationship between corrosion development and implant-related hypersensitivity. The hypothesis is that implant-derived wear particles caused by corrosion or peri-implantitis initiate a foreign body inflammatory reaction in the joint capsule and along implant–bone interfaces, which results in bone loss and aseptic loosening. Witt & Swann reported 13 cases of failed total hip replacements, and indicated that tissue reaction in response to metal-wear debris may contribute to the early failure of orthopedic implants [24]. A titanium oxide surface rapidly reforms in response to wear damage, which is referred to as repassivation of titanium [24,25]. Previous study has suggested that titanium and other elements released from titanium implants have been detected in tissues near the implants [26,27]. Regarding the pathophysiological influence of titanium ions, previous research has suggested that released titanium ions may combine with biomolecules, such as native proteins, to form a protein-material complex, becoming an immunogenic antigen, and eliciting a delayed type IV hypersensitivity with the local presence of abundant macrophages and T lymphocytes, and the absence of B lymphocytes [28,29]. Otherwise, recent study has suggested that metal nanoparticles serve as ion carriers to enable metal sensitization, and have a role of potentially new trigger for metal allergy [30]. The understanding of initial trigger for metal allergy is controversial issue, however, our results would support these studies suggesting implant failure is caused by titanium-induced hypersensitivity.

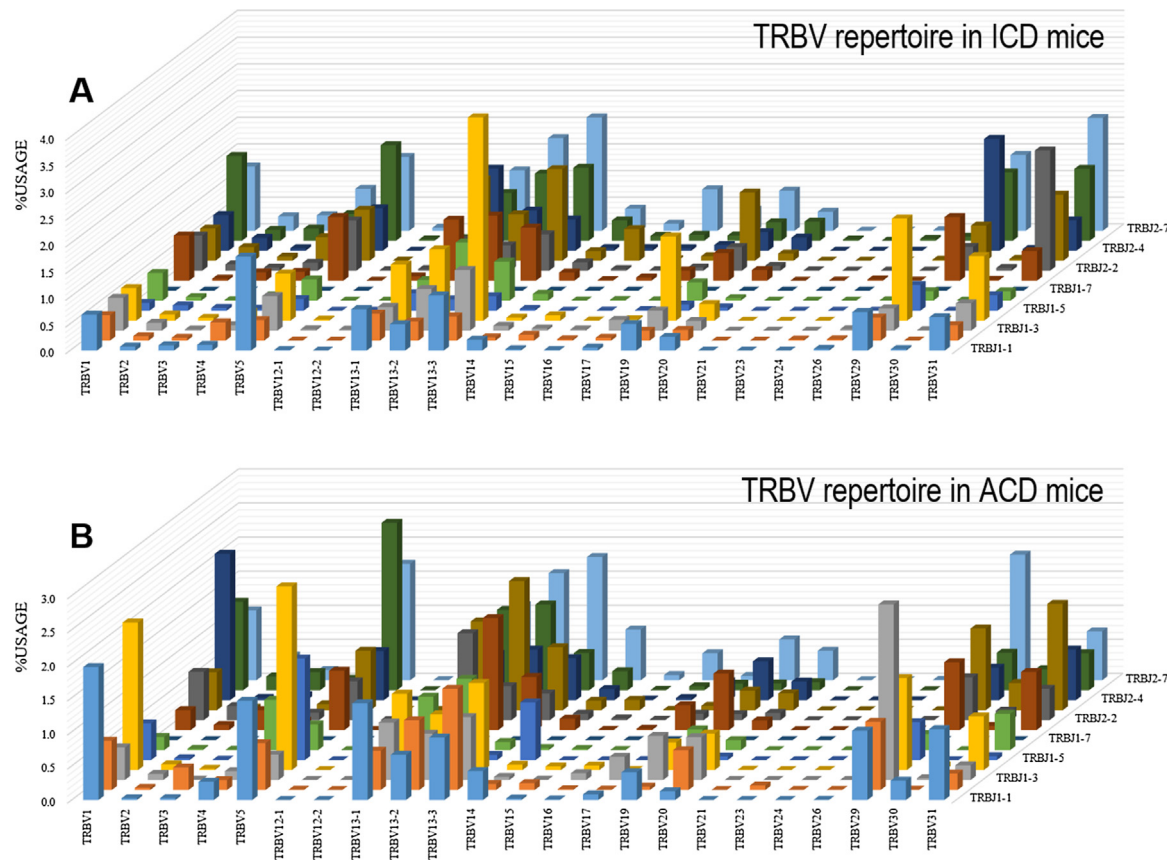


Fig. 8. 3D Graphic analysis of TRBV repertoire in ICD and ACD mice at Day 7 after final challenge. Some TRBV/TRBJ subfamilies were frequently used in all combinations while others were expressed at a low frequency.

