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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

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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 $TiCl_3$ plus lipopolysaccharide solution into the mouse groin followed by two challenges with $TiCl_3$ 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.


## 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,

*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

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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 μg/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

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