ICD is a form of non-specific inflammatory dermatitis, mainly caused by the toxicity of chemicals to skin cells with the activation of innate immunity [31]. It has been suggested that titanium particles have cytotoxic effects on osteoblasts *in vitro* [32]. Consistent with this, the pathological reaction of ICD mice in this study also suggests that titanium ions may be cytotoxic *in vivo*. Hapten-induced effects mediated by innate immunity are essential for the activation of cutaneous APCs [33]. Indeed, the innate immune response is a prerequisite for the pathogenesis of ACD [34]. When epidermal keratinocytes are partially separated by the stimuli of heavy metal ions, spongiotic dermatitis can develop in both ICD and ACD [7]. The dermal spongiosis is a characteristic histopathological feature in acute eczema [35]. Inflammatory reactions in the footpad were relatively less severe in ICD mice than in ACD mice at 7 days after challenge (Fig. 3B, E).

ACD is defined as a delayed-type hypersensitivity response, in which skin inflammation is mediated by antigen-specific T cells [26]. It can be elicited by either CD4- or CD8-positive T cells depending on the pathway by which the antigen is processed [36,37]. In this study, the accumulation of large numbers of CD4-positive T cells was observed in ACD mice, but not in ICD mice (Figs. 5, 6). The CD4/CD8T cell phenotype observed was consistent with other metal hypersensitivity models in mice, such as Ni, Pd, and Cr hypersensitivity [7–9,13]. The role of metal-specific CD4⁺/CD8⁺ or Th1/Th2 cell subsets in the pathogenesis of allergic contact dermatitis in man is largely unknown. Our results suggest that CD4⁺ T cells, rather than cytotoxic CD8⁺ effector T cells, play an important role in the titanium-specific immune response, however, further experiments such as transferring into T-cell deficient mice would be required to clarify the T-cell clonality responsible for titanium hypersensitivity.

In the elicitation phase of ACD, keratinocytes are stimulated by metal ions, which subsequently generate several proinflammatory cytokines that activate epithelial tissues and induce memory metal-specific T cells [38]. Infiltrated metal-specific effector T cells are also activated, which produce cytokines that activate keratinocytes and induce further inflammatory cell infiltration. At 7 days after challenge, the epithelial ridges become elongated and broadened as hyperkeratosis, and the abundant infiltration of dermal lymphocytes and vascular dilatations can be seen (Fig. 3). The pathological features in our titanium ion-induced ACD mice at 7 days after challenge resemble the clinical features of ACD in humans. Thus, our model faithfully mimics the immune response of metal allergy-inflamed human skin.

We further investigated the cytokine expression profiles in the footpad in response to titanium ion-induced hypersensitivity in mice. The expression level of *IFN* γ was significantly higher in ACD mice than in control and ICD mice. In our titanium ion-induced ACD model, the spongiosis of keratinocytes was affected by infiltrating T cells (Fig. 3). In this study, the mRNA levels of *Fas*/*FasL* were not significantly higher than those of control and ICD mice, however, histological apoptotic changes, such as the spongiosis of keratinocytes, were observed in ACD mice. These results suggest that upregulation of IFN- γ produced by accumulating Th1 cells and consequent keratinocyte apoptosis may be involved in the pathogenesis of titanium hypersensitivity.

5. Conclusions

We established a mouse model of titanium-induced allergy, and have provided evidence of a titanium-specific immune response with involvement of NK T cells and MAIT cells. This novel mouse

model is useful for the study of the pathogenesis of titanium allergy, and the intriguing results obtained from this study will provide new insight into the immune response to titanium-induced delayed-type hypersensitivity. Further studies using this mouse model of titanium allergy will contribute to the foundation knowledge required for reliable diagnosis of titanium hypersensitivity. We thus speculate that the regulation of innate lymphocytes might be a new diagnostic or therapeutic target for titanium allergy.

Conflicts of interest

The authors state no conflicts of interest.

Acknowledgments

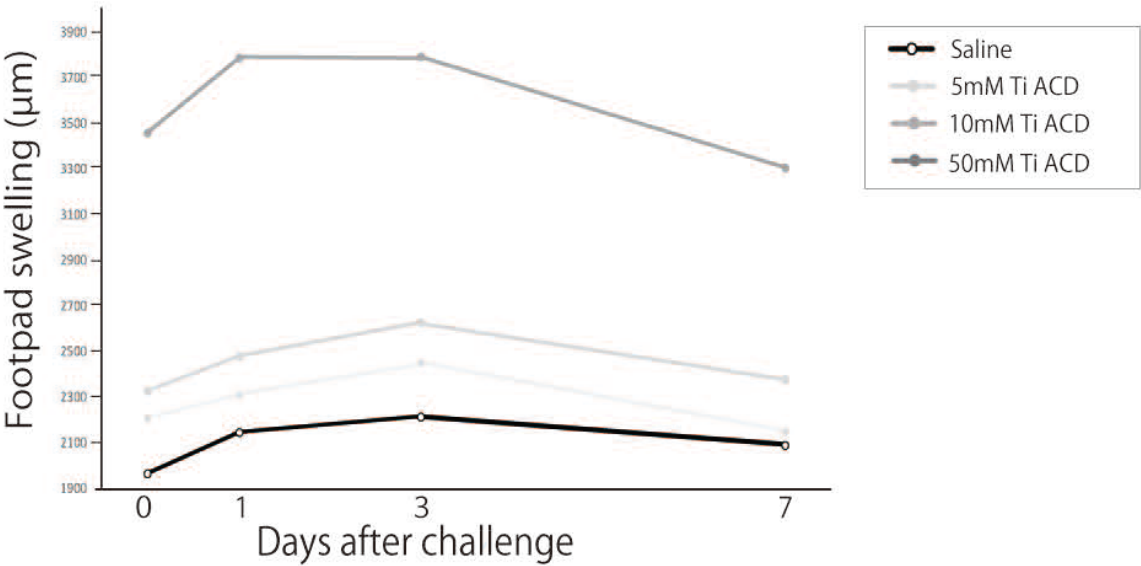
This work was supported by the Japan Society for the Promotion of Science KAKENHI Grant-in-Aid for Scientific Research C Grant No. 15K11329.

Appendix A. Supplementary data

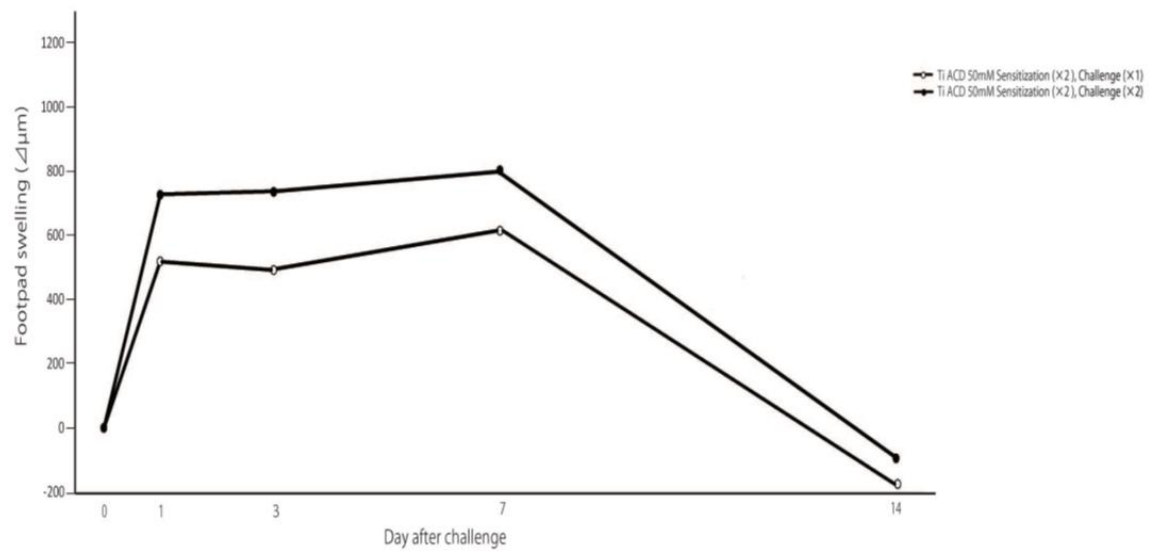
Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ajoms.2017.08.001>.

References

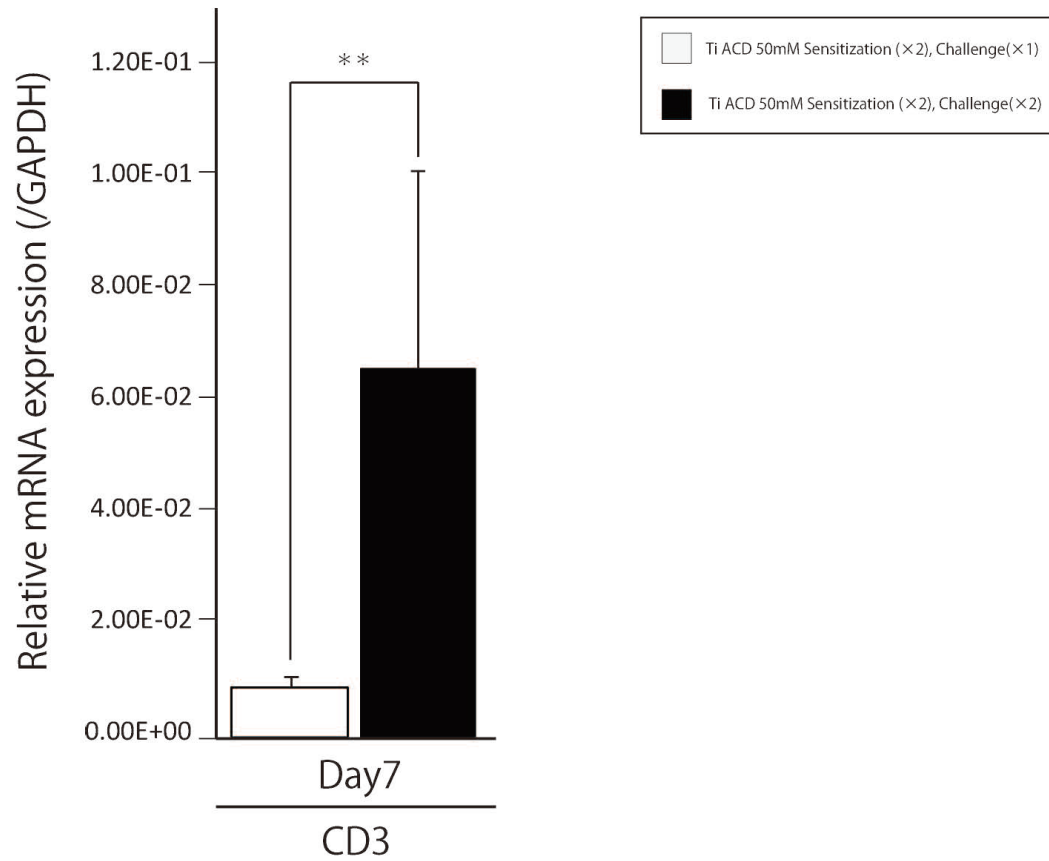
- [1] Mareci D, Chelariu R, Gordin DM, Ungureanu G, Gloriant T. Comparative corrosion study of Ti-Ta alloys for dental applications. *Acta Biomater* 2009;5:3625–39.
- [2] Siddiqi A, Payne AG, De Silva RK, Duncan WJ. Titanium allergy: could it affect dental implant integration. *Clin Oral Implants Res* 2011;22:673–80.
- [3] Hallab N, Merritt K, Jacobs JJ. Metal sensitivity in patients with orthopaedic implants. *J Bone Joint Surg Am* 2001;83-a:428–36.
- [4] Zeng Y, Feng W, Li J, Lu L, Ma C, Zeng J, et al. A prospective study concerning the relationship between metal allergy and post-operative pain following total hip and knee arthroplasty. *Int Orthop* 2014;38:2231–6.
- [5] Forte G, Petrucci F, Bocca B. Metal allergens of growing significance: epidemiology, immunotoxicology, strategies for testing and prevention. *Inflamm Allergy Drug Targets* 2008;7:145–62.
- [6] Kim HJ, Shin JU, Lee J, Lee H, Jin S, Kim SH, et al. Positive reactions to nickel on a patch test do not predict clinical outcome of nickel alloy-based atrial septal defect occluder implantation. *Dermatology* 2015;230:184–8.
- [7] Shigematsu H, Kumagai K, Kobayashi H, Eguchi T, Kitaura K, Suzuki S, et al. Accumulation of metal-specific T cells in inflamed skin in a novel murine model of chromium-induced allergic contact dermatitis. *PLoS One* 2014;9:e85983.
- [8] Kobayashi H, Kumagai K, Eguchi T, Shigematsu H, Kitaura K, Kawano M, et al. Characterization of T cell receptors of Th1 cells infiltrating inflamed skin of a novel murine model of palladium-induced metal allergy. *PLoS One* 2013;8:e76385.
- [9] Eguchi T, Kumagai K, Kobayashi H, Shigematsu H, Kitaura K, Suzuki S, et al. Accumulation of invariant NKT cells into inflamed skin in a novel murine model of nickel allergy. *Cell Immunol* 2013;284:163–71.
- [10] Hashizume H, Seo N, Ito T, Takigawa M, Yagi H. Promiscuous interaction between gold-specific T cells and APCs in gold allergy. *J Immunol* 2008;181:8096–102.
- [11] Budinger L, Neuser N, Totzke U, Merk HF, Hertl M. Preferential usage of TCR-Vbeta17 by peripheral and cutaneous T cells in nickel-induced contact dermatitis. *J Immunol* 2001;167:6038–44.
- [12] Silvennoinen-Kassinen S, Karvonen J, Ikaheimo I. Restricted and individual usage of T-cell receptor beta-gene variables in nickel-induced CD4+ and CD8+ cells. *Scand J Immunol* 1998;48:99–102.
- [13] Kitaura K, Yamashita H, Ayabe H, Shini T, Matsutani T, Suzuki R. Different somatic hypermutation levels among antibody subclasses disclosed by a new next-generation sequencing-based antibody repertoire analysis. *Front Immunol* 2017;8:389.
- [14] Tilloy F, Treiner E, Park SH, Garcia C, Lemonnier F, De La Salle H, et al. An invariant T cell receptor alpha chain defines a novel TAP-independent major histocompatibility complex class Ib-restricted alpha/beta T cell subpopulation in mammals. *J Exp Med* 1999;189:1907–21.
- [15] Taniguchi M, Harada M, Kojo S, Nakayama T, Wakao H. The regulatory role of Valpha14 NKT cells in innate and acquired immune response. *Annu Rev Immunol* 2003;21:483–513.
- [16] Martin E, Treiner E, Duban L, Guerri L, Hln Laude, Cc Toly, et al. Stepwise development of MAIT cells in mouse and human. *PLoS Biol* 2009;7:e1000054.
- [17] Reantragoon R, Corbett AJ, Sakala IG, Gherardin NA, Furness JB, Chen Z, et al. Antigen-loaded MR1 tetramers define T cell receptor heterogeneity in mucosal-associated invariant T cells. *J Exp Med* 2013;210:2305–20.
- [18] Tang XZ, Jo J, Tan AT, Sandalova E, Chia A, Tan KC, et al. IL-7 licenses activation of human liver intrasinusoidal mucosal-associated invariant T cells. *J Immunol* 2013;190:3142–52.
- [19] Dusseaux M, Martin E, Serriari N, Peguillet I, Premel V, Louis D, et al. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. *Blood* 2011;117:1250–9.
- [20] Shimamura M, Huang YY, Goji H, Endo S, Migishima R, Yokoyama M. Regulation of immunological disorders by invariant Valpha19-Jalpha33 TCR-bearing cells. *Immunobiology* 2011;216:374–8.
- [21] Zitter H, Plenck Jr H. The electrochemical behavior of metallic implant materials as an indicator of their biocompatibility. *J Biomed Mater Res* 1987;21:881–96.
- [22] Bozzini B, Carlino P, D'Urzo L, Pepe V, Mele C, Ventura F. An electrochemical impedance investigation of the behaviour of anodically oxidised titanium in human plasma and cognate fluids, relevant to dental applications. *J Mater Sci Mater Med* 2008;19:3443–53.
- [23] Huber M, Reinisch G, Trettenhahn G, Zweymuller K, Lintner F. Presence of corrosion products and hypersensitivity-associated reactions in periprosthetic tissue after aseptic loosening of total hip replacements with metal bearing surfaces. *Acta Biomater* 2009;5:172–80.
- [24] Witt JD, Swann M. Metal wear and tissue response in failed titanium alloy total hip replacements. *J Bone Joint Surg Br* 1991;73:559–63.
- [25] Lalor PA, Revell PA, Gray AB, Wright S, Railton GT, Freeman MA. Sensitivity to titanium. A cause of implant failure? *J Bone Joint Surg Br* 1991;73:25–8.
- [26] Olmedo DG, Guglielmotti MB, Cabrini RL. An experimental study of the dissemination of Titanium and Zirconium in the body. *J Mater Sci Mater Med* 2002;13:793–6.
- [27] Olmedo DG, Paparella ML, Brandizzi D, Cabrini RL. Reactive lesions of peri-implant mucosa associated with titanium dental implants: a report of 2 cases. *Int J Oral Maxillofac Surg* 2010;39:503–7.
- [28] Holgers KM, Roupe G, Tjellstrom A, Bjursten LM. Clinical, immunological and bacteriological evaluation of adverse reactions to skin-penetrating titanium implants in the head and neck region. *Contact Dermatitis* 1992;27:1–7.
- [29] Hallab NJ, Mikecz K, Vermes C, Skipor A, Jacobs JJ. Orthopaedic implant related metal toxicity in terms of human lymphocyte reactivity to metal-protein complexes produced from cobalt-base and titanium-base implant alloy degradation. *Mol Cell Biochem* 2001;222:127–36.
- [30] Hirai T, Yoshioka Y, Izumi N, Ichihashi K, Handa T, Nishijima N, et al. Metal nanoparticles in the presence of lipopolysaccharides trigger the onset of metal allergy in mice. *Nat Nanotechnol* 2016;11:808–16.
- [31] Nosbaum A, Vocanson M, Rozieres A, Hennino A, Nicolas JF. Allergic and irritant contact dermatitis. *Eur J Dermatol* 2009;19:325–32.
- [32] Pioletti DP, Takei H, Kwon SY, Wood D, Sung KL. The cytotoxic effect of titanium particles phagocytosed by osteoblasts. *J Biomed Mater Res* 1999;46:399–407.
- [33] Honda T, Egawa G, Grabbe S, Kabashima K. Update of immune events in the murine contact hypersensitivity model: toward the understanding of allergic contact dermatitis. *J Invest Dermatol* 2013;133:303–15.
- [34] Martin SF, Esser PR, Weber FC, Jakob T, Freudenberg MA, Schmidt M, et al. Mechanisms of chemical-induced innate immunity in allergic contact dermatitis. *Allergy* 2011;66:1152–63.
- [35] Saint-Mezard P, Rosieres A, Krasteva M, Berard F, Dubois B, Kaiserlian D, et al. Allergic contact dermatitis. *Eur J Dermatol* 2004;14:284–95.
- [36] Saint-Mezard P, Berard F, Dubois B, Kaiserlian D, Nicolas JF. The role of CD4+ and CD8+ T cells in contact hypersensitivity and allergic contact dermatitis. *Eur J Dermatol* 2004;14:131–8.
- [37] Sumiwi YA, Soesatyio MH, Sosroseno W. The role of CD4+ T cells in the induction of contact hypersensitivity to mercury in a murine model. *Cutan Ocul Toxicol* 2010;29:30–3.
- [38] Kumagai K, Horikawa T, Shigematsu H, Matsubara R, Kitaura K, Eguchi T, et al. Possible immune regulation of Natural Killer T Cells in a murine model of metal ion-induced allergic contact dermatitis. *Int J Mol Sci* 2015;12:17.



Supplement Fig. 1. Footpad swelling after challenge with each dose of TiCl3 in the preliminary examination of this study.



Supplement Fig. 2. Footpad swelling in twice sensitized–once or twice challenged ACD mice.



Supplement Fig. 3. mRNA expression of CD3 in twice sensitized–once or twice challenged ACD mice.

Supplement Table 1. Top 30 Ranking TRAV clonotypes in ICD mice at Day 7 after final challenge

Rank	V	J	CDR3	Reads%
1	TRAV11	TRAJ18	CVVGDRGSALGRLHF	5.86
2	TRAV4-4/DV10	TRAJ13	CAASNSGTYQRF	1.70
3	TRAV14-1	TRAJ57	CAASARGGSAKLIF	1.32
4	TRAV3D-3	TRAJ15	CAVSAGQGGRALIF	1.07
5	TRAV1	TRAJ33	CAVRDSNYQLIW	0.93
6	TRAV4D-3	TRAJ33	CAAEGSNYQLIW	0.77
7	TRAV7-6	TRAJ52	CGGYTGANTGKLTF	0.74
8	TRAV14-2	TRAJ40	CAAKLNTGNYKYVF	0.73
9	TRAV7D-2	TRAJ30	CAAKDTNAYKVIF	0.72
10	TRAV5D-4	TRAJ18	CAASVKGSALGRLHF	0.65
11	TRAV7D-3	TRAJ33	CAVPMDSNYQLIW	0.55
12	TRAV6-7/DV9	TRAJ39	CALDENAGAKLTF	0.54
13	TRAV7D-2	TRAJ15	CAASIQGGRALIF	0.44
14	TRAV16D/DV11	TRAJ17	CAMRAPNSAGNKLTF	0.44
15	TRAV7-5	TRAJ6	CAKSGGNYKPTF	0.43
16	TRAV6-2	TRAJ43	CVLGDDNNNAPRF	0.38
17	TRAV14D-1	TRAJ6	CAAITSGGNYKPTF	0.37
18	TRAV4D-3	TRAJ12	CAAEVSGGYKVVF	0.33
19	TRAV13-2	TRAJ52	CAIDSGANTGKLTF	0.31
20	TRAV7-1	TRAJ6	CAVTSGGNYKPTF	0.31
21	TRAV12N-2	TRAJ12	CALRGTGGYKVVF	0.29
22	TRAV11	TRAJ18	CVVVDRGSALGRLHF	0.28
23	TRAV13-2	TRAJ40	CAITGNYKYVF	0.28
24	TRAV10D	TRAJ22	CAAGSSSGSWQLIF	0.27
25	TRAV7-5	TRAJ34	CAMRVSSNTDKVVF	0.27
26	TRAV8D-1	TRAJ15	CATPYQGGRALIF	0.26
27	TRAV6D-7	TRAJ57	CALVNQGGSAKLIF	0.25
28	TRAV7D-2	TRAJ30	CAARDTNAYKVIF	0.25
29	TRAV10	TRAJ27	CAASKNTNTGKLTF	0.24
30	TRAV7-6	TRAJ40	CGGKDTGNYKYVF	0.24

Supplement Table 2. Top 30 Ranking TRAV clonotypes in ACD mice at Day 7 after final challenge

Rank	V	J	CDR3	Reads%
1	TRAV11	TRAJ18	CVVGDRGSALGRLHF	4.11
2	TRAV13D-1	TRAJ52	SSTGANTGKLTF	2.19
3	TRAV13-1	TRAJ22	CAMERSSSGSWQLIF	1.39
4	TRAV12-3	TRAJ50	CALRASSSFSKLVF	1.38
5	TRAV6-7/DV9	TRAJ50	CALGDPGSSSFSKLVF	1.21
6	TRAV16D/DV11	TRAJ15	CAMREGQGGRALIF	1.08
7	TRAV6-1	TRAJ13	CVLGANSPTYQRF	1.06
8	TRAV7-3	TRAJ9	CAVRRNMGYKLTF	0.90
9	TRAV10	TRAJ13	CAAYSGTYQRF	0.87
10	TRAV5-4	TRAJ53	CAARLSGGSNYKLTF	0.85
11	TRAV9D-4	TRAJ43	CARNNNNAPRF	0.82
12	TRAV7D-2	TRAJ30	CAASDTNAYKVIF	0.79
13	TRAV4D-3	TRAJ4	CAAGLSGSFNKLTF	0.70
14	TRAV6-4	TRAJ22	CAISSGSWQLIF	0.69
15	TRAV4D-4	TRAJ30	CAGDTNAYKVIF	0.69
16	TRAV4D-3	TRAJ43	CAAERNNNNAPRF	0.68
17	TRAV4D-3	TRAJ31	CAAEEGRIFF	0.65
18	TRAV13D-1	TRAJ22	CAMDASSGSWQLIF	0.61
19	TRAV3D-3	TRAJ17	CAVSETNSAGNKLTF	0.57
20	TRAV13D-1	TRAJ57	CAMVNQGGSACLIF	0.52
21	TRAV7-2	TRAJ40	CAVNTGNYKYVF	0.45
22	TRAV4D-4	TRAJ43	CAAERNNNNAPRF	0.43
23	TRAV1	TRAJ33	CAVRDSNYQLIW	0.41
24	TRAV7D-2	TRAJ30	CAAKDTNAYKVIF	0.40
25	TRAV10D	TRAJ13	CAAYSGTYQRF	0.40
26	TRAV7D-4	TRAJ15	CAASHYQGGRALIF	0.36
27	TRAV4-4/DV10	TRAJ42	CAAAPPSNAKLTF	0.35
28	TRAV9-1	TRAJ22	CAVSFVSSGSWQLIF	0.34
29	TRAV10	TRAJ40	CAFNTGNYKYVF	0.33
30	TRAV6D-4	TRAJ43	CALVNNNAPRF	0.32

21 **Supplement Table 3. Top 30 Ranking TRBV clonotypes in ICD mice at Day 7 after final**
 22 **challenge**

Rank	V	J	CDR3	Reads%
1	TRBV29	TRBJ1-3	CASSFGNTLYF	1.21
2	TRBV1	TRBJ1-1	CTCSADQKGDTEVFF	0.98
3	TRBV1	TRBJ2-4	CTCSADRGSQNTLYF	0.66
4	TRBV29	TRBJ2-7	CASSLGDRDSYEQYF	0.65
5	TRBV5	TRBJ1-1	CASSPRANTEVFF	0.63
6	TRBV13-2	TRBJ2-3	CASGDRGGGAETLYF	0.63
7	TRBV13-3	TRBJ1-2	CASSRDSTNSDYTF	0.61
8	TRBV14	TRBJ1-5	CASSFSTGEGDNQAPLF	0.53
9	TRBV31	TRBJ2-3	CAWSLAWSQSGETLYF	0.53
10	TRBV1	TRBJ1-4	CTCSAGTGSNERLFF	0.51
11	TRBV13-1	TRBJ2-2	CASSDKYTGQLYF	0.51
12	TRBV4	TRBJ1-6	CASSSNRVYNSPLYF	0.49
13	TRBV13-3	TRBJ2-7	CASRQLGYEQYF	0.49
14	TRBV29	TRBJ1-3	CASSLGNTLYF	0.48
15	TRBV13-2	TRBJ1-2	CASGDAGTGRPDYTF	0.46
16	TRBV29	TRBJ2-3	CASSFTGASAETLYF	0.45
17	TRBV1	TRBJ2-4	CTCSADWSQNTLYF	0.44
18	TRBV5	TRBJ2-5	CASSQDWGPTQDTQYF	0.42
19	TRBV13-2	TRBJ2-1	CASGDGGADYAEQFF	0.40
20	TRBV29	TRBJ1-2	CASSPRDNNSDYTF	0.39
21	TRBV20	TRBJ1-3	CGARSQNSGNTLYF	0.38
22	TRBV13-1	TRBJ2-3	CASRAGTSAETLYF	0.38
23	TRBV13-3	TRBJ1-4	CASSPGGISNERLFF	0.38
24	TRBV31	TRBJ2-3	CAWNAWTSaETLYF	0.38
25	TRBV1	TRBJ2-7	CTCSADLGGSYEQYF	0.36
26	TRBV20	TRBJ1-4	CGAGTANERLFF	0.36
27	TRBV19	TRBJ2-1	CASSIDRGMDAEQFF	0.35
28	TRBV20	TRBJ1-2	CGATSGTANSDYTF	0.35
29	TRBV31	TRBJ1-6	CAWAGTGGDSPLYF	0.33
30	TRBV31	TRBJ1-4	CAWSPISNERLFF	0.33

23

Supplement Table 4. Top 30 Ranking TRBV clonotypes in ACD mice at Day 7 after final challenge

Rank	V	J	CDR3	Reads%
1	TRBV13-3	TRBJ1-4	CASSDVNERLFF	2.29
2	TRBV31	TRBJ2-2	CAWSQRARDTGQLYF	1.72
3	TRBV29	TRBJ1-4	CASSFVGSNERLFF	0.78
4	TRBV29	TRBJ2-4	CQNTLYF	0.71
5	TRBV13-3	TRBJ1-1	CASSGTGTEVFF	0.55
6	TRBV29	TRBJ2-4	CASSLSGSQNTLYF	0.51
7	TRBV15	TRBJ2-3	CASSLGGEAETLYF	0.46
8	TRBV13-3	TRBJ2-7	CASRDRRSSYEQYF	0.44
9	TRBV31	TRBJ2-7	CAGASSYEQYF	0.41
10	TRBV13-2	TRBJ1-6	CASGDAGAYNSPLYF	0.40
11	TRBV19	TRBJ1-4	CASSLVISNERLFF	0.39
12	TRBV31	TRBJ2-5	CAWSSTGGNQDTQYF	0.38
13	TRBV19	TRBJ1-4	CASRGTVSNERLFF	0.38
14	TRBV5	TRBJ1-1	CASSQPGGQANTEVFF	0.37
15	TRBV19	TRBJ2-3	CASSINQILGGRAETLYF	0.36
16	TRBV16	TRBJ2-7	CASSLGTGDYEQYF	0.35
17	TRBV13-1	TRBJ2-4	CASSDGRGRDTLYF	0.35
18	TRBV5	TRBJ2-5	CASSQDSWADTQYF	0.31
19	TRBV31	TRBJ1-4	CAWSLGNERLFF	0.29
20	TRBV13-3	TRBJ1-3	CASTGQNTLYF	0.28
21	TRBV19	TRBJ1-4	CASSMPSGQGARERLFF	0.28
22	TRBV31	TRBJ2-7	CAWSEGSYEQYF	0.26
23	TRBV13-3	TRBJ2-3	CASGTGGSSAETLYF	0.23
24	TRBV31	TRBJ2-3	CAWSLGGSAETLYF	0.22
25	TRBV13-3	TRBJ2-2	CASSDGDGTGQLYF	0.20
26	TRBV13-1	TRBJ2-3	CASSARDRGRAETLYF	0.20
27	TRBV31	TRBJ1-1	CAWSRGTAEVFF	0.19
28	TRBV19	TRBJ2-3	CASSIGSAETLYF	0.18
29	TRBV5	TRBJ2-2	CASSQGLGDTGQLYF	0.18
30	TRBV13-1	TRBJ1-4	CASSEGQSNERLFF	0.